



Christian Ehnholm  
*Editor*

# Cellular Lipid Metabolism



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# Preface

The key to every biological problem must in the end be sought in the cell and yet, although we know a lot about the mechanism by which cells operate, there is still a shortage in our understanding of how lipids affect cell biology. For years lipids have fascinated cell biologists and biochemists because they have profound effects on cell function. Encoded within lipid molecules is the ability to spontaneously form macroscopic, two-dimensional membrane systems. In addition to their function as physical and chemical barriers separating aqueous compartments, membranes are involved in many regulatory processes, such as secretion, endocytosis, and signal transduction. The functional interaction between lipids and proteins is essential for such membrane activities.

Lipids serve as one of the major sources of energy, both directly and when stored in adipose tissues. They also act as thermal insulators in the subcutaneous tissues and serve as electrical insulators in myelinated nerves, allowing the rapid propagation of waves of depolarization. Some lipids act as biological modulators and signal transducers (e.g., pheromones, prostaglandins, thromboxanes, leukotrienes, steroids, platelet-activating factor, phosphatidylinositol derivatives) and as vehicles for carrying fat-soluble vitamins.

Research on cell biology is at present in a very active phase and molecular genetics is helping us to recognize and exploit the unity of all living systems and to reveal the fundamental mechanisms by which the cell operates.

The challenge in composing a book on *Cellular lipid metabolism* has been to select concepts that are important for our understanding in areas that have changed or in which new concepts have emerged. Recognizing that it is impossible to be comprehensive, I have tried to ensure that this book provides a survey of cell biology in areas that I consider important.

This book was planned to be a resource for scientists at post-doctoral level and above, in other words, a rather specific publication to highlight recent findings in cell biology and biochemistry but also to include important findings made in the past and give a good overview. I contacted the best experts in 13 fields and the chapters represent their specialized contributions. They represent analyses at the molecular level and reveal the principles by which cellular lipid metabolism functions.

There are still large areas of ignorance in cell biology and numerous intriguing observations that cannot be explained. In this volume we try to expose them and to stimulate readers to contemplate and discover ways of solving the open questions.

I hope this book will be of interest to all researchers in the area of cell biology, lipid metabolism and atherosclerosis, providing a useful review of accomplishments and a stimulating guide for future studies.

Helsinki, February 2009

Christian Ehnholm

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# Chapter 1

## The Lipid Droplet: a Dynamic Organelle, not only Involved in the Storage and Turnover of Lipids

Sven-Olof Olofsson, Pontus Boström, Jens Lagerstedt, Linda Andersson, Martin Adiels, Jeanna Perman, Mikael Rutberg, Lu Li, and Jan Borén

**Abstract** Neutral lipids such as triglycerides are stored in cytosolic lipid droplets. These are dynamic organelles and consist of a core of neutral lipids surrounded by amphipathic lipids and proteins. The surface is complex and contains proteins involved in lipid biosynthesis and turnover and proteins involved in sorting and trafficking events in the cell. Lipid droplets are formed at microsomes as primordial droplets, which increase in size by fusion. In this chapter, we review the assembly and fusion of lipid droplets. We also discuss a possible mechanism to explain the link between lipid accumulation in muscle cells and the development of insulin resistance. Triglycerides are secreted as milk globules from the epithelial cells of the mammary glands, as chylomicrons from enterocytes, and as very low-density lipoproteins (VLDL) from hepatocytes. We review the processes involved in the formation of milk globules and VLDL, and we discuss the clinical consequences of overproduction of VLDL.

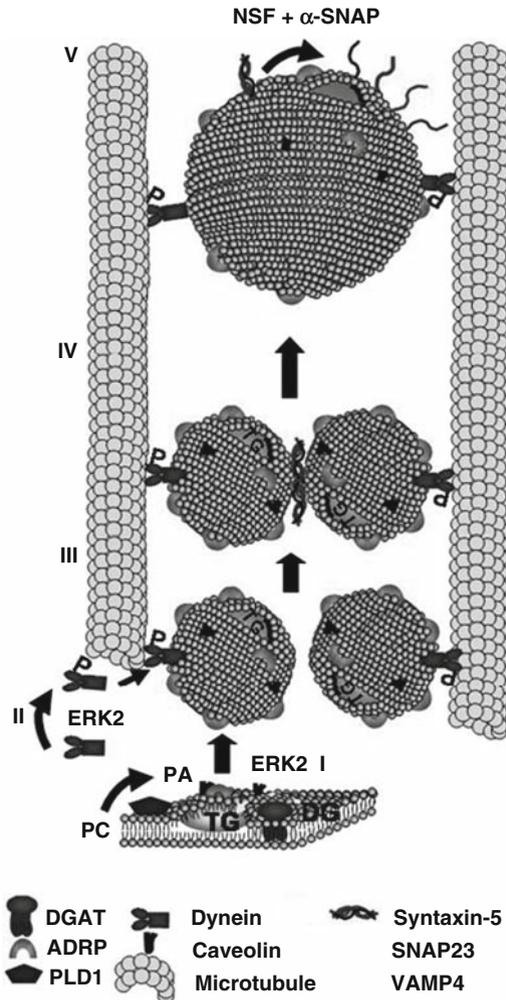
**Abbreviations** ADRP, adipocyte differentiation related protein; ATGL, adipose triglyceride lipase; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; ERGIC, ER Golgi intermediate compartment; ERK2, extracellular signal regulated kinase 2; GPAT, glycerol-3-phosphate acyltransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NSF, N-ethylmaleimide-sensitive factor; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PC, phosphatidylcholine; PLD1, phospholipase D1; SNAP23, synaptosomal-associated protein of 23 kDa;  $\alpha$ -SNAP,  $\alpha$ -soluble NSF adaptor protein; SNARE, SNAP receptor; VAMP, vesicle-associated protein; VLDL, very low-density lipoprotein

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## 1.1 Introduction

Neutral lipids, such as triglycerides and cholesterol esters, are stored in the cells within so-called cytosolic lipid droplets. The neutral lipids form the core of the lipid droplet and are surrounded by an outer layer of amphipathic lipids, such as phospholipids and cholesterol (Brown 2001; Martin and Parton 2006; Fig. 1.1). The surface of the lipid droplet is generally considered to be a monolayer of lipids (Robenek et al. 2005).



**Fig. 1.1** A model for the formation of lipid droplets. Diglycerides (*DG*) are catalyzed by DGAT to form triglycerides (*TG*) in the microsomal membrane. *TG* have limited solubility in the amphipathic monolayer, and therefore oil out between the leaflets of the membrane to form a *TG* lens, which will become the core of the lipid droplet (*I*). Lipid droplet assembly also requires the production of phosphatidic acid (*PA*) from phosphatidylcholine (*PC*) catalyzed by PLD1 and an active ERK2 (*II*).

Specific proteins that are essential for the formation, structure and function of the lipid droplet are bound to its surface (Brasaemle 2007; Martin and Parton 2006). As a result of recent advances in our knowledge about the structure and function of lipid droplets, they are now considered to be dynamic organelles that can interact with other organelles and have a key role in the cellular turnover of lipids (Martin and Parton 2006).

In today's increasingly overweight society, the problems associated with excess levels of triglycerides are now well recognized. Accumulation of triglycerides, particularly in the liver and muscles, is highly correlated with the development of insulin resistance and type 2 diabetes, which are important risk factors for arteriosclerosis and cardiovascular diseases (Taskinen 2003). Triglycerides are stored to a variable extent in most cells, but they are only efficiently secreted by certain organs, i.e. liver and intestine (Olofsson and Boren 2005) and mammary glands (McManaman et al. 2007). In this article, we review the storage and secretion of triglycerides.

## 1.2 Lipid Droplets Form as Primordial Structures at Microsomal Membranes

The nature of the site of assembly of lipid droplets has not been conclusively determined. Results from a cell-free system indicate that they can be formed from a microsomal fraction enriched in markers for the endoplasmic reticulum (ER) and Golgi apparatus but lacking markers for the plasma membrane (Marchesan et al. 2003). An ER localization of the assembly of lipid droplets is also suggested by results showing that lipid droplets are associated with adipocyte differentiation related protein (ADRP)-enriched regions of the ER (Robenek et al. 2006). However, other results (Ost et al. 2005) indicate that the plasma membrane may be a source of droplets: triglycerides accumulate in the plasma membrane of adipocytes and this accumulation seems to be a precursor for the formation of cytosolic lipid droplets.

### 1.2.1 Microsomal Membrane Proteins Involved in Lipid Droplet Formation

The formation of lipid droplets is highly linked to the biosynthesis of triglycerides (Marchesan et al. 2003). Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the first step, i.e. the formation of lyso-phosphatidic acid. GPAT exists in several

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**Fig. 1.1** (continued) The assembly process first forms a primordial droplet with a diameter <0.5  $\mu\text{m}$ . ERK2 phosphorylates the motor protein dynein, which is then sorted to droplets allowing them to transfer on microtubules (II). This allows long-distance transport of the droplet in the cell and is also required for lipid droplet fusion (III–V). The fusion process is catalyzed by the SNAREs SNAP23, syntaxin-5 and VAMP4 (IV). After the fusion, the four-helix bundle formed by the SNARE domains of these three SNAREs is recognized by  $\alpha$ -SNAP which, together with the ATPase NSF, unwinds the bundle, allowing new fusions to occur (V)

isoforms. GPAT1 and GPAT2 are present on mitochondria and GPAT3 and GPAT4 are present on ER (Gonzalez-Baro et al. 2007; for a review, see Coleman et al. 2000). The mitochondrial isoforms of GPAT were first identified and cloned and most information is from studies of these isoforms. Overexpression of GPAT1 has been shown to increase the accumulation of triglycerides in the cell and promote the formation of steatoses (Gonzalez-Baro et al. 2007). GPAT3 has also been cloned (Cao et al. 2006). This isoform is highly upregulated during adipocyte differentiation and overexpression leads to lipid accumulation in the cell (Cao et al. 2006).

The formation of phosphatidic acid from lyso-phosphatidic acid is catalyzed by 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT). This enzyme exists in several isoforms of which 1 and 2 are confined to microsomes. AGPAT is a membrane-spanning protein that catalyzes the reaction on the cytosolic side of the ER (for reviews with references, see for example Agarwal and Garg 2003; Leung 2001).

When associated with microsomal membranes, the amphipathic enzyme phosphatidic acid phosphohydrolase hydrolyzes phosphatidic acid, forming diacylglycerol (for reviews, see Carman and Han 2006; Coleman et al. 2000). Diacylglycerol acyltransferase (DGAT), an integral membrane protein of microsomes (Stone et al. 2006), then catalyzes the conversion of diacylglycerol to triglycerides. There are two mammalian forms of DGAT: DGAT1 and DGAT2. DGAT1 is a multifunctional enzyme (Yen et al. 2005) whereas DGAT2 has been shown to be more potent and specific for triglyceride synthesis (Stone et al. 2004). In addition to its localization on the ER membrane, DGAT2 has also been identified on lipid droplets (Kuerschner et al. 2008). However, DGAT2 is a membrane protein that spans the bilayer twice; and it remains to be clarified how it is integrated into the amphipathic monolayer that surrounds lipid droplets. Alternatively, there may be a very tight interaction between lipid droplets and the ER allowing the DGAT2 product formed in the microsomal membrane to enter into the droplets. Such a tight interaction has been demonstrated and shown to be dependent on the GTPase Rab18 (for a review, see Martin and Parton 2006).

Lipid droplet assembly is also dependent on phospholipase D (PLD) activity and the formation of phosphatidic acid (Marchesan et al. 2003). Using intact cells, we showed that the active isoform is PLD1 and not PLD2 (Andersson et al. 2006), which is consistent with the localization of the two isoforms: PLD1 is present in ER and Golgi membranes (Andersson et al. 2006; Freyberg et al. 2001) while PLD2 is confined to the plasma membrane (Andersson et al. 2006; Du et al. 2004).

The observation that most of the identified enzymes associated with lipid droplet assembly are localized on microsomes supports the idea that lipid droplets are formed at the ER and/or Golgi apparatus.

### ***1.2.2 Model for the Assembly of Lipid Droplets***

The lipid droplets formed at the isolated microsomal membranes in a cell-free system have a diameter of 0.1–0.4  $\mu\text{m}$  (Marchesan et al. 2003). This corresponds well to the size of the smallest droplets observed in cells by electron microscopy

(Marchesan et al. 2003). The newly formed droplets recovered from the cell-free system contain ADRP and are rich in caveolin and vimentin; and we propose that they represent the first primordial structures formed during the assembly process (Marchesan et al. 2003).

Although no experimental results have been obtained to date demonstrating how lipid droplets are formed, a tentative model for their assembly has been proposed (see for example Brown 2001; Fig. 1.1). Triglycerides (formed from diglycerides and acyl-CoA by the DGAT reaction in the microsomal membranes) are highly hydrophobic and have limited solubility in the monolayer of the membrane. The formed triglycerides will therefore “oil out” as a separate phase between the two leaflets, forming a lens structure that is the core of the lipid droplets. One problem is that the formed triglycerides may rapidly diffuse laterally in the ER and Golgi membranes and saturate these organelles before the oiling out occurs. However, this could be prevented if the regions of triglyceride synthesis are sealed off from the rest of the organelle.

### 1.3 Lipid Droplet Size Increases by Fusion

We have shown that droplets can increase in size by a fusion process, which is independent of triglyceride biosynthesis (Bostrom et al. 2005; Fig. 1.1). Approximately 15% of all droplets in the cells are engaged in fusion events at any given time (Bostrom et al. 2005) and thus fusion is a frequently occurring event that represents an important mechanism by which lipid droplets increase in size.

Lipid droplets are transported relatively long distances on microtubules (Welte et al. 1998) and motor proteins such as dynein have been shown to be present on droplets (Bostrom et al. 2005). We demonstrated that dynein is sorted to the droplets following phosphorylation by the cytosolic protein extracellularly regulated kinase 2 (ERK2; Andersson et al. 2006; Fig. 1.1). Both dynein and microtubules are essential for the fusion between droplets (Andersson et al. 2006; Bostrom et al. 2005).

#### 1.3.1 *SNAREs are Involved in Lipid Droplet Fusion*

We have shown that the fusion between lipid droplets is catalyzed by N-ethylmaleimide-sensitive factor adaptor protein receptors (SNAREs), the synaptosomal-associated protein of 23 kDa (SNAP 23), syntaxin-5 and vesicular-associated protein 4 (VAMP4; Fig. 1.1). In addition, the fusion requires the ATPase N-ethylmaleimide-sensitive factor (NSF) and  $\alpha$ -soluble NSF adaptor protein ( $\alpha$ -SNAP; Fig. 1.1).

The role of these proteins has been extensively described for the fusion process between transport vesicles and target membranes (see for example Jahn and Scheller 2006). The SNAREs present on the target membrane (t- or Q-SNAREs) interact with a SNARE on the transport vesicle (v- or R-SNARE) to form a SNARE complex that causes fusion. A central feature in this process is the formation of a superhelix bundle, formed by four  $\alpha$ -helical SNARE domains from the different SNAREs. The formation of the four-helix bundle forces the two membranes together, promoting their fusion.

After the fusion, the SNARE complex is unwound by NSF and  $\alpha$ -SNAP (for reviews of the SNARE system, see Hong 2005; Jahn and Scheller 2006).

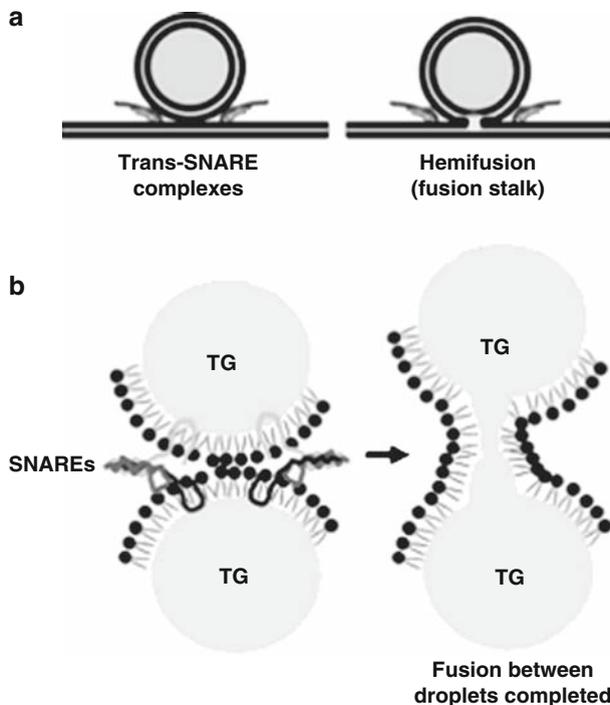
The four-helix bundle is mostly stabilized by hydrophobic interactions, except in the zero-plane, where an arginine side-chain from one SNARE domain (R-SNARE) interacts with the glutamine side-chains from the other three SNARE domains (Q-SNAREs). On the basis of the homology of the SNARE domains, Q-SNAREs are subdivided into Qa, Qb and Qc; and a complete and functioning SNARE complex has the structure QabcR. The most well known t-SNARE complex is formed by a syntaxin (Qa-SNARE) and SNAP25 or SNAP23 (Qbc SNAREs). The R-SNARE is present on the transport vesicle and belongs to the VAMP family of SNAREs (for reviews, see Hong 2005; Jahn and Scheller 2006).

### ***1.3.2 Model for the Fusion Between Lipid Droplets***

As oils in water fuse spontaneously, unprotected triglycerides would fuse to form large hydrophobic regions that may influence the function of the cell. We hypothesize that this spontaneous fusion is reduced by protecting the triglycerides with amphipathic structures such as phospholipids and proteins. We also propose that the SNARE system could restore the fusion capacity of intact droplets and, moreover, provide a way to control the fusion process.

SNAP23 is a covalently palmitoylated SNARE and thus can anchor in the monolayer surrounding the lipid droplet by the palmitic acid residues (Bostrom et al. 2007). Syntaxin-5 and VAMP4 are tail-anchored proteins (High and Abell 2004). Such proteins are synthesized on ribosomes present in the cytosol and inserted into bilayer membranes through interactions with chaperone proteins (High and Abell 2004). It is reasonable to assume that syntaxin-5, which has a very hydrophobic C-terminus, could similarly be inserted into the surface of lipid droplets. However, the end of the C-terminus of VAMP4 is less hydrophobic (Bostrom et al. 2007) and thus its incorporation into the surface of lipid droplets may require other mechanisms (e.g. interaction with specific proteins, or the formation of hair-pin structures in which the hydrophobic regions dip down into the hydrophobic part of the droplet). It has been suggested that bilayer regions in the lipid droplet surface could allow the focal insertion of SNARE proteins (Sollner 2007).

The surface of the droplet is an amphipathic monolayer, whereas a vesicle is surrounded by a bilayer. Thus, it is likely that there are differences between the fusion of lipid droplets and the fusion of a transport vesicle with a target membrane (Fig. 1.2). The stalk hypothesis has been proposed to describe the fusion process between bilayers as an ordered sequence of transition states (Jahn and Scheller 2006; Fig. 1.2). We postulate that fusion between lipid droplets requires fewer steps and is complete at a stage equivalent to the creation of a “fusion stalk”, i.e. when the two outer monolayers of the bilayers have fused and there is a continuum between the hydrophobic portions of the two membranes. For lipid droplets, this would



**Fig. 1.2** A model for the fusion between lipid droplets. We propose that the fusion between lipid droplets (which involves two monolayers of amphipathic structures) corresponds to the first step in the fusion between transport vesicles and target membranes (which involves two bilayers). **a** According to the stalk hypothesis, a ‘fusion stalk’ is formed by a fusion of the outer leaflets, which allows the hydrophobic parts of the two membranes to contact each other (adapted from Jahn and Scheller 2006). **b** Formation of a four-helix bundle between syntaxin-5 (*Qa*), SNAP23 (*Qbc*) and VAMP4 (*R*) forces the two monolayers of the lipid droplets to fuse with each other, which results in a connection between the hydrophobic phases. This is equivalent to the creation of a fusion stalk in **a** (adapted from Bostrom et al. 2007)

correspond to a fusion of the monolayers surrounding the two droplets connecting the two hydrophobic cores (Bostrom et al. 2007; Fig. 1.2).

## 1.4 Lipid Droplets and the Development of Insulin Resistance

The accumulation of lipids in muscle (Falholt et al. 1988; Krssak et al. 1999; Machann et al. 2004) is highly correlated with the development of insulin resistance and type 2 diabetes (for reviews, see Goossens 2007; Kovacs and Stumvoll 2005; Sell et al. 2006; Yki-Jarvinen 2002; Yu and Ginsberg 2005). The accumulation of triglycerides in muscle occurs when the inflow of fatty acids exceeds the capacity of the cell to use them (by oxidation or biosynthesis).

The glucose transporter GLUT4 is of central importance for the insulin-regulated uptake of glucose in skeletal muscle (for reviews, see Dugani and Klip 2005; Huang and Czech 2007; Pilch 2008; Watson and Pessin 2006, 2007). GLUT4 exists mainly in intracellular compartments and is translocated to the plasma membrane in response to insulin. Thus, insulin results in an increase in the plasma membrane pool of GLUT4 and a subsequent increase in glucose uptake.

The sorting of GLUT4 in the cell is complex and not fully understood. It has been the subject of recent reviews (see for example Dugani and Klip 2005; Huang and Czech 2007; Pilch 2008; Watson and Pessin 2006, 2007) and the reader is referred to these reviews for details and references. One important step is the accumulation of GLUT4 in GLUT4-specific vesicles (GSV). Insulin promotes the transport of these vesicles to the plasma membrane and their subsequent fusion with the plasma membrane. The fusion process involves three SNARE proteins: syntaxin-4, VAMP2 and SNAP23 – the protein involved in lipid droplet fusion.

We showed that incubation of muscle cells with fatty acids results in an increased formation of lipid droplets and increased insulin resistance, measured as a reduced response to insulin for both glucose uptake and GLUT4 translocation (Bostrom et al. 2007). Furthermore, we found that fatty acid treatment decreases the plasma membrane pool of SNAP23 and increases the intracellular pool of SNAP23, in part due to a sequestering of SNAP23 on the increasing lipid droplet pool (Bostrom et al. 2007). Moreover, the fatty acid-induced insulin resistance can be reversed by increasing the pool of SNAP23 in the cell (Bostrom et al. 2007). These results indicate that SNAP23 has a central role in the development of lipid-induced insulin resistance. They also indicate that it is not the lipid droplets per se that promote the development of insulin resistance, but the influence of the fatty acids on SNAP23. The effect of fatty acids on the insulin signal is well established and is proposed to be mediated by fatty acid metabolites, such as diglycerides (for reviews, see Morino et al. 2006; Savage et al. 2007), ceramides (reviewed by Summers 2006) and partially oxidized fatty acids (Koves et al. 2008). However, the exact mechanisms involved are not known.

## 1.5 Lipid Droplet-Associated Proteins

### 1.5.1 *PAT Proteins*

In addition to those discussed above, numerous proteins have been identified in association with lipid droplets. The quantitatively most important are the so-called PAT proteins (Dalen et al. 2007; Londos et al. 1999; reviewed by Brasaemle 2007), named after the three first identified species of the family: *perilipin*, *ADRP* and *tail interacting protein 47* (TIP47). The name PAT could also reflect the existence of a *perilipin* amino-terminal domain with high degree of homology between the family members. There are two recent additions to this family: *lipid storage droplet protein 5* (LSDP5), also known as *OXPAT*, *myocardial lipid droplet protein* (MLDP) or *PAT-1* (Dalen et al. 2007; Wolins et al. 2006), and *S3-12* (Dalen et al. 2004; Wolins et al. 2003).

In addition to the highly homologous N-terminal PAT domain, there are reports of a C-terminal PAT domain, which shows very low homology between the family members (Lu et al. 2001; Miura et al. 2002). The PAT domains do not seem to be important for the binding of the protein to lipid droplets, but ADRP mutation studies indicate that the middle domain – including  $\alpha$ -helical regions between amino acids 189 and 205 – directs ADRP to droplets (Nakamura and Fujimoto 2003).

### 1.5.1.1 Perilipin

Perilipin was initially identified as a protein kinase A (PKA)-phosphorylated protein in lipolytically stimulated adipocytes (Greenberg et al. 1991, 1993). The dominating sites of expression are adipocytes and steriogenic cells (Londos et al. 1995). There are three isoforms (perilipin A, B, C), which are formed from a single gene through alternative splicing (Lu et al. 2001). Perilipin A is by far the most abundant and best investigated isoform (Londos et al. 1999). The gene for human perilipin is located on chromosome 15q26 at a locus that has been linked to diabetes, hypertriglyceridemia and obesity (for a review, see Tai and Ordovas 2007).

Perilipin has an important and dualistic role in the turnover of triglycerides in lipid droplets. It protects the degradation of triglycerides when expressed in cells that lack natural expression of the protein (Brasaemle et al. 2000). Moreover, perilipin A knockout mice show a substantial increase in basal lipolysis and reduction in adipose mass, and are resistant to diet-induced obesity (Martinez-Botas et al. 2000; Tansey et al. 2001). Perilipin also promotes triglyceride degradation:  $\beta$ -adrenergic stimulation does not promote lipolysis in perilipin A knockout mice; and cells derived from these mice fail to show translocation of hormone-sensitive lipase (HSL) to the lipid droplet (Martinez-Botas et al. 2000; Tansey et al. 2001; reviewed by Londos et al. 2005). A proposed model, based on these and more direct results (Granneman et al. 2007), states that concomitant phosphorylation of perilipin and HSL results in translocation of HSL to the lipid droplet where it catalyzes the hydrolyzation of triglycerides (Granneman et al. 2007).

In addition to its effect on HSL, perilipin seems to have an important role in the activation of the first step in the degradation of triglycerides, i.e. the step catalyzed by the newly discovered adipose triglyceride lipase (ATGL) and its co-activator, a perilipin-interacting protein CGI-58 (or Abhd5; Lass et al. 2006). It is suggested that phosphorylation of perilipin A results in its dissociation from CGI-58, which then associates with ATGL on lipid droplets to allow lipolysis (Granneman et al. 2007; Miyoshi et al. 2007).

### 1.5.1.2 ADRP

Although perilipin has a central role in the turnover of triglycerides in adipocytes, there is no experimental evidence to indicate that it participates in the assembly of lipid droplets. Indeed, ADRP seems to play a central role in the assembly process

even in perilipin-expressing cells such as the adipocyte. Thus, ADRP is expressed in increasing amounts early in adipocyte differentiation, but is later replaced by perilipin (Brasaemle et al. 1997).

ADRP was identified as a protein related to adipocyte differentiation (Jiang and Serrero 1992) and was originally thought to be a fatty acid-binding protein (Serrero et al. 2000). However, it later proved to be one of the major PAT proteins with a striking homology to perilipin in the N-terminus (Brasaemle 2007; Londos et al. 1999). In contrast to perilipin, ADRP is expressed ubiquitously (Brasaemle et al. 1997). This expression is highly related to the amount of neutral lipid in the cell (Heid et al. 1998) and overexpression of ADRP results in an increased formation of droplets (Imamura et al. 2002; Magnusson et al. 2006; Wang et al. 2003). The regulation of ADRP levels in the cell is complex. ADRP is regulated at the transcriptional level by peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ; Dalen et al. 2006; Edvardsson et al. 2006; Targett-Adams et al. 2003), but also through post-translational degradation by the proteasomal system (Masuda et al. 2006; Xu et al. 2005), which occurs when there are low levels of lipids in the cell. Thus, accumulation of intracellular triglycerides appears to stabilize ADRP and prevents it from being sorted to degradation.

Knockout of ADRP results in a rather modest phenotype (a reduced amount of triglycerides in the liver and resistance to diet-induced hepatosteatosis; no effect on adipocyte differentiation and lipolysis; Chang et al. 2006). One reason for this modest phenotype is that TIP47 is directed to the droplets and replaces ADRP (Sztalryd et al. 2006). ADRP $-/-$  cells treated with siRNA against TIP47 retain the ability to form lipid droplets, although to a lesser extent, and added fatty acids are to a greater extent directed to phospholipid biosynthesis (Sztalryd et al. 2006). Interestingly, the combined knockdown of ADRP and TIP47 in cultured liver cells results in large lipid droplets with high turnover of triglycerides and insulin resistance (Bell 2006).

### 1.5.1.3 TIP47

TIP47 was initially described as a ubiquitously expressed cytosolic and endosomal 47-kDa protein involved in the intracellular transport of mannose 6-phosphate receptors between the *trans*-Golgi and endosomes (Diaz and Pfeffer 1998; Krise et al. 2000). TIP47 is believed to act as an effector for the Rab9 protein in this process, causing budding of vesicles directed to lysosomes (Carroll et al. 2001). TIP47 is also present on lipid droplets (Wolins et al. 2001). In contrast to ADRP, which is always associated with droplets and is degraded in the absence of neutral lipid, cytosolic TIP47 is shifted to lipid droplets in the presence of increased levels of fatty acids (Wolins et al. 2001).

The C-terminus of TIP47 has been crystallized and its structure determined (Hickenbottom et al. 2004). It has an  $\alpha/\beta$  domain of novel topology and four helix bundles resembling the low-density lipoprotein (LDL) receptor-binding domain of apolipoprotein E (Hickenbottom et al. 2004) and the N-terminal domain of apolipoprotein A-I (Ajees et al. 2006; Lagerstedt et al. 2007). These results suggest an analogy between PAT proteins and plasma apolipoproteins.

#### 1.5.1.4 LSDP5 and S3-12

LSDP5 is mainly expressed in tissues with high rates of  $\beta$ -oxidation, such as muscle, heart, liver and brown adipose tissue (Wolins et al. 2006), and shows a high degree of homology with ADRP and TIP47 (Dalen et al. 2007; Scherer et al. 1998). Forced overexpression of LSDP5 in cultured cells results in a substantial increase in the accumulation of triglycerides in response to fatty acid treatment (Wolins et al. 2006), which might be explained by a decrease in both basal and stimulated lipolysis (Dalen et al. 2007). Thus, LSDP5 seems to protect the triglyceride core of lipid droplets from degradation in a similar manner to perilipin. LSDP5 is transcriptionally regulated by PPAR $\alpha$  in striated muscle and liver and by PPAR $\gamma$  in white adipose tissue (Dalen et al. 2007; Wolins et al. 2006; Yamaguchi et al. 2006).

S3-12 is mainly expressed in white adipose tissue; and it shares only a weak sequence homology with the PAT proteins (Wolins et al. 2003). S3-12 expression is transcriptionally regulated by PPAR $\gamma$  (Dalen et al. 2004).

### 1.5.2 Other Lipid Droplet-Associated Proteins

Vimentin has been shown to be present as cages around the lipid droplets (Franke et al. 1987). Knockdown by anti-sense RNA has been shown to result in a decrease in the formation of droplets (Lieber and Evans 1996). However, this finding was not verified in vimentin  $-/-$  mice (Colucci-Guyon et al. 1994).

Caveolin is also present on lipid droplets protein (Brasaemle et al. 2004; Liu et al. 2004) and, again, its function is not fully understood. One suggestion is that it may reflect an involvement of caveolin-rich plasma membranes in the assembly of lipid droplets (Ost et al. 2005). Caveolin has also been linked to triglyceride lipolysis in the droplets (for a review, see Martin and Parton 2006).

A number of other proteins have been identified on the lipid droplets by proteomics (Brasaemle et al. 2004; Cermelli et al. 2006; Liu et al. 2004). These include proteins involved in lipid biosynthesis (e.g. acyl-CoA synthetase, lanosterol synthetase) and turnover (e.g. ATGL, CGI-58, HSL) as well as in sorting and trafficking events (e.g. the Rab proteins; Grosshans et al. 2006). In addition, there are several other proteins with functions that are yet to be elucidated.

## 1.6 Lipid Droplets and the Secretion of Triglycerides from the Cell

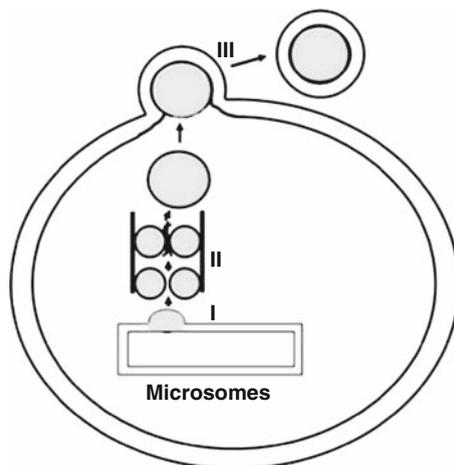
Triglycerides are secreted as milk globules from the epithelial cells in mammary glands, as chylomicrons from enterocytes in the intestine and as very low-density lipoproteins (VLDL) from hepatocytes in the liver. The mechanism involved in the secretion of milk globules is very different from that involved in the secretion of

chylomicrons and VLDL; and here we illustrate the two different mechanisms by focusing on the secretion of milk globules and VLDL.

### 1.6.1 The Assembly and Secretion of Milk Globules

Milk globules have been extensively reviewed (e.g. Heid and Keenan 2005; Mather and Keenan 1998; McManaman and Neville 2003; McManaman et al. 2007). The secreted milk globule consists of a core of triglycerides covered by a membrane that has a tripartite structure. The outer portion, or primary membrane, has a typical bilayer structure and seems to originate from the apical plasma membrane of the mammary epithelial cell. This is supported by the finding that secreted milk globules contain plasma membrane-specific proteins such as the plasma membrane calcium-transporting ATPase 2 (Reinhardt and Lippolis 2006). The material that covers the triglyceride core originates from the ER and has the appearance of a monolayer of lipid and proteins (Heid and Keenan 2005; Mather and Keenan 1998; McManaman and Neville 2003; McManaman et al. 2007).

The formation of milk globules starts in the ER by the formation of ADRP- and TIP47-containing lipid droplets, most likely by the same mechanism as other droplets (Fig. 1.3). The primordial milk globule has a diameter of less than 0.5  $\mu\text{m}$  and it is thought that these newly formed droplets increase in size by fusion (Fig. 1.3; see reviews cited above). A recent proteomics study showed that milk globules appear to contain several SNARE proteins, such as SNAP23, VAMP2, syntaxin-3 and Ykt6 (Reinhardt and Lippolis 2006). The presence of significant



**Fig. 1.3** The formation and secretion of milk globules from mammary glands. Primordial lipid droplets  $<0.5 \mu\text{m}$  in diameter are formed at microsomal membranes (I), transported on microtubules and increase in size by fusion (II) The mature lipid droplet is enveloped by the bi-layer of the plasma membrane and is then secreted (III)

levels of SNAP23 was confirmed by Western blot experiments; and thus the fusion process may be the same as that described for droplets in other cells. However, several of these proteins are abundant in the plasma membrane of the cell and the view that the outer bilayer of the milk globule structure is derived from the apical plasma membrane (see discussion below) could explain the presence of the SNARE proteins on milk globules. The mechanism by which lipid droplets are transported to the plasma membrane requires clarification.

Milk globules are secreted via a mechanism that differs completely from that used for the other types of triglyceride secretion; and two potential mechanisms have been proposed. First, it has been suggested that secretory vesicles fuse on the surface of the milk globule, leading to an intracytoplasmic vacuole containing both casein micelles and lipid droplets enveloped with secretory vesicle membrane. The content of such vacuoles is then released from the cell by exocytosis. Mather and Keenan (1998) discuss concerns about the data supporting this mechanism. The alternative and currently favored mechanism is that the milk globule approaches the plasma membrane where it is enveloped and, ultimately, pinched off (see Fig. 1.3; reviewed by Heid and Keenan 2005). The mechanism by which the milk globule interacts with the plasma membrane has not been elucidated in detail. However, butyrophilin and xanthine oxidoreductase are present both in milk globules and on the apical region of the plasma membrane of mammary gland epithelial cells; and it has been suggested that these proteins together with ADRP form a tripartite structure that is of importance for this interaction and the secretion of the milk globule (for reviews, see Heid and Keenan 2005; McManaman et al. 2007). There is still debate about the molecular details for the secretion of milk globules and indeed whether the three proteins interact (for a discussion, see McManaman et al. 2007).

### ***1.6.2 ApoB100: the Structural Protein of VLDL***

Liver and intestinal cells express apolipoprotein B (apoB), which is essential for the formation of triglyceride-containing lipoproteins and triglyceride secretion (Davidson and Shelness 2000b; Gibbons et al. 2004; Olofsson and Asp 2005; Olofsson et al. 1999, 2000). ApoB is a large amphipathic protein (Segrest et al. 2001), which exists in two forms: apoB100 and apoB48. In humans, apoB100 is expressed in the liver and is required for the formation of VLDL whereas apoB48 is expressed in the intestine and is lipidated to form chylomicrons. Here, we focus on apoB100 and the assembly of VLDL.

ApoB100 has a pentapartite structure consisting of one globular N-terminal structure, two domains of amphipathic  $\beta$ -sheets and two domains of amphipathic  $\alpha$ -helices (Segrest et al. 2001). The N-terminal domain is of vital importance for the formation of VLDL as it interacts with microsomal triglyceride transfer protein (MTP), which catalyzes the transfer of lipids to apoB during the formation of lipoproteins (see below; Dashti et al. 2002).

ApoB differs from other apolipoproteins in that it is nonexchangeable, i.e. it cannot equilibrate between different lipoproteins but remains bound to the particle

on which it was secreted into plasma. This is generally thought to be explained by the presence of antiparallel  $\beta$ -sheets with a width of approximately 30 Å, which form very strong lipid-binding structures (Segrest et al. 2001).

The three-dimensional structure of apoB100 is not known in detail, but its overall organization on LDL has been elucidated (Chatterton et al. 1995). ApoB has an elongated structure encompassing the entire particle. The C-terminus folds back over the preceding structure and crosses it at amino acid residue 3500 (arginine). The arginine binds to a tryptophan (residue 4396) preventing the C-terminus from sliding over the binding site for the LDL receptor (residues 3359–3369; Boren et al. 1998, 2001). A number of known mutations involving these amino acids break the arginine–tryptophan interaction and result in reduced binding of LDL to its receptor (Boren et al. 1998, 2001).

### ***1.6.3 ApoB100 and the Secretory Pathway***

Secretory proteins such as apoB are synthesized on ribosomes attached to the surface of the ER (Fig. 1.4). During its biosynthesis, the “nascent” polypeptide is translocated through a channel (for reviews, see Johnson and Haigh 2000; Johnson and van Waes 1999) to the lumen of the ER, where it is folded into its correct structure with the help of chaperone proteins. Correctly folded proteins are sorted into exit sites to leave the ER by transport vesicles. If the correct tertiary structure is not achieved, the protein is retained in the ER, retracted through the membrane channel and sorted to proteasomal degradation (Ellgaard and Helenius 2001, 2003; Ellgaard et al. 1999; Johnson and Haigh 2000; Johnson and van Waes 1999; Kostova and Wolf 2003; Lippincott-Schwartz et al. 2000).

ApoB100 exits the ER in vesicles that bud off from specific sites on the ER membrane and form the ER Golgi intermediate compartment (ERGIC), which is involved in protein sorting. ER-specific proteins are returned to the ER from the ERGIC and thereby prevented from entering the later part of the secretory pathway to be secreted. The ERGIC matures into *cis*-Golgi, which undergoes “cisternal maturation” to form the medial and *trans*-Golgi apparatus. During this maturation, the proteins that will be secreted are transferred through the Golgi stack. Finally, the proteins are transported from the *trans*-Golgi to the plasma membrane for secretion (for reviews, see Elsner et al. 2003; Kartberg et al. 2005).

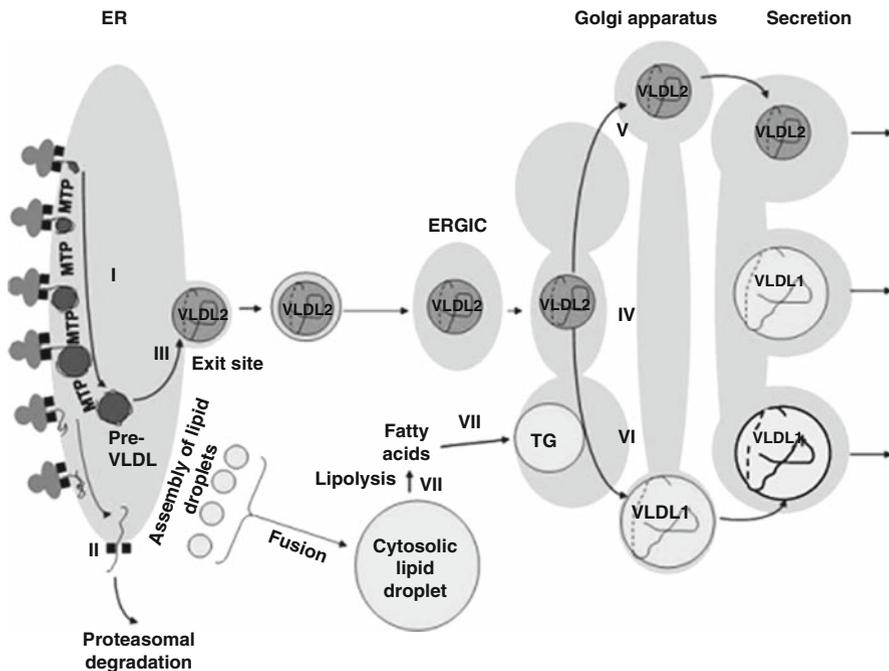
### ***1.6.4 The Assembly of VLDL***

The assembly of VLDL involves three types of particles: a primordial lipoprotein (pre-VLDL), a triglyceride-poor form of VLDL (VLDL2) and a triglyceride-rich atherogenic form of VLDL (VLDL1; Stillemark-Billton et al. 2005).

### 1.6.4.1 The Primordial Lipoprotein

The assembly process starts when the growing apoB100 is co-translationally lipidated by MTP in the lumen of the ER (Fig. 1.4). By analyzing the content of the secretory pathway, we revealed a dense form of an apoB100-containing lipoprotein (Boström et al. 1988; Stillemark-Billton et al. 2005). This lipoprotein is not secreted from the cell but is a precursor to VLDL2 and VLDL1. We propose that it represents a partially lipidated form of apoB100; and we refer to it as a primordial lipoprotein (or pre-VLDL). It differs from the VLDL2 analog formed by apoB48 (see below), which is a mature particle that is avidly secreted from the cell (Stillemark-Billton et al. 2005).

The appearance of the apoB100 primordial lipoprotein is highly dependent on the C-terminal region of apoB100 (Stillemark-Billton et al. 2005). Moreover, the



**Fig. 1.4** The assembly and secretion of VLDL. ApoB100 is co-translationally lipidated in the ER by the transfer protein MTP to form a partially assembled primordial particle (pre-VLDL; *I*). If apoB100 is not co-translationally lipidated, it is retracted to the cytosol and sorted to proteasomal degradation (*II*). Pre-VLDL is either retained and degraded, or further lipidated to form a small triglyceride-poor form of VLDL (VLDL2; *III*). VLDL2 reaches the Golgi apparatus (*IV*) where it can either be secreted (*V*) or converted to the large triglyceride-rich form, VLDL1, by a bulk addition of triglycerides (*VI*). The cytosolic lipid droplets supply the apoB with triglycerides. The triglycerides in the droplets are thought to be hydrolyzed (*VII*) and the released fatty acids are re-esterified into new triglycerides that are added onto apoB (*VIII*)

particle is highly associated with chaperone proteins such as BiP (binding proteins) and PDI (protein disulfide isomerase). A potential explanation is that regions in the C-terminal of apoB100 sense the degree of lipidation and anchor the partially lipidated particle to chaperones, which retain the particle in the ER. Once the level of lipidation is sufficient to allow apoB100 to fit on the particle and the C-terminal to fold correctly, the chaperones dissociate and the particle can transfer out of the ER. Thus, the primordial lipoprotein is either retained in the cell and degraded (if not sufficiently lipidated) or further lipidated to form VLDL2 (Fig. 1.4; Olofsson and Asp 2005; Olofsson et al. 1999, 2000; Stillemark-Billton et al. 2005).

#### 1.6.4.2 VLDL2

VLDL2 formation is highly dependent on the size of apoB. Bona fide VLDL2 is only formed with apoB100; and truncated forms of apoB result in the formation of denser particles (Stillemark et al. 2000). Indeed, there is an inverse relation between the density of the lipoproteins formed and the size of apoB. The lipoprotein formed by apoB48 (i.e. the apoB expressed in the intestine) has the density of a high-density lipoprotein (HDL; Stillemark-Billton et al. 2005) and we propose that it is a VLDL2 analog. Both bona fide VLDL2 and VLDL2 analogs can either be secreted directly or further lipidated to form VLDL1 and secreted (Fig. 1.4).

#### 1.6.4.3 VLDL1

VLDL1 is formed from VLDL2. Thus a precursor product relationship between VLDL2 and VLDL1 is seen in pulse-chase experiments in cell cultures (Stillemark-Billton et al. 2005) and also in turnover experiments in humans (Adiels et al. 2007).

The formation of VLDL1 involves a second type of lipidation in which VLDL2 or the VLDL2 analog receives a bulk load of lipids in the Golgi apparatus (Stillemark-Billton et al. 2005). In contrast to the formation of VLDL2, apoB only needs to have a minimum size of apoB48 to allow the conversion to VLDL1 (Stillemark-Billton et al. 2005). Our recent results indicate that a sequence very close to the C-terminal end of apoB48 is required for this lipidation (Beck et al., personal communication).

The formation of VLDL1 is dependent on the GTPase ADP ribosylation factor 1 (ARF-1), a protein that is required in the anterograde transport from ERGIC to *cis*-Golgi (Asp et al. 2000). This is consistent with results showing that VLDL1 formation occurs in the Golgi apparatus (Stillemark et al. 2000) and indicates that the formation of VLDL1 requires a transfer of apoB100 from the ER to the Golgi apparatus (Fig. 1.4). Thus, one would expect a time delay of approximately 15 min between the biosynthesis of apoB100 and the major addition of lipids to form the VLDL1 particle. Indeed, in turnover studies in humans, we confirmed the presence of two steps in the assembly of VLDL1 with a 15 min difference between the secretion of newly formed apoB100 and newly formed triglycerides (Adiels et al. 2005b).

It is not known how lipids are added to VLDL2 during the formation of VLDL1, but it has been suggested that the formation of lipid droplets in the lumen of the secretory pathway plays a central role in VLDL assembly, i.e. lipid droplets fuse with apoB to form VLDL (Alexander et al. 1976). This is an interesting hypothesis as it links the formation of the core of VLDL to the process by which the core of a cytosolic lipid droplet is assembled. As discussed above, it has been proposed that the assembly of a droplet starts in the hydrophobic portion of the microsomal membrane with the formation of a triglyceride lens, which is then released to the cytosol. A triglyceride lens may also bud into the lumen of the secretory pathway, thereby giving rise to a luminal droplet that becomes the core of VLDL. This hypothesis remains to be tested experimentally (Brown 2001; Murphy and Vance 1999; Olofsson et al. 1987).

Several authors have demonstrated that fatty acids used for the biosynthesis of VLDL triglycerides are derived from triglycerides stored in cytosolic lipid droplets (Gibbons et al. 2000; Salter et al. 1998; Wiggins and Gibbons 1992) and that the enzymes involved in the release of such fatty acids have an influence on the formation of VLDL (Dolinsky et al. 2004a, b; Gilham et al. 2003; Lehner and Vance 1999; Trickett et al. 2001).

## ***1.6.5 Regulation of VLDL Assembly***

### **1.6.5.1 Insulin**

Several possibilities to explain why insulin inhibits VLDL1 formation have been summarized by Taskinen (2003). One potential reason is that insulin inhibits lipolysis in adipose tissue and thereby decreases the inflow of fatty acids to the liver. The assembly of VLDL and in particular VLDL1 is highly dependent on the triglyceride level in the hepatocytes; and thus an insulin-induced reduction in the triglyceride level results in a reduced formation of VLDL1. This is supported by the observation that the insulin-dependent inhibition of VLDL1 assembly is not working in patients with high amount of lipids in the liver (Adiels et al. 2007). Further support for an influence on the lipolysis in the adipose tissue was obtained from studies using nicotinic acid analogs (reviewed by Taskinen 2003).

### **1.6.5.2 Lipid Droplets**

Insulin is known to promote the formation of lipid droplets (Andersson et al. 2006). Thus, it is possible that insulin diverts triglycerides from the bulk lipidation of VLDL2 to the formation of lipid droplets. Indeed, we have observed other situations where promoting the formation of lipid droplets inhibits VLDL1 assembly. For example, increased levels of ADRP in the cell result in increased storage of neutral lipids in lipid droplets and reduce their entry into the assembly pathway (Magnusson et al. 2006). Such a manipulation results in decreased VLDL1 production, despite increased

levels of triglycerides in the cell. In addition, increasing the rate of fusion between droplets (e.g. by treatment with epigallocatechin gallate ) results in decreased VLDL secretion and an increased number of droplets in the liver cell (Li et al. 2005).

### **1.6.5.3 ApoB100 Degradation**

It is generally believed that the secretion of apoB is regulated post-transcriptionally by co- and post-translational degradation. It has long been known that apoB100 undergoes intracellular degradation (for reviews, see Davidson and Shelness 2000a; Olofsson et al. 1999; Shelness and Sellers 2001). The degradation is dramatically reduced when the supply of fatty acids (and the biosynthesis of triglycerides) is increased (Borén et al. 1993; Boström et al. 1986). The intracellular degradation of apoB100 occurs at three different levels (Fisher and Ginsberg 2002; Fisher et al. 2001): (i) close to the biosynthesis of apoB (co- or post-translationally) by a mechanism that involves retraction of the apoB molecule from the lumen of the ER to the cytosol (through the same channel as it entered during its biosynthesis), ubiquitination and subsequent proteasomal degradation (Fisher et al. 2001; Liang et al. 2000; Mitchell et al. 1998; Pariyarath et al. 2001), (ii) post-translationally by an unknown mechanism that seems to occur in a compartment separate from the rough ER and is referred to as post-ER presecretory proteolysis (PERPP; Fisher et al. 2001) and (iii) by reuptake from the unstirred water layer around the outside of the plasma membrane (Williams et al. 1990) via the LDL receptor. The LDL receptor has been shown to have an important role in regulation of the secretion of apoB100-containing lipoproteins (Horton et al. 1999; Twisk et al. 2000).

The intracellular degradation of apoB seems to be a consequence of a failure to form the correct particle. To avoid degradation, apoB100 needs to form pre-VLDL during translation and pre-VLDL must be converted to VLDL2 (see Sect. 1.6.4.1). Both are depending on the amount of lipids that are loaded on to apoB. The formation of VLDL1 is not necessary for apoB100 secretion but allows increased secretion of triglycerides from the liver. The importance of triglycerides for the assembly and secretion of VLDL1 is supported by our observations from turnover studies in vivo, which have demonstrated that the secretion of VLDL1 apoB100 increases with increasing concentrations of liver lipids (Adiels et al. 2005a)

## ***1.6.6 Clinical Implications of VLDL1 Production***

Insulin resistance is a major risk factor for the development of premature atherosclerosis. In part, this could be explained by the increased production of VLDL1. Overproduction of VLDL1 has a central role in the development of the atherogenic dyslipidemia of diabetes (Taskinen 2003), which is characterized by low levels of high-density lipoproteins, the appearance of small dense LDL (sdLDL) and high levels of plasma triglycerides and apoB.

A model has been proposed in which LDL, in particular sdLDL, is retained by the intercellular substance of the arterial wall (Tabas et al. 2007; Williams and Tabas 1995, 1998). Through modification, this LDL is targeted to endocytosis into macrophages via scavenger receptors (Tabas et al. 2007). This results in the accumulation of neutral lipids [primarily cholesterol esters (Mattsson et al. 1993) but also triglycerides (Mattsson et al. 1993)] within lipid droplets in these cells. Such lipid-loaded macrophages, or foam cells, are a characteristic feature of atherosclerotic lesions and are highly involved in the progression of such lesions.

## 1.7 Conclusions

Triglycerides are stored in lipid droplets, dynamic organelles equipped with a protein machinery that allows their participation in sorting and trafficking processes. Here, we have presented recent breakthroughs in our understanding of how lipid droplets are assembled. Additional studies are required to determine how and where in the cell the primordial lipid droplets are formed. Of key importance is the identification of a link between the accumulation of lipid droplets and the development of insulin resistance, but further research is required to clarify the precise mechanism and proteins involved. We propose that these investigations may identify targets that could be modulated to reduce the accumulation of lipid droplets and hence reverse the associated complications.

Triglycerides can be secreted both as milk globules and lipoproteins (chylomicrons, VLDL). We have reviewed the assembly and secretion of milk globules. Further research is required to clarify how a newly assembled milk globule interacts with the plasma membrane during the budding of the globule. We have also reviewed the assembly of VLDL and described a model for its assembly. As overproduction of VLDL1 is a key feature of insulin resistance and type 2 diabetes, it is important to further elucidate how VLDL formation is regulated in humans and to clarify the role of cytosolic lipid droplets in the formation of VLDL1.

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# Chapter 2

## Oxysterols and Oxysterol-Binding Proteins in Cellular Lipid Metabolism

Vesa M. Olkkonen

*This chapter is dedicated to my parents, Raimo and Riitta Olkkonen*

**Abstract** Oxysterols are 27-carbon oxidized derivatives of cholesterol or by-products of cholesterol synthesis with multiple biological activities. The major oxysterols arise either via cholesterol oxidation by cytochrome P450 enzymes or via non-enzymatic autoxidation processes. Oxysterols are present in healthy tissues or plasma at very low levels but are found enriched in pathological structures such as atherosclerotic lesions. Their concentrations in serum are suggested to reflect increased *in vivo* lipid peroxidation due to oxidative stress. The suggested physiologic functions of oxysterols include transcriptional control of lipid metabolism mediated by liver X receptors and sterol regulatory element binding proteins, as well as modulation of a variety of signaling and differentiation events. The recently characterized family of cytoplasmic oxysterol-binding homologues (ORP proteins) is conserved throughout the eukaryotic kingdom. These proteins are suggested to act as lipid sensors/transporters with important roles in mediating the impacts of oxysterols and possibly other signaling lipids to the machineries governing cellular lipid metabolism, vesicle transport, and signaling cascades.

### 2.1 Oxysterols, Their Synthesis and Catabolism

Oxysterols are naturally occurring 27-carbon oxidized derivatives of cholesterol or by-products of cholesterol synthesis with multiple biological activities (Björkhem and Diczfalusy 2002; Javitt 2008; Schroepfer 2000). Kandutsch, Chen, and Heiniger (1978) formulated the so-called oxysterol hypothesis of cholesterol homeostasis. This hypothesis contended that oxysterols, rather than cholesterol itself, mediate the feedback regulation of cholesterol synthesis. However, a wealth of evidence has subsequently accumulated that cholesterol plays a pivotal role in its own feedback regulation (reviewed by Gill et al. 2008). In addition to the proposed role in feedback

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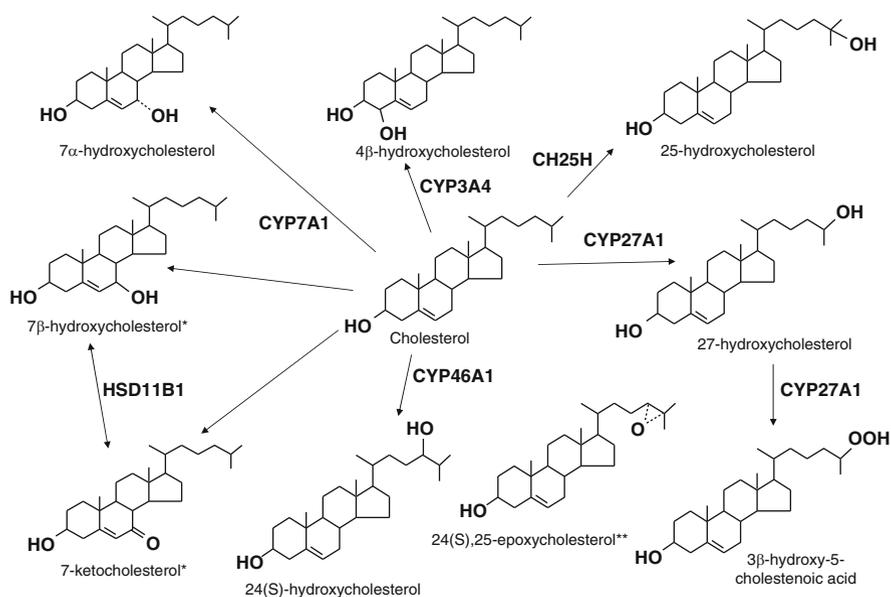
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regulation of cholesterol metabolism, oxysterols have been extensively studied in the context of the oxidative hypothesis of atherosclerosis (Steinberg et al. 1989). Although these sterols are detected in healthy human or animal tissues or plasma at very low concentrations as compared to cholesterol, they are enriched in low-density lipoprotein (LDL) subfractions associated with increased cardiovascular disease risk, macrophage foam cells, and atherosclerotic lesions, evoking the idea that they might play a role in the pathology of atherosclerosis. However, normal physiologic functions of these compounds have more recently emerged. Oxysterols act as ligands of liver X receptors (LXR; Tontonoz and Mangelsdorf 2003) and regulate the intracellular transport and processing of sterol regulatory element binding proteins (SREBP; Goldstein et al. 2006). Thereby these compounds have the potential to act as endogenous regulators of gene expression in lipid metabolism. Furthermore, new intracellular receptors for oxysterols have been identified and novel functions of oxysterols in cell signaling discovered, enhancing the interest in these compounds.

### ***2.1.1 Oxysterols that Arise Through Enzymatic Cholesterol Oxidation***

The most common modifications of cholesterol that occur in oxysterols are hydroxyl, keto, hydroperoxy, or epoxy moieties (Fig. 2.1). Oxysterols are markedly more hydrophilic and therefore more mobile within cells than cholesterol, but they do incorporate into the cellular membranes (Lange et al. 1995; Morel et al. 1996; Theunissen et al. 1986). A number of the most abundant oxysterols are generated in cells as products of enzyme-mediated oxidative processes (Fig. 2.1). The quantitatively most important of these are 27-, 24(S)-, and 7 $\alpha$ -hydroxycholesterol (OHC; for nomenclature see Table 2.1), which arise in reactions catalyzed by mitochondrial or endoplasmic reticulum cholesterol hydroxylases belonging to the cytochrome P450 family (Luoma 2007; Russell 2000).

27-OHC [also called, more appropriately, 25(R),26-hydroxycholesterol] has an important function in hepatocytes: It serves as an intermediate in the alternative, so-called acid pathway of bile acid biosynthesis (Björkhem and Eggertsen 2001). However, other cell types including macrophages and endothelial cells are also capable of converting cholesterol to 27-OHC, a process that provides one means of removing excess cellular cholesterol (Babiker et al. 1997). This oxysterol is generated by the mitochondrial sterol-27-hydroxylase encoded by *CYP27A1*, genetic defects of which, found in a disease called cerebrotendinous xanthomatosis, result in tissue xanthomas, deposits containing both cholesterol and cholestanol, and an increased risk of premature atherosclerosis (Björkhem et al. 1994; Panzenboeck et al. 2007). Sterol-27-hydroxylase is also capable of converting the primary product 27-OHC further into 3 $\beta$ -hydroxy-5-cholestenoic acid (Fig. 2.1). 27-OHC is a potent suppressor of cholesterol biosynthesis: Synthesis of this oxysterol was



**Fig. 2.1** Structure and origin of some common oxysterols. Most of the oxysterols displayed are generated via cholesterol oxidation by enzymes that belong to the cytochrome P450 family (CYP). *CH25H*, cholesterol 25-hydroxylase, is a di-iron enzyme; *HSD11B1* stands for 11 $\beta$ -hydroxysteroid dehydrogenase type 1; *7-ketocholesterol\** and *7 $\beta$ -hydroxycholesterol\** arise through non-enzymatic oxidation of cholesterol and *24(S),25-epoxycholesterol\*\** from a shunt of the cholesterol biosynthetic pathway (courtesy of Kati Hyvärinen)

**Table 2.1** Nomenclature and abbreviations of the oxysterols discussed in this chapter

Abbreviation	Common name	Systematic name
$\alpha$ -EPOX	5 $\alpha$ ,6 $\alpha$ -Epoxycholesterol	Cholestan-5 $\alpha$ ,6 $\alpha$ -epoxy-3 $\beta$ -ol
$\beta$ -EPOX	5 $\beta$ ,6 $\beta$ -Epoxycholesterol	Cholestan-5 $\beta$ ,6 $\beta$ -epoxy-3 $\beta$ -ol
4 $\beta$ -OHC	4 $\beta$ -Hydroxycholesterol	Cholest-5-en-3 $\beta$ ,4 $\beta$ -diol
7 $\alpha$ -OHC	7 $\alpha$ -Hydroxycholesterol	Cholest-5-en-3 $\beta$ ,7 $\alpha$ -diol
7 $\beta$ -OHC	7 $\beta$ -Hydroxycholesterol	Cholest-5-en-3 $\beta$ ,7 $\beta$ -diol
7-KC	7-Ketocholesterol	Cholest-5-en-3 $\beta$ -ol-7-one
	7-Hydroperoxycholesterol	7 $\beta$ -Hydroperoxycholest-5-en-3 $\beta$ -ol
20(R)-OHC	20(R)-Hydroxycholesterol	(20R)-Cholest-5-en-3 $\beta$ ,20-diol
20(S)-OHC	20(S)-Hydroxycholesterol	(20S)-Cholest-5-en-3 $\beta$ ,20-diol
22(R)-OHC	22(R)-Hydroxycholesterol	(22R)-Cholest-5-en-3 $\beta$ ,22-diol
24(S)-OHC	24(S)-Hydroxycholesterol	(24S)-Cholest-5-en-3 $\beta$ ,24-diol
24(S),25-EPOX	24(S),25-Epoxycholesterol	(24S,25)-Epoxycholest-5-en-3 $\beta$ -ol
25-OHC	25-Hydroxycholesterol	Cholest-5-en-3 $\beta$ ,25-diol
27-OHC	27-Hydroxycholesterol	(25R)-Cholest-5-en-3 $\beta$ ,26-diol
	3 $\beta$ -Hydroxy-5-cholestenoic acid	3 $\beta$ -Hydroxy-5-cholesten-25(R)-26-carboxylic acid
		Cholestan-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol

shown to be required for the rapid inactivation of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase upon cellular cholesterol loading (Lange et al. 2008). Furthermore, both 27-OHC and cholestenic acid can act as ligands of the liver X receptors (see Sect. 2.2.3.1).

Sterol-27-hydroxylase is also able to use also 7- and 8-dehydrocholesterol as its substrates, generating their 27-hydroxymetabolites capable of liganding the LXRs (Wassif et al. 2003). These metabolites were found in vivo in the serum of patients suffering of Smith-Lemli-Opitz syndrome (Yu and Patel 2005) caused by genetic defects of  $3\beta$ -hydroxysterol- $\Delta 7$  reductase (*DHCR7*). *CYP27A1* was also found to act in vitro on other intermediates of the cholesterol biosynthetic process, lanosterol, zymosterol, and desmosterol, bringing up the idea that in vivo there may be a large family of previously unstudied 27-hydroxylase products of cholesterol precursor forms, termed oxysteroids (Javitt 2004).

Cholesterol-24-hydroxylase (encoded by *CYP46A1*) is almost exclusively expressed in neurons of the central nervous system (CNS), and the product of the enzyme, 24(S)-OHC, is transferred into the circulation. This process has been suggested to play an important role in the maintenance of the CNS sterol balance (Björkhem et al. 1997; Lütjohann et al. 1996). Increased plasma levels of 24(S)-OHC are associated with Alzheimer's disease (AD) and non-Alzheimer dementia, but the concentration of the sterol falls below control levels at the late stages of AD, most likely due to extensive neuron loss (Bretillon et al. 2000; Kolsch et al. 2003; Lütjohann et al. 2000). Furthermore, changes in plasma 24(S)-OHC levels are found in patients with multiple sclerosis (Leoni et al. 2002). Serum 24(S)-OHC concentration apparently reflects the rate of cholesterol turnover in the brain, and it has been suggested as a new diagnostic indicator of diseases affecting the CNS.

Hepatic cholesterol-7 $\alpha$ -hydroxylase (encoded by *CYP7A1*) carries out the initial enzymatic step in the major, so-called neutral pathway of bile acid synthesis (reviewed by Björkhem and Eggertsen 2001). 7 $\alpha$ -OHC is thus produced in relatively large quantities in the liver and part of it leaks into the circulation, where it is one of the most abundant oxysterols. 25-OHC has been extensively used for in vitro studies of cellular sterol metabolism, due to its potent inhibitory effect on cholesterol biosynthesis and LDL receptor expression (for a review, see Brown and Jessup 1999), as well as the fact that it is readily available commercially. 25-OHC is present in mammalian tissues or plasma at very low quantities, but has been suggested to have important tissue-specific functions (Chen et al. 2002). 25-OHC can arise via cholesterol autoxidation but it is also generated enzymatically by a di-iron enzyme, cholesterol-25-hydroxylase (CH25H; Lund et al. 1998). Furthermore, also *CYP27A1* is capable of generating this oxysterol (Lund et al. 1993). 22(R)-OHC and 20(R)-OHC are formed in steroidogenic tissues as intermediates in the synthesis of steroid hormones (Sugano et al. 1996). A further endogenous enzymatically formed oxysterol important for the control of cellular sterol homeostasis is 24(S),25-epoxycholesterol generated by a shunt of the cholesterol biosynthetic process by the same enzymes that catalyze the synthesis of cholesterol (see Sect. 2.2.3.3; Nelson et al. 1981; Rowe et al. 2003).

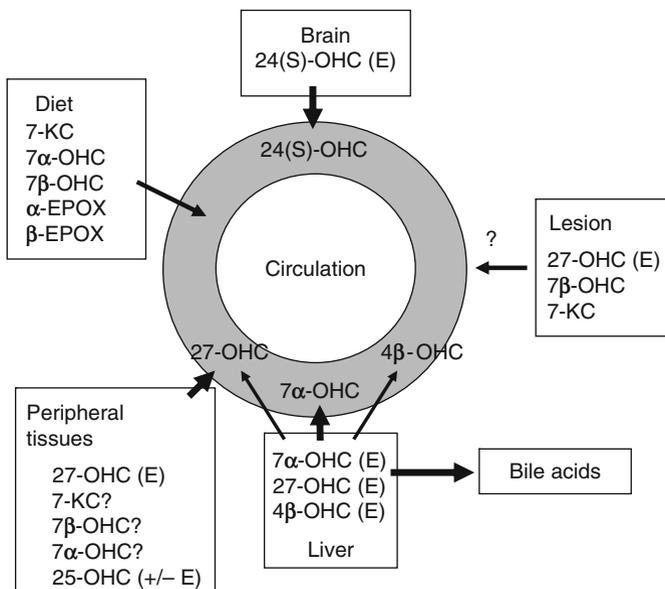
### ***2.1.2 Oxysterols Generated via Non-Enzymatic Oxidative Events***

Certain oxysterols are generated in non-enzymatic, lipid peroxide or free radical mediated processes, often termed cholesterol autoxidation. It is somewhat unclear to what extent these compounds arise within our body and how much of them are obtained via nutrition. Approximately 1% of the sterol present in the western diet is estimated to be in oxidised form (van de Bovenkamp et al. 1988). The most common oxysterols found in food are 7-ketocholesterol (7-KC), 7 $\alpha$ -OHC, 7 $\beta$ -OHC, and cholesterol-5 $\alpha$ ,6 $\alpha$ - and -5 $\beta$ ,6 $\beta$ -epoxides ( $\alpha$ - and  $\beta$ -EPOX). Sterols modified in the 7-position, as well as some of the side-chain hydroxylated oxysterols, are also suggested to arise in vivo as a result of non-enzymatic oxidation events (for a review, see Brown and Jessup 1999).

### ***2.1.3 Oxysterols in the Circulation***

Cholesterol autoxidation that occurs readily during sample preparation has formed a major problem in the analysis of plasma or tissue oxysterol concentrations, resulting in substantial variation in the reported oxysterol concentrations of human plasma (Björkhem 1986; Smith et al. 1981). The most abundant oxysterol in plasma is 27-OHC, the concentration of which is approximately 80  $\mu\text{mol/mol}$  cholesterol. The next in abundance are 24(S)-OHC, 4 $\beta$ -OHC and 7 $\alpha$ -OHC, followed by  $\beta$ -EPOX and cholestan-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol (reviewed by Brown and Jessup 1999). The concentration of 4 $\beta$ -OHC is markedly increased in the serum of patients treated with certain antiepileptic drugs, due to induction of the drug metabolizing enzyme cytochrome P450 CYP3A4, which converts cholesterol to this oxidized derivative (Bodin et al. 2002; Fig. 2.1). 4 $\beta$ -OHC is markedly stable, since it is a poor substrate for the hydroxylases that further modify oxysterols thus routing them to the bile acid biosynthetic pathways. Of the most abundant oxysterols in plasma (apart from 7-KC and the 5,6-epoxides, which are esterified to a lower extent, and 3 $\beta$ -hydroxy-5-cholestanic acid that is entirely transported in the lipoprotein-free fraction), 70–90% are found as fatty acyl esters associated with lipoproteins (Brown and Jessup 1999; Dzeletovic et al. 1995). Concerning atherosclerosis, it is interesting that increased oxysterol concentrations have been detected in low-density lipoprotein (LDL) subfractions associated with an increased risk of coronary artery disease (CAD). The oxysterol levels in the desialylated LDL subfraction of CAD patients and in circulating LDL immuno-complexes were reported to be 2- to 3-fold higher than in the normally sialylated LDL (Tertov et al. 1992, 1996). Further, oxysterols were found approximately 4-fold enriched in electronegative LDL (LDL<sup>-</sup>) as compared to normal LDL, corresponding to approximately 5% of the total amount of sterol in the LDL<sup>-</sup> (Chang et al. 1997; Sevanian et al. 1997). The origin of the most abundant oxysterols in human circulation is summarized in Fig. 2.2.

Can oxysterols in human serum be used as an indicator CVD risk, or could they even represent an actual risk factor? Salonen et al. (1997) reported that an



**Fig. 2.2** Sources of the most common oxysterols found in human circulation and tissues. Enzymatic origin is indicated with (E). Adapted with permission from Taylor & Francis Healthsciences (Olkkonen and Lehto 2004)

increased plasma concentration of  $7\beta$ -OHC was the strongest predictor for thickening of carotid artery walls in an analysis of more than 30 variables. Zieden et al. (1999) compared established and possible new risk factors between Swedish and Lithuanian 50-year-old men. The latter had a 4-fold higher risk of coronary death and displayed significantly higher plasma levels of  $7\beta$ -OHC than the Swedes, while the levels of the established risk factors did not differ significantly between the study groups. Zhou et al. (2000) reported significantly higher plasma concentrations of several oxysterols in a group of patients with angina and 80% stenosis in their coronary arteries than in age- and sex-matched controls. Yasunobu et al. (2001) studied the concentrations of auto-antibodies against oxidized (Ox)LDL and the serum oxysterol content in patients subjected to coronary angiography. When angiographically normal subjects were compared with the stenotic group, both the OxLDL antibodies and the levels of 25-OHC, 27-OHC, and  $7\beta$ -OHC were significantly higher in the stenotic group. Rimner et al. (2005) reported parallel findings: Plasma 7-KC,  $\beta$ -EPOX, and  $7\beta$ -OHC were found to be significantly increased in patients with stable coronary artery disease as compared to a control group. Familial combined hyperlipidemia (FCH) is the most common inherited disorder of lipid metabolism, characterized by reduced plasma high-density lipoprotein (HDL) cholesterol, elevated apolipoprotein B concentrations, and a preponderance of small dense LDL subfractions. FCH is associated with increased cardiovascular risk, perhaps due to oxidative damage to lipids accumulating in the plasma of the subjects. Arca et al. (2007)

recently reported increased levels of 7-KC and 7 $\beta$ -OHC in the plasma of FCH patients that persisted after correction for hyperlipidemia and were independent of the presence of clinical atherosclerosis. Furthermore, increased serum 7-KC levels were reported in patients with type 2 diabetes mellitus, associated especially with a group with multiple coronary risk factors (Endo et al. 2008), and elevated oxysterol levels were found in end-stage renal disease patients (Siems et al. 2005). These and a number of other investigations suggest that the serum concentrations of certain oxysterols can be used as indicators of increased in vivo lipid peroxidation and thus of oxidative stress.

### 2.1.4 Catabolism of Oxysterols

A majority of the oxysterols in plasma are associated with lipoproteins and internalized by cells via various cell surface lipoprotein receptors. The bulk of this internalization occurs in the liver, an organ that excretes sterols as such or in the form of bile acids. Thus, the liver is the major site of oxysterol clearance. In addition to *CYP7A1*, hepatocytes express oxysterol-7 $\alpha$ -hydroxylase (encoded by *CYP7B1*), which carries out 7 $\alpha$ -hydroxyl modification of both exogenous 27-OHC and that produced by hepatic CYP27A1, thus routing it to the bile acid synthetic pathway (Rose et al. 1997). 27-OHC and 24(S)-OHC are also to some extent 7 $\alpha$ -hydroxylated by CYP7A1 (Norlin et al. 2000a, b) in reactions that are, however, inefficient. An additional cytochrome P450 species, CYP39, carries out more efficiently the 7 $\alpha$ -hydroxylation of 24(S)-OHC (Li-Hawkins et al. 2000). In the bile acid synthetic pathway, 3 $\beta$ -hydroxy- $\Delta^5$  steroids undergo oxidation and isomerization to produce the corresponding 3-oxo- $\Delta^4$  steroids, followed by saturation of the double bond formed in the A-ring and reduction of the oxo-group into a 3 $\alpha$ -hydroxy group. Degradation of the steroid side-chain is initiated by the 27-hydroxylation (see above) either early or late in the process. Synthesis of the primary bile acid cholic acid requires a further 12 $\alpha$ -hydroxylation carried out by another P450 enzyme CYP8B1 (for a review, see Björkhem and Eggertsen 2001).

The central non-enzymatically formed oxysterol, 7-ketocholesterol, can be hydroxylated by CYP27A1 to form 27-hydroxy-7-ketocholesterol, which is further metabolized into aqueous-soluble products, apparently bile acids (Brown et al. 2000b; Lyons and Brown 2001b). On the other hand, 7-KC is reduced to 7 $\beta$ -OHC by hepatic 11 $\beta$ -hydroxysteroid dehydrogenase type 1, HSD11B1 (Hult et al. 2004; Schweizer et al. 2004; Fig. 2.1). This enzyme shows species-specific differences in its function: in human and rat it interconverts 7-KC and 7 $\beta$ -OHC, while substrates of the hamster enzyme also include 7 $\alpha$ -OHC (Schweizer et al. 2004). The interconversion processes enable rapid hepatic catabolism of 7-KC into aqueous-soluble bile acids, and provide an obvious explanation to the findings that 7-KC added exogenously into the circulation is rapidly metabolized and does not accumulate in the vascular walls (Lyons and Brown 2001a; Lyons et al. 1999). In addition, oxysterols such as 7-KC and 25-OHC are substrates for the steroid/sterol sulfotransferase

SULT2B1b, which modifies them at the 3-position and apparently has a detoxifying function (Fuda et al. 2007; Li et al. 2007).

## 2.2 Biological Activities of Oxysterols

### 2.2.1 *Effects of Oxysterol Administration on Cells in Vitro*

In a number of studies, the effects of OxLDL administration on cultured cells have been at least in part attributed to the biological activities of oxysterols present in these oxidatively modified lipoproteins. The most common approach of generating OxLDL employs  $\text{Cu}^{2+}$  ions. This method of LDL modification results primarily in the formation of oxysterols derivatised in the 7-position, the most abundant of which is 7-KC (Brown and Jessup 1999).

Treatment of cultured macrophages with OxLDL leads to foam cell formation and a marked intracellular sterol accumulation. In such experiments the amount of oxysterols may account for even 50% of the total sterol content of the cells. Macrophages loaded with OxLDL or ones specifically enriched with 7-KC display impaired ability to efflux cholesterol to apolipoprotein A-I (Gelissen et al. 1996; Kritharides et al. 1995). Why is this? Cholesterol and oxysterols esterified with the oxidized fatty acids of OxLDL tend to accumulate within the lysosomes of the macrophages (Brown et al. 2000a). This type of sterol esters may also in vivo form lipid deposits that are difficult to remove from cells and may facilitate the development of atherosclerotic lesions. Furthermore, the oxysterols present in OxLDL, mainly 7-KC, 7 $\beta$ -OHC and  $\beta$ -EPOX, have cytotoxic properties and induce apoptotic death of several cell types, including those relevant for atherogenesis, monocytes/macrophages, endothelial cells, and smooth muscle cells (Larsson et al. 2006; Lemaire-Ewing et al. 2005; Lordan et al. 2007; Luthra et al. 2007; Ohtsuka et al. 2006; Rimner et al. 2005). The extremely unstable and cytotoxic 7-hydroperoxycholesterol arises as an intermediate in the oxidation reactions targeting the sterol 7-position, and is apparently an important cytotoxic compound in OxLDL (Chisolm et al. 1994). Interestingly, ATP-binding cassette transporter G1 (ABCG1) is capable of protecting macrophages and other cell types from apoptosis induced by oxidized LDL, 7-KC or 7 $\beta$ -OHC, apparently by promoting the efflux of oxysterols to HDL particles (Engel et al. 2007; Terasaka et al. 2007). Other oxysterols such as 25-OHC can also be removed from cells by ABCA1 (Tam et al. 2006, Terasaka et al. 2007).

Several oxysterols have been reported to impair the selective barrier function of endothelial cells (Boissonneault et al. 1991) and to inhibit nitric oxide (NO) synthesis, thus having an adverse effect on vasodilatation (Deckert et al. 1998; Millanvoeye-Van Brussel et al. 2004). Oxysterols (mainly 7 $\beta$ -OHC, 25-OHC, 7-KC) induce the expression and secretion of proinflammatory cytokines/chemokines such as interleukins 6 and 8 and monocyte chemoattractant protein-1 (Erridge et al. 2007; Knutsen Rydberg et al. 2003; Lemaire-Ewing et al. 2005, 2008; Leonarduzzi et al. 2005; Sung et al. 2008).

Furthermore, oxysterols have the capacity to impact cell differentiation: As examples, 7-KC induces monocyte differentiation into macrophages and further into foam cells (Hayden et al. 2002), 22(S)-OHC, and 20(S)-OHC enhance stem cell differentiation into osteoblasts in vitro and bone healing in vivo (Aghaloo et al. 2007; Richardson et al. 2007) while they inhibit the adipogenic differentiation of the stem cells (Kha et al. 2004; Kim et al. 2007), and 25-OHC modulates the differentiation of Leydig cells (Chen et al. 2002).

The findings based on the use of either OxLDL or pure oxysterol species on cultured cells underscore the potency of oxysterols as effector molecules impacting multiple biologic processes. Many of the effects are obviously harmful in terms of cardiovascular status. One must, however, keep in mind that the adverse effects of oxysterols have often been studied in vitro at pharmacologic concentrations. Furthermore, the studies have in most cases been carried out using single, pure oxysterol species. When applied as mixtures similar to those found in vivo, together with cholesterol or as fatty acyl esters, the adverse cellular effects of oxysterols are markedly alleviated (Biasi et al. 2004; Hietter et al. 1984; Leonarduzzi et al. 2004; Monier et al. 2003). However, certain oxysterol combinations may also have synergistic toxic effects (Larsson et al. 2006).

### ***2.2.2 Oxysterols in Atherosclerotic Lesions***

The concentrations of oxysterols in atherosclerotic lesions in both humans and in animal models are typically two orders of magnitude higher than in plasma; The lesion oxysterols are almost completely in esterified form. Several studies demonstrate that 27-OHC is the most abundant oxysterol in human lesions. Its concentration correlates with the cholesterol content of the lesions and the severity of atherosclerosis (Brown and Jessup 1999; Carpenter et al. 1993, 1995; Garcia-Cruset et al. 1999, 2001; Vaya et al. 2001). This may reflect activation of the mitochondrial sterol-27-hydroxylase in cells within the lesion as an attempt to solubilize and remove the accumulating sterol excess. Also, 7 $\beta$ -OHC and 7-KC, which arise through non-enzymatic oxidation processes, are abundant in lesions. The portion of these oxysterols is especially high in fatty streaks, perhaps reflecting a strong oxidative activity in lesions at this stage, or selective accumulation of the 7-modified oxysterols in macrophage foam cells, which are the principal component of fatty streaks (Maor et al. 2000; Mattsson-Hulten et al. 1996). The observations on the deposition of oxysterols in lesions, together with the documented cytotoxic and pro-apoptotic effects of these compounds, as well as their impact on macrophage differentiation (see Sect. 2.2.1), have made researchers believe that they may have pathophysiological importance in the development of atherosclerotic lesions: They may inflict endothelial injury, promote formation of the necrotic core of atheroma and vascular calcification, and possibly facilitate plaque rupture (Colles et al. 2001; Guyton et al. 1990; Li et al. 2001; Marchant et al. 1996; Saito et al. 2008; Sevanian et al. 1995).

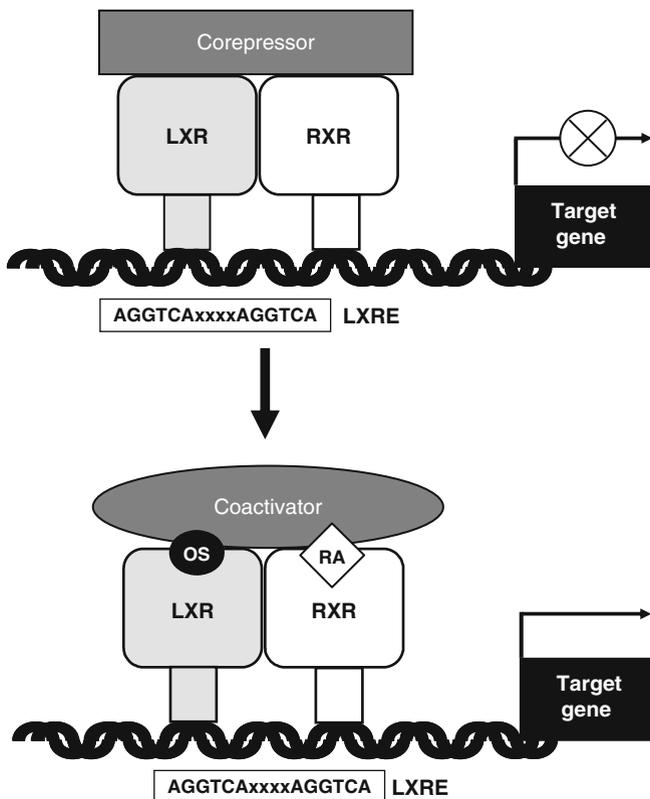
## 2.2.3 Oxysterols as Regulators of Cellular Lipid Metabolism

### 2.2.3.1 Oxysterols Act as Liver X Receptor Agonists

The interest of the scientific community towards oxysterols has greatly intensified since the discovery that they act as ligands of the nuclear receptor proteins designated Liver X receptor- $\alpha$  (LXR $\alpha$ ) and LXR $\beta$  (also known as NR1H3 and NR1H2, respectively; Apfel et al. 1994; Willy et al. 1995). While LXR $\beta$  is expressed at relatively even levels in all tissues, LXR $\alpha$  is expressed at high levels in the liver and less abundantly in the adrenal glands, intestine, adipose tissue, macrophages, lung, and kidney (Repa and Mangelsdorf 2000). Oxysterols at physiological concentrations were found to bind to and activate the LXRs in vitro (Forman et al. 1997; Janowski et al. 1996; Lehmann et al. 1997). There has been controversy as to whether naturally occurring oxysterol agonists such as 22(R)-OHC, 24(S),25-EPOX, 24(S)-OHC, 27-OHC, and 3 $\beta$ -hydroxy-5-cholestenoic acid execute the same LXR activating function in vivo as they do in vitro (Björkhem and Diezfelusy 2002). Recent investigations employing genetic manipulation of oxysterol metabolism have shed light on this question, supporting the notion that oxysterols indeed act as endogenous LXR agonists in vivo (Chen et al. 2007).

The LXRs bind to the promoters of specific target genes as heterodimers with the retinoid X receptor (RXR; NR2B1) and impact gene expression via recruitment of transcriptional coactivators or corepressors (Fig. 2.3). The binding is of the permissive type, i.e., ligand binding to either LXR or RXR is sufficient to activate target gene transcription. The sequence elements recognized by the LXRs are direct repeat four (DR4) motifs, termed LXR responsive elements (LXREs; Repa and Mangelsdorf 2000; Tontonoz and Mangelsdorf 2003). Genes regulated by the LXRs are involved in sterol absorption in the intestine, the reverse cholesterol transport process, bile acid synthesis, biliary neutral sterol secretion, hepatic lipogenesis, and synthesis of nascent high-density lipoproteins (Li and Glass 2004; Tontonoz and Mangelsdorf 2003). In addition, new functions have recently assigned to the LXRs: LXR $\alpha$  was reported to silence cholesterol biosynthesis by direct suppression of two genes encoding cholesterologenic enzymes (Wang et al. 2008b). The most extensively studied LXR target genes are those encoding ATP-binding cassette transporters A1 (ABCA1; Costet et al. 2000; Repa et al. 2000b) and G1 (ABCG1; Kennedy et al. 2001; Sabol et al. 2005; Venkateswaran et al. 2000), which mediate the efflux of cholesterol from macrophages to lipid-poor apolipoprotein A1 and to spherical HDL particles, respectively. The lipidation of apolipoprotein A1 by ABCA1 is crucial for the formation of nascent high-density lipoproteins (for a review, see Oram and Vaughan 2006). A recent report suggests that, in addition to control of *ABCA1* transcription, LXR $\beta$ /RXR dimers bind directly to the ABCA1 protein, regulating its activity in an oxysterol-dependent manner (Hozoji et al. 2008). In general, the LXRs are regarded antiatherogenic due their central role as regulators of genes involved in the reverse cholesterol transport pathway. Importantly, the stimulation of the LXRs is also reported to inhibit macrophage

inflammatory responses, another mechanism that alleviates atherogenesis (Castrillo et al. 2003; Joseph et al. 2003; Zelcer and Tontonoz 2006). Further, Bensinger et al. (2008) reported that ligation of LXR during T-cell activation inhibits mitogen-driven expansion, while loss of LXR $\beta$  conferred the cells proliferative advantage. In contrast to the potentially atheroprotective properties of the LXRs, they also activate genes responsible for lipogenesis, such as those encoding sterol regulatory element binding protein 1c (SREBP-1c; Repa et al. 2000a; Yoshikawa et al. 2001) and fatty acyl synthetase (FAS; Joseph et al. 2002). Therefore, LXR stimulation by pharmacologic agonists increases plasma triglycerides representing a cardiovascular risk factor. Development of LXR agonists that selectively activate reverse cholesterol transport in the absence of effects on lipogenesis is therefore an important goal of the pharmaceutical industry.



**Fig. 2.3** Function of liver X receptors (*LXR*). Within the nucleus, LXR–retinoid X receptor (*RXR*) heterodimers are bound to LXR response elements (*LXRE*) in the promoters of target genes. In the absence of LXR or RXR ligands, the complex recruits corepressors that silence gene expression. When ligands of either LXR (oxysterols, *OS*) or RXR (retinoic acid or its derivatives, *RA*) or both are bound, the co-repressor complexes are exchanged for co-activator complexes and target gene transcription is induced

### 2.2.3.2 Oxysterols Regulate the Maturation of Sterol Regulatory Element Binding Proteins

Cholesterol biosynthesis and cellular uptake, as well as fatty acid biosynthesis, are controlled by transcription factors named sterol regulatory element binding proteins (SREBPs), and their cholesterol-sensing accessory factor, the SREBP cleavage activating protein (SCAP)(Eberle et al. 2004; Goldstein et al. 2006). Of the three SREBP isoforms produced from two genes, SREBP-1c is particularly abundant in the liver where its expression is regulated by insulin and glucagon, and it plays a major role in controlling hepatic lipogenesis and glucose utilization. SREBP-2, also expressed at relatively high levels in the liver, is predominantly responsible for control of cholesterol metabolism. The third family member, SREBP-1a, is mainly expressed in tissues with a high proliferative capacity, and functions in both cholesterol and triglyceride metabolism. The SREBPs are synthesized as precursors anchored to ER membranes and form complexes with SCAP. When the cellular cholesterol level is low, SREBP-SCAP complexes move to the Golgi complex, where SREBPs undergo a two-step proteolytic processing to release the N-terminal fragment, a basic helix–loop–helix leucine zipper transcription factor. These fragments enter the nucleus and bind to sterol regulatory elements (SRE) in the promoter regions of a number of genes whose products mediate the synthesis of cholesterol and fatty acids. When sterol builds up in cells, SCAP senses cholesterol in the ER membranes and interacts with the Insig (*Insulin-induced gene*) proteins. As a result the SREBP-SCAP complex is retained in the ER (Yabe et al. 2002; Yang et al. 2002). The SREBP machinery is sensitive to both cholesterol and oxysterols, of which 25-OHC is most commonly used in experimental set-ups. While SCAP does not bind 25-OHC (Adams et al. 2004; Brown et al. 2002), the Insig proteins were recently found to directly bind this oxysterol and to mediate the regulatory effect of 25-OHC on SREBP processing. Upon oxysterol binding, the Insigs apparently undergo a conformational change that increases their affinity for SCAP, resulting in an inhibition of ER to Golgi transport of SCAP–SREBP complexes and SREBP activation (Radhakrishnan et al. 2007; Sun et al. 2007).

### 2.2.3.3 24(S),25-Epoxycholesterol, a Signal for Sterol Biosynthetic Pathway Activity

Unlike most oxysterols, 24(S),25-epoxycholesterol [24(S),25-EPOX] is not derived from preformed cholesterol but is generated as a side product of the cholesterol biosynthetic process by the same enzymes that catalyze the synthesis of cholesterol (Nelson et al. 1981; Rowe et al. 2003). This compound is a potent feedback regulator of cholesterol biosynthesis, suppressing SREBP-2 processing (Janowski et al. 2001) and repressing the cellular HMGCoA reductase activity (Mark et al. 1996). Furthermore, it is the most potent oxysterol activator of the LXRs (Chen et al. 2007; Janowski et al. 1999; Lehmann et al. 1997; Wong et al. 2004), and its role in cellular sterol homeostatic regulation has in the past few years been substantially

elucidated. The cellular level of 24(S),25-EPOX can be manipulated with two methods: (i) by using inhibitors of the cholesterol biosynthetic pathway enzyme 2,3-oxidosqualene cyclase (OSC), resulting in elevation of cellular 24(S),25-EPOX levels, or (ii) by overexpressing this enzyme, which reduces the cellular content of 24(S),25-EPOX. With these tools and other approaches the groups of A. Brown and M. Huff have come up with a number of novel and highly important findings: Wong et al. (2004) showed that the treatment of macrophages with statins, inhibitors of HMGCoA reductase and thus of cholesterol synthesis, results in reduced expression of LXR target genes due to concomitant reduction in the synthesis of 24(S),25-EPOX. They also demonstrated that the synthesis of 24(S),25-EPOX parallels that of cholesterol and acts as a signal that fine-tunes the acute control of cellular cholesterol homeostasis and protects cells against accumulation of newly synthesized cholesterol. This occurs via its regulatory impact on both the LXRs which control cholesterol efflux from cells and the SREBP machinery responsible for cellular cholesterol biosynthesis and uptake (Wong et al. 2007a, 2008). Beyea et al. (2007) demonstrated that partial inhibition of OSC in THP-1 macrophages reduced cholesterol synthesis and increased the expression of several LXR target genes, *ABCA1*, *ABCG1*, and *APOE*. Importantly, OSC inhibition did not stimulate lipoprotein lipase (*LPL*) or fatty acid synthase (*FAS*), and the observed induction of the lipogenic transcription factor SREBP-1c was counteracted by a block in its conversion to the active nuclear form. Rowe et al. (2003) suggested that induction of LXRs by partial inhibition of OSC could be used in cardiovascular therapy. However, this idea is undermined by the recent finding that, even though 24(S),25-EPOX facilitates *ABCA1* and *ABCG1* expression and cholesterol efflux from normal macrophages in culture, it strongly inhibits cholesterol efflux from macrophage foam cells, possibly due to inhibition of cholesterol ester hydrolase function (Ouimet et al. 2008).

24(S),25-EPOX also seems to have a specific role in CNS sterol homeostasis: It was shown to be produced by astrocytes in vitro and to be taken up by neurons in which it exerted downstream effects on gene regulation (Wong et al. 2007b). The authors presented the interesting hypothesis that 24(S),25-EPOX acts as a signal from astrocytes that reduces the energetically costly cholesterol biosynthetic capacity of neurons, enabling the neurons to divert resources into other cellular processes.

As a conclusion, the endogenous cellular oxysterols can be regarded as signaling molecules employed as indicators of the cellular sterol status, which, via their effects on the LXR and SREBP machineries of sterol homeostatic control, prevent cellular sterol accumulation and have a potentially atheroprotective impact.

#### **2.2.3.4 27-Hydroxycholesterol, a Selective Estrogen Receptor Modulator**

Estrogen receptors  $\alpha$  (NR3A1) and  $\beta$  (NR3A2) are nuclear receptors that, in addition to reproductive functions, mediate estrogen regulation of a number of other physiologic processes (Deroo and Korach 2006). One potentially important target of estrogen receptors is the cardiovascular system, where the action of estrogens is believed to be beneficial and to explain in part the fact that women in reproductive

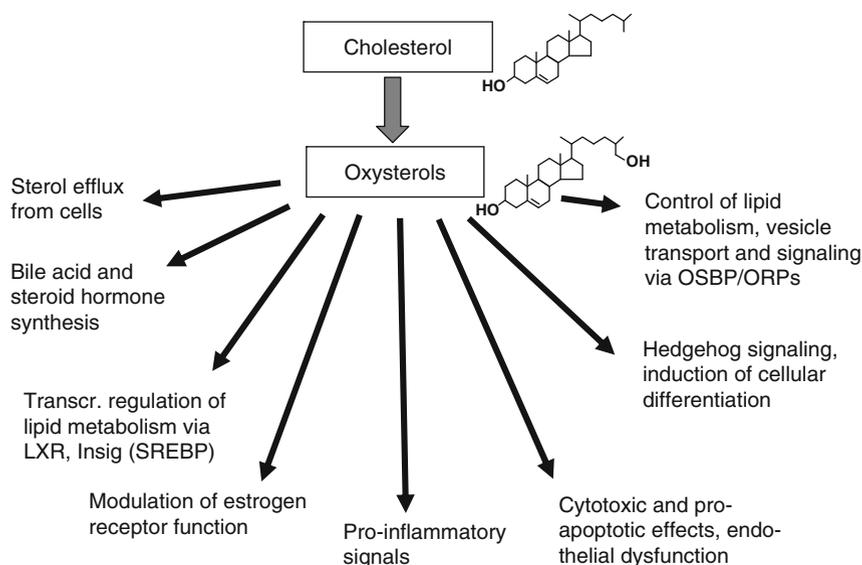
age are markedly protected from cardiovascular diseases as compared to men. It was recently reported that 27-OHC, which accumulates in atherosclerotic lesions (see Sect. 2.2.2), antagonized the estrogen-dependent production of nitric oxide by vascular cells, resulting in reduced vasorelaxation of rat aorta, a potentially deleterious effect (Umetani et al. 2007). Furthermore, increasing 27-OHC levels repressed carotid artery re-endothelialization. Interestingly, 27-OHC also had cell type-specific proestrogenic actions (DuSell et al. 2008; Umetani et al. 2007), indicating that it acts as an endogenous selective estrogen receptor modulator (SERM). The above findings suggest that 27-OHC may contribute to the loss of estrogen protection from vascular disease.

### **2.2.3.5 The Niemann–Pick C1 Protein Binds both Cholesterol and Oxysterols**

Niemann–Pick C (NPC) disease is an inborn error of metabolism characterized by accumulation of free cholesterol and sphingolipids within late endocytic compartments of cells (Sturley et al. 2004). The disease is caused by mutations in either of two proteins, NPC1 or NPC2. Of these, NPC1 is a large multi-spanning membrane protein localized in late endosomes. NPC1 has a consensus cholesterol binding motif consisting of five trans-membrane helices, but the mechanism by which NPC1 facilitates cholesterol egress from late endocytic compartments is unknown. Recently, NPC1 was found to bind not only cholesterol but also 25-OHC and most likely also other oxysterols such as 24(S)-OHC and 27-OHC (Infante et al. 2008a). The authors found that cholesterol and 25-OHC bind not to the trans-membrane helix motif but to a luminal loop of NPC1 (Infante et al. 2008b). The relevance of this sterol binding for the function of NPC1 remained unclear in experiments employing cultured fibroblasts, but one can envision that the oxysterol interaction may in vivo play a regulatory role in the function of NPC1 in routing cholesterol, glycolipids, and phospholipids out of late endosomes.

### **2.2.4 Oxysterols Regulate Hedgehog Signaling**

The Hedgehog (Hh) signaling pathway plays a central role in the patterning of multicellular embryos, in post-embryonic development, and in adult tissue homeostasis, including the physiology of stem cells (Varjosalo and Taipale 2007). Corcoran and Scott (2006) reported that cholesterol or certain oxysterols are required for Sonic hedgehog pathway signal transduction and proliferation of medulloblastoma cells. Later, Dwyer et al. (2007) demonstrated convincingly that naturally occurring oxysterols exert their osteoinductive effects (see Sect. 2.2.1) through activation of the Hh signaling pathway. Furthermore, Kim et al. (2007) reported that the inhibition of bone marrow stromal cell differentiation into adipocytes by 20(S)-OHC occurs through a mechanism dependent on the Hh pathway. These findings introduce an



**Fig. 2.4** Summary of the most prominent biological activities of oxysterols. The functions are not assigned to oxysterols in general, but each activity is associated with specific oxysterol species (for details and references, see text)

important, novel role of oxysterols as developmental regulators The most prominent biological activities of oxysterols are summarized in Fig. 2.4.

## 2.3 Cytoplasmic Oxysterol-Binding Proteins

### 2.3.1 Identification of Oxysterol-Binding Protein-Related Proteins

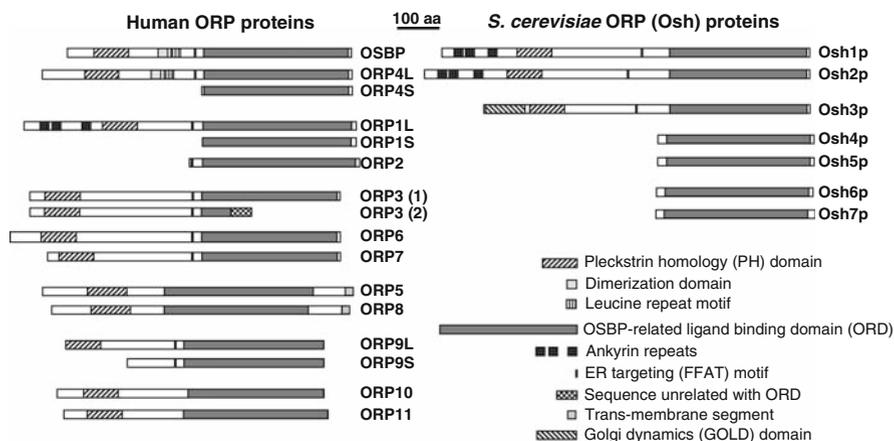
During early studies of feedback inhibition of cholesterol synthesis, oxysterols such as 25-OHC were found to be more potent than cholesterol in reducing the activity of HMGCoA reductase, a rate-controlling enzyme in cholesterol biosynthesis (Brown and Goldstein 1974; Kandutsch and Chen 1974; Kandutsch et al. 1978). These findings prompted a search for protein factors mediating the effects of oxysterols on cellular lipid metabolism. Protein fractions with oxysterol binding activity were isolated from different sources (Beseme et al. 1986, 1987; Defay et al. 1982; Kandutsch et al. 1977; Kandutsch and Shown 1981; Kandutsch and Thompson 1980). Taylor and co-workers identified a cytosolic oxysterol-binding protein (OSBP) whose sterol binding specificity correlated with the ability of these compounds to suppress the activity HMGCoA reductase (Taylor and Kandutsch 1985; Taylor et al. 1984). OSBP was purified (Dawson et al. 1989b; Taylor et al. 1989) and

cDNAs were cloned from rabbit (Dawson et al. 1989a) and human (Levanon et al. 1990). OSBP was found to be a cytoplasmic protein that is translocated from a cytosolic or vesicular compartment to membranes of the Golgi apparatus upon treatment of cells with 25-OHC. Discovery of the SREBPs (see Sect. 2.2.3.2; Hua et al. 1993; Wang et al. 1993; Yokoyama et al. 1993) turned major interest in the field away from OSBP. Furthermore, the LXRs (see Sect. 2.2.3.1) were soon identified as oxysterol-binding transcription factors with central roles in the control of cellular sterol and lipid metabolism (Forman et al. 1997; Janowski et al. 1996; Lehmann et al. 1997). However, work aimed to characterize the function of OSBP continued in the laboratory of N. Ridgway. After some years, novel interest in this protein and its relatives was evoked due to: (i) identification of OSBP-related gene/protein families in eukaryotic organisms from yeast to man, and (ii) functional studies revealing clues for important roles of OSBP homologues in cellular lipid metabolism, vesicle transport, and cell signaling.

Proteins displaying homology with the carboxy-terminal ligand binding domain of OSBP (designated OSBP-related ligand binding domain; ORD) are present in practically all eukaryotic organisms (Lehto and Olkkonen 2003; Yan and Olkkonen 2008). They are called either OSBP-related proteins (ORP) or OSBP-like proteins (OSBPL). In humans (Jaworski et al. 2001; Lehto et al. 2001) and mice (Annis et al. 2002) the gene family consists of 12 members. Most ORP messages are expressed ubiquitously. However, there are marked quantitative differences in the tissue- and cell type-specific mRNA expression patterns of the family members, and alterations of ORP expression levels occur during cell differentiation processes (Gregorio-King et al. 2001; Johansson et al. 2003; Lehto et al. 2004). The cellular functions of ORPs have mainly been investigated in mammalian cells and in the yeast *Saccharomyces cerevisiae* (Schulz and Prinz 2007; Yan and Olkkonen 2008). However, reports have also been published on ORP family members in *Drosophila melanogaster* (Alphey et al. 1998), *Caenorhabditis elegans* (Sugawara et al. 2001), *Dictyostelium discoideum* (Fukuzawa and Williams 2002), rainbow trout (Ramachandra et al. 2007), the parasitic protist *Cryptosporidium parvum* (Zeng and Zhu 2006), as well as in several plants (Avrova et al. 2004; Li et al. 2008; Skirpan et al. 2006). The presence of the gene family throughout the eukaryotic kingdom provides evidence for a fundamental function of the ORPs that originated early in eukaryotic evolution. The human and *S. cerevisiae* ORP proteins are depicted in Fig. 2.5.

### 2.3.2 *Structure and Ligands of ORPs*

The ORPs minimally comprise an OSBP-related ligand-binding domain (ORD), but in mammals a majority of them carry an amino-terminal extension containing a pleckstrin homology (PH) domain that is in several cases known to bind membrane phosphoinositides (Johansson et al. 2005; Lehto et al. 2005; Levine and Munro 1998, 2002). The proteins consisting of an ORD only are here designated “short ORPs”, while those carrying a PH domain are called “long ORPs”.

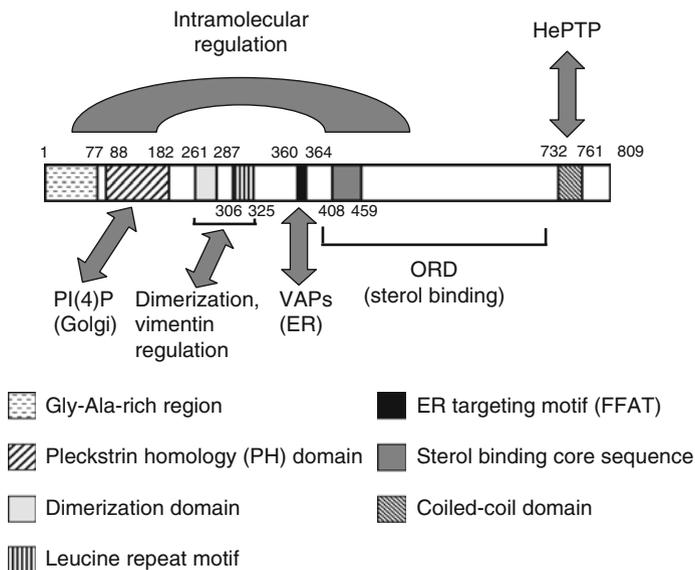


**Fig. 2.5** The human and *S. cerevisiae* OSBP-related protein (ORP) families. The major structural elements are identified. In humans, splice variation leading to changes in mRNA and protein structure is found for several ORPs (see NCBI database; Collier et al. 2003). Therefore, the amino acid (aa) scale given is not exact but indicative; and the proteins depicted do not represent individual splice variants but rather groups formed by several variants

Identification of ligands for the ORDs of the ORPs is crucial for elucidation of the functions of these proteins. The first ORP high-resolution structure, that of a short yeast *S. cerevisiae* ORP, Osh4p/Kes1p, revealed that Osh4p is a sterol-binding protein (Im et al. 2005). It was crystallized in complex with five different sterols, and has a sterol binding pocket formed by 19  $\beta$ -strands in an antiparallel arrangement. The sheet bends to an almost complete roll that is, in the presence of bound ligand, closed by a lid containing an amphipathic  $\alpha$ -helix connected by a flexible linker. Sterols bind within the pocket oriented with the  $3\beta$ -hydroxyl group at the bottom of the hydrophobic binding tunnel. The sterol side-chain interacts with the lid, stabilizing its closed conformation. Importantly, many of the interactions of the bound sterol are mediated via water molecules within the pocket, giving the ligand interaction substantial flexibility. This provides a plausible explanation to the ability of the pocket to accommodate structurally different sterols and possibly also other types of lipid ligands. The structure of Osh4p suggested that this protein and its homologues might act as sterol transporters or mediators of sterol signals (Im et al. 2005).

Of the mammalian ORPs, OSBP, ORP4 (also designated OSBP2), ORP1, and ORP2 have been shown to bind oxysterols (Moreira et al. 2001; Suchanek et al. 2007; Taylor et al. 1984; Wang et al. 2002; Yan et al. 2007a). Moreover, ORP8 was reported to show affinity for 25-OHC (Yan et al. 2008). Based on evidence obtained through the use of UV cross-linkable sterol derivatives in live cells, Suchanek et al. (2007) suggested that a majority of the other human ORPs may bind sterols. However, this data must be interpreted with caution since it is extremely difficult to verify the specificity of the live cell cross-linking signals. The structure of Osh4p

(Im et al. 2005) was used as template for molecular homology modeling of mammalian ORP2 (Suchanek et al. 2007) and OSBP (Wang et al. 2008a). In the former study, analysis of site-specific mutants designed based on the model suggested that ORP2 has a sterol-binding pocket similar to that of Osh4p. Interestingly, Wang et al. (2008a) showed evidence that the intact ORD of OSBP is not required for binding of 25-OHC or cholesterol, which is also known to be a ligand of OSBP (Wang et al. 2005c). Specific and high-affinity sterol binding was also detected with a 51-amino-acid (aa) protein fragment that corresponds to the amino-terminal end of the ORD and comprises part of the modeled lid region (Fig. 2.6). One interpretation of the data is that the 51-aa segment forms the actual sterol binding site, and the  $\beta$ -barrel structure merely protects the bound sterol. The authors also found that a glycine/alanine-rich region at the amino-terminal end of OSBP, together with the PH domain, controls the binding of cholesterol but not of 25-OHC, suggesting interactions of the amino-terminal PH domain region and the ORD, most likely regulated by conformational changes triggered by ligand interactions. At the carboxy-terminal end of the ORD of OSBP and several other ORPs, there is a predicted



**Fig. 2.6** Structural elements important for OSBP function. The domains and sequence motifs are identified at the bottom. The pleckstrin homology (PH) domain specifies Golgi targeting mediated by PI(4)P and the ER targeting motif (FFAT) interacts with the VAP proteins, functions relevant for the control of sphingomyelin synthesis. OSBP forms homodimers and heterodimers with ORP4L, the latter process being connected with regulation of vimentin intermediate filament organization by ORP4L. A coiled-coil domain near the carboxy-terminus of OSBP interacts with HePTP, a tyrosine phosphatase that regulates the activity of extracellular signal regulated kinases, ERK. The Gly/Ala-rich region at the amino-terminus works with the PH domain to control cholesterol binding by the ORD, while occupancy of the ORD by oxysterol ligands regulates the Golgi-targeting function of the PH domain

coiled-coil forming region. Wang et al. (2008a) demonstrated that this region is required for the interaction of OSBP with a phosphatase acting on the ERK. The coiled-coil motif may play an important role in the protein–protein interactions of other ORPs as well (see Sect. 2.3.7).

In addition to sterol ligands, the ORDs of ORP1, ORP2, ORP9, and ORP10 have been suggested to show affinity for phosphoinositides (PIPs; Fairn and McMaster 2005a, b; Hynynen et al. 2005), but it is unclear whether these interactions involve a pocket such as that found in yeast Osh4p. Most likely, they represent binding of positively charged amino acid clusters on the protein surface to negatively charged membrane phosphoinositides (see Im et al. 2005). The data by Raychaudhuri et al. (2006) demonstrates that PIPs facilitate the transfer of sterols between membranes by Osh4p, suggesting that the PIP interaction plays an important regulatory role in the sterol sensor/transporter function of the short ORPs.

### 2.3.3 Subcellular Distribution of ORPs

ORPs are in principle cytosolic proteins with the capacity to associate peripherally with cellular membranes. To obtain clues of ORP function, one of the avenues of research has been the analysis of their subcellular localization and of the determinants that specify the membrane targeting of the proteins. The Golgi targeting of OSBP (Ridgway et al. 1992) is specified by a pleckstrin homology (PH) domain in the amino-terminal part of the protein (Fig. 2.6; Lagace et al. 1997; Levine and Munro 1998). PH domains are also present in the N-terminal region of ten “long” mammalian OSBP homologues. The amino-terminal PH domain-containing extensions present in these ORPs contain predominant targeting information: (i) ORP9, the Golgi complex (Wyles and Ridgway 2004), (ii) ORP1L, late endosomes (Johansson et al. 2003, 2005), and (iii) ORP3,6, and 7, plasma membrane (Lehto et al. 2004). Binding of 25-OHC to the carboxy-terminal domain of OSBP has been suggested to induce a conformational change that unmasks the PH domain, thus inducing a shift of the protein to Golgi membranes. The interaction of the OSBP PH domain with phosphatidylinositol-4-phosphate [PI(4)P] is crucial for targeting of this protein to the Golgi complex and essential for its function (Lagace et al. 1997; Levine and Munro 1998, 2002). Similarly, the PH domain of *S. cerevisiae* Osh1p displays Golgi-targeting specificity and interacts with PI(4)P (Roy and Levine 2004). The Golgi localization of the OSBP and Osh1p PH domains also depends on ADP-ribosylation factors, ARFs (Levine and Munro 2002; Roy and Levine 2004), small GTPases essential for transport vesicle formation (Kahn et al. 2005).

The long variant of mammalian ORP1, ORP1L (Johansson et al. 2003), and two of the long ORPs in *S. cerevisiae*, Osh1p and Osh2p (Beh et al. 2001; Schmalix and Bandlow 1994), have at their very amino-terminus a region containing ankyrin repeats (ANK), motifs typically involved in protein–protein interactions (Fig. 2.5). The ANK region of ORP1L interacts with the GTP-bound active form of the late endosomal (LE) small GTPase Rab7 and plays an important role in targeting of

ORP1L to these compartments. The ANK repeat region of yeast Osh1p was reported to target the protein to the nucleus–vacuole junction (NVJ; Levine and Munro 2001). Since Osh1p was shown to interact with the NVJ protein component Nvj1p (Kvam and Goldfarb 2004), this protein is most likely recognized by the Osh1p ANK region. Findings by Johansson et al. (2003) suggest that the ORP1L ANK region and PH domain synergize in targeting the protein to LE. The ANK repeat region is most likely used to achieve specific membrane targeting of select ORP family members via protein–protein interactions.

Eight of the mammalian ORPs (OSBP, ORP1, 2, 3, 4, 6, 7, 9) possess a sequence motif denoted FFAT (two phenylalanines in an acidic tract) with the consensus sequence EFFDaxE, in the region between the PH domain and the ORD (Fig. 2.5). This motif binds to VAMP-associated proteins (VAP), trans-membrane proteins of the endoplasmic reticulum (ER), conferring the proteins the ability to associate with ER membranes (Kaiser et al. 2005; Loewen et al. 2003). Furthermore, ORP5 and ORP8 have a carboxy-terminal trans-membrane segment that, at least in the case of ORP8, specifies ER targeting (Yan et al. 2008).

The VAPs act as ER docking receptors for several factors involved in the regulation of lipid metabolism, including the ORPs, Goodpasture antigen binding protein (GBPB)/ceramide transporter (CERT; Hanada et al. 2003), the retinal degeneration B (rdgB)/Nir proteins (Amarilio et al. 2005; Lev 2004), and *S. cerevisiae* Opi1p, a transcriptional repressor of inositol synthesis (Loewen et al. 2004). In the case of Opi1p, the association with a yeast VAP homologue, Scs2p, is regulated by the phospholipid composition of the ER: phosphatidic acid (PA) facilitates Opi1p binding to Scs2p in the ER. Addition of inositol that results in phosphatidylinositol synthesis consumes ER PA, leading to the detachment and nuclear translocation of Opi1p. One can envision that also the ORPs could bind to VAP proteins dependent on the presence/absence of a lipid signal, such as ER phospholipid composition or occupancy of the ORD by a ligand. These signals could induce shuttling of ORPs from the ER to another compartment, specified by the targeting determinants in the amino-terminal PH domain region. After executing a given function at the non-ER target organelle, the ORP could undergo a conformational change and return to the ER. This functional cycle could involve transfer of the bound ORD ligand between the two membranes, but binding of the ligand could also serve a signaling function.

Junctions at which ER membranes are closely juxtaposed with other organelles (ER junctions) have been implicated in several crucial cellular processes such as inter-compartmental lipid transport, store-operated  $\text{Ca}^{2+}$  entry, excitation–contraction coupling in striated muscle, and coupling of  $\text{Ca}^{2+}$  transport between the ER and mitochondria (reviewed by Levine 2004). *S. cerevisiae* Osh1p localizes, in addition to the Golgi complex, to the nucleus–vacuole junction, an ER junction characteristic of yeast, and Osh2p as well as Osh3p seem to target ER–plasma membrane junctions (Kvam and Goldfarb 2004; Levine and Munro 2001; Loewen et al. 2003). In a scheme arising from these findings, ORPs interact with the two (ER, non-ER) membrane compartments simultaneously via the FFAT motif and the amino-terminal PH domain region. The model predicts a role of long ORPs in the generation of ER

junctions with other organelles or the regulation of some aspects of their function, possibly lipid transport through these membrane contact sites (Levine 2004; Olkkonen and Levine 2004; Peretti et al. 2008).

### ***2.3.4 Function of OSBP in Lipid Metabolism***

Of the mammalian ORPs, the founder member of the family, OSBP, has been characterized most extensively. It is translocated from a cytosolic or vesicular/ER localization to the Golgi complex upon addition of its high-affinity ligand 25-OHC (Ridgway et al. 1992). OSBP overexpression in Chinese hamster ovary (CHO) cells results in increased cholesterol biosynthesis and a reduction of cholesterol esterification (Lagace et al. 1997). Manipulations of the cellular sterol status were reported impact the Golgi localization of OSBP, suggesting that the Golgi sterol content or the flux of cholesterol through the Golgi complex are sensed by OSBP (Mohammadi et al. 2001; Ridgway et al. 1998; Storey et al. 1998). Furthermore, OSBP undergoes cholesterol-sensitive phosphorylation of specific serine residues, dephosphorylation accompanying the Golgi association of the protein. 25-OHC does not affect the phosphorylation status of OSBP, nor does phosphorylation affect the binding of 25-OHC by OSBP, indicating that the subcellular localization of the protein may be regulated by phosphorylation only in the absence of a “master” 25-OHC signal (Ridgway et al. 1998). The mechanisms by which OSBP affects cholesterol metabolism, and whether these impacts are oxysterol-dependent, remain unclear. A study employing siRNA-mediated silencing of OSBP demonstrated that the endogenous OSBP in HeLa cells plays no role in the suppression of cholesterol biosynthesis by 25-OHC (Nishimura et al. 2005). Instead, the effect of 25-OHC on cellular cholesterol homeostasis is apparently mediated through a direct interaction with the Insigs, proteins that retain SREBP-SCAP complexes in the ER when sterols are abundant (see Sect. 2.2.3.2; Radhakrishnan et al. 2007; Sun et al. 2007).

OSBP overexpression in CHO cells in the presence of 25-OHC was reported to enhance the synthesis of sphingomyelin (Lagace et al. 1999). Mutant OSBP with an amino acid substitution in the PH domain displayed enhanced association with ER membranes and was found to arrest a fluorescent ceramide analogue in the ER, indicating that the function of OSBP may involve transport of ceramide from the ER to Golgi sites where sphingomyelin synthase is located (Wyles et al. 2002). Solid evidence for this hypothesis was provided by the finding that the Golgi translocation and activation of ceramide transport protein, CERT (Hanada et al. 2003), are abolished when OSBP expression is knocked down through RNA interference (Perry and Ridgway 2006). The authors proposed that OSBP acts as a sterol sensor whose function is to integrate sphingomyelin biosynthesis with the cellular sterol status. Consistently, Peretti et al. (2008) reported new evidence that OSBP, together with VAPs, CERT, and the FFAT-motif containing phosphatidylcholine/phosphatidylinositol transfer protein Nir2, co-ordinates lipid transport between the ER and the Golgi

apparatus and thus the Golgi lipid composition as well as the functional properties of this organelle. This finding may be connected with the recent observation that OSBP has the capacity to modulate the intracellular trafficking and processing of amyloid precursor protein (Zerbinatti et al. 2008).

We recently showed that adenoviral overexpression of rabbit OSBP in mouse liver leads to an increase of plasma very-low-density lipoprotein (VLDL) and liver tissue triglycerides (TG; Yan et al. 2007b). The increase of plasma TG was attributed to an enhancement of hepatic TG secretion. Investigation of the underlying mechanism revealed up-regulation of SREBP-1c expression and increase of the active nuclear form of this lipogenic transcription factor in the OSBP-transduced liver. Furthermore, we found evidence that siRNA-mediated silencing of OSBP in cultured hepatocytes attenuated the insulin induction of SREBP-1c and fatty acid synthetase (FAS), as well as TG synthesis. OSBP overexpression was also found to inhibit phosphorylation of the extracellular signal-regulated kinases (ERK). As changes in ERK activity were reported to impact the stability of nuclear SREBP-1c (Botolin et al. 2006), this observation provides a putative mechanistic explanation to the OSBP overexpression phenotype. The above findings suggest a new role of OSBP as a regulator of TG metabolism and its control by insulin signaling cascades.

Bowden and Ridgway (2008) demonstrated that reducing the cellular amount of OSBP by RNA interference increased the cellular content and cholesterol efflux activity of ABCA1. No change was detected in the *ABCA1* mRNA level; and OSBP silencing was shown to increase the half-life of the ABCA1 protein, the effect depending on an intact OSBP sterol-binding domain. Even though the underlying mechanism remained unclear, this finding underscores the multiple sterol-dependent regulatory functions of OSBP.

### **2.3.5 Evidence for the Involvement of Mammalian OSBP Homologues in Lipid Metabolism**

The physiologic functions of the OSBP homologues in mammals are as yet poorly understood. The closest relative of OSBP, ORP4/OSBP2, exists as two major variants (Wang et al. 2002): ORP4L (long) and ORP4S (short). Like OSBP, ORP4 was shown to bind the oxysterols 25-OHC and 7-KC (Moreira et al. 2001; Wang et al. 2002). Both ORP4S and ORP4L were reported to localize on vimentin intermediate filaments in CHO cells (Wyles et al. 2007). Unlike OSBP, the subcellular localization of the ORP4 variants was not affected by treatment of cells with 25-OHC. Interestingly, overexpression of ORP4S or ORP4L mutants in leucine repeat motif induced abnormal bundling or aggregation of the vimentin filaments and significant inhibition of the esterification of LDL-derived cholesterol, suggesting a functional role of this protein in cholesterol transport to the ER (Wang et al. 2002; Wyles et al. 2007). The authors envisioned that ORP4 could use vimentin filaments as a scaffold or tracks for transport of cholesterol or regulatory oxysterols between endocytic compartments and the ER. They also provided evidence that ORP4L heterodimerizes with OSBP.

Interestingly, even though OSBP does not localize on vimentin filaments, OSBP leucine repeat mutants caused a collapse of vimentin filaments similar to corresponding ORP4L mutants, probably reflecting dimerization and functional interplay of OSBP with ORP4L (Wyles et al. 2007). These findings have important implications, considering that each cell type expresses simultaneously a large number of different ORPs, which could form homo- and heterodimers with distinct functional properties.

There is increasing evidence for functions of the closely related ORP1 and ORP2 in cellular lipid metabolism. Overexpression of the long ORP1 variant, ORP1L, or its ankyrin repeat region leads to enhanced recruitment of microtubule-dependent dynein/dynactin motor complexes on late endosomes (LE) and clustering of the endosomes in the juxtanuclear region (see Sect. 2.3.3). Since the ORD of ORP1L binds both PIPs (Fairn and McMaster 2005a) and sterols (Suchanek et al. 2007), and also the PH domain interacts with PIPs (Johansson et al. 2005), ORP1L can be envisioned to act as a lipid sensor that, in complex with Rab7 and its other effector protein RILP (Johansson et al. 2007), modulates the motility and/or distribution of LE according to lipid cues. Our latest work demonstrates that macrophage ORP1L overexpression in LDL receptor-deficient mice increases the size of atherosclerotic lesions (Yan et al. 2007a). The transgenic macrophages were shown to display a defect in cholesterol efflux to spherical HDL and reduced expression of ABCG1 and apolipoprotein E (apoE), as well as increased expression of phospholipid transfer protein (PLTP). These changes in gene expression in response to ORP1L overexpression provide putative explanations to the observed enhancement of atherogenesis and provide compelling evidence for a functional role of ORP1L in macrophage sterol metabolism. However, the relationship between ORP1L function in LE motility and the latter findings remains so far poorly understood.

Overexpression of ORP2, a short human ORP, in CHO or HeLa cells results in an up-regulation of cellular cholesterol efflux (Hynynen et al. 2005; Laitinen et al. 2002). Furthermore, the transport of newly synthesized cholesterol from the ER to the cell surface was enhanced by overexpressed ORP2, as determined by an assay based on cyclodextrin extraction of plasma membrane cholesterol (Hynynen et al. 2005). The HeLa cells expressing ORP2 also showed, probably as a homeostatic response to cholesterol loss, up-regulation of LDL receptor expression and LDL uptake, as well as increased HMGCoA reductase activity. The results were consistent with enhancement of intracellular cholesterol transport by ORP2, or to impaired ability of the cellular plasma membrane to sequester cholesterol. The mechanism underlying the findings is so far unclear, but since we know that ORP2 binds several sterols, we find it possible that ORP2 could (at least in the overexpression situation) transport sterols between subcellular membrane compartments. Interestingly, we also observed in the ORP2 expressing CHO cells a defect in neutral lipid (both triglyceride and cholesterol ester) storage, associated with altered phospholipid fatty acid composition especially under conditions of lipoprotein starvation (Käkelä et al. 2005). Together with our recent unpublished findings, this suggests that a central physiologic function of endogenous cellular ORP2 may involve neutral lipid metabolism.

### **2.3.6 Functional Interplay of ORPs with the Transcriptional Regulators of Lipid Metabolism**

One is tempted to speculate that ORPs could modulate the availability of oxysterol ligands to the LXRs or the Insigs. Overexpression of ORP1L was found to mildly enhance the LXR-mediated transactivation of a reporter gene, dependent on the presence of LXR agonist, either 22(R)OHC or a synthetic non-sterol agonist (Johansson et al. 2003). However, the mechanism underlying this effect remained unclear. We recently demonstrated (Yan et al. 2007a) that transgenic macrophages overexpressing ORP1L display a defect in cholesterol efflux to spherical HDL and reduced expression of ABCG1 and apoE, as well as increased expression of PLTP (see Sect. 2.3.5). The affected genes are subject to transcriptional regulation by the LXRs. Moreover, ORP1L overexpression in cultured mouse macrophages attenuated the response of the *ABCG1* mRNA to the LXR agonist 22(R)OHC, which was also shown to be a ligand of ORP1L. One possible interpretation of the results is that ORP1L modulates the LXR ligand interactions, thereby affecting the expression of LXR target genes and the development of atherosclerosis. However, we find it equally probable that other, more indirect mechanisms may account for the observed phenotypic effect. The strongest evidence for functional interplay between ORPs and the LXRs is found in our recent study (Yan et al. 2008) demonstrating that silencing of ORP8 expression in THP-1 macrophages induces the transcription of *ABCA1* and, as a plausible consequence, cholesterol efflux to apolipoprotein A-I. This effect was reproduced using a luciferase reporter driven by the *ABCA1* promoter. ORP8 silencing synergized with a synthetic LXR agonist and was significantly suppressed when a mutant *ABCA1* promoter devoid of a functional LXR response (DR4) element was used. Interestingly, ORP8 was found to be abundant in the macrophages of human coronary artery lesions, bringing up the possibility that ORP8 might play a role in the development of atherosclerosis.

Our recent finding that hepatic OSBP overexpression enhances lipogenesis putatively via up-regulation of SREBP-1c (see Sect. 2.3.4; Yan et al. 2007b) suggests a functional link between the ORPs and the SREBPs. However, it is unclear if the underlying mechanism involves modulation of Insig function. A change in ERK activity and a resulting change in the stability of nuclear SREBP-1c (Botolin et al. 2006) provide another putative explanation for the findings. The functional connections between the ORP and the LXR and SREBP systems of lipid homeostatic control are an attractive topic for future investigations, relevant for the mechanisms underlying the development of dyslipidemias and atherosclerosis.

### **2.3.7 Function of Yeast Osh Proteins in Sterol Metabolism**

Baker's yeast *S. cerevisiae* is used extensively as a model organism in molecular cell biology due to its amenability to a spectrum of powerful genetic approaches. Sterol homeostasis in *S. cerevisiae* shares a number of similarities with that in

mammalian cells (Henneberry and Sturley 2005). The predominant sterol in yeast is ergosterol, the structure of which differs only slightly from cholesterol. A majority of the enzymes responsible for the synthesis of ergosterol are localized in the ER but, just like cholesterol in mammalian cells (Ikonen 2008), ergosterol concentrates at the plasma membrane (Zinser et al. 1991). Under anaerobic conditions yeast sterol biosynthesis is inhibited and the cells are able to take up sterol from the growth medium. Sterol uptake can also be achieved in specific genetic set-ups (Schulz and Prinz 2007). Sterol transport between the yeast ER and plasma membrane is reported to occur via non-vesicular mechanisms (Baumann et al. 2005; Li and Prinz 2004). *S. cerevisiae* lacks homologues of the putative sterol carriers found in mammalian cells, but has, however, seven ORPs named Osh1–7p. Three of them (Osh1–3p) belong to the category of long ORPs and four (Osh4–7p) are of the short subtype (Beh et al. 2001; Schmalix and Bandlow 1994).

Even before the Osh4p structure was solved, several studies provided evidence for the involvement of yeast Osh proteins in sterol metabolism. Jiang et al. (1994) investigated strains mutant for *OSH1*, *OSH4/KES1*, and *OSH5/HES1*. In the double or triple mutants they discovered pleiotropic sterol-related phenotypes, including tryptophan transport defects and nystatin resistance, as well as mild reductions of membrane ergosterol levels. Beh et al. (2001) determined the phenotypic effects of all 127 permutations of *OSH* deletion alleles. The results demonstrated that the individual *OSH* genes are not essential, but deletion of all seven is lethal, suggesting that the genes have a shared function essential for viability. The viable combinations of *OSH* deletions displayed distinct sterol-related defects and depletion of all seven proteins resulted in cellular sterol accumulation, providing evidence for a disturbance of sterol homeostatic control. Elimination of *OSH* function resulted in a redistribution of ergosterol from the plasma membrane to intracellular locations, vacuolar fragmentation, and cellular accumulation of lipid droplets (Beh and Rine 2004). Furthermore, the authors reported disturbances of endocytosis, cell budding, and cell wall deposition in cells with *OSH* defects. These findings suggest that the function of *S. cerevisiae* Osh proteins involves the subcellular sterol distribution, the other phenotypic effects possibly being secondary to this. Alternatively, disturbance of cellular membrane dynamics by Osh depletion could lead to an abnormal sterol distribution. In support of the former hypothesis, Raychaudhuri et al. (2006) presented evidence for function of the Osh proteins (Osh3–5p) in sterol transport from the yeast plasma membrane to the esterification compartment, the ER. Consistent with the *in vivo* findings, the authors showed that Osh4p is capable of extracting sterols from donor liposomes and transferring them to acceptor vesicles *in vitro*. The sterol transfer was more rapid between membranes that contain PIPs, suggesting that interaction of ORPs with the negatively charged PIP headgroups on membrane surfaces facilitates the sterol transport function. Consistently, the authors found that the transport of sterol from the plasma membrane to the ER is slowed down in mutant strains with defects in PI(4)P or PI(4,5)P<sub>2</sub> synthesis. Therefore, also the interactions of several short mammalian ORPs with PIPs (Fair and McMaster 2005a, b; Hynynen et al. 2005) may play an important role in the ability of these proteins to extract sterol from cellular membranes. It is unclear whether the yeast ORPs investigated by Raychaudhuri et al. (2006) act as true sterol transporters – lack of all

seven Osh proteins reduced sterol transport to the ER only 5-fold. According to an alternative hypothesis, the ORPs affect sterol transport indirectly by affecting the ability of the cellular plasma membrane to sequester these lipids (Sullivan et al. 2006).

Work by the group of H. Yang shows that the association of *S. cerevisiae* Osh6p and Osh7p with cellular membranes is regulated by the AAA family ATPase Vps4p (Wang et al. 2005a, b). The carboxy-terminal coiled-coil domain of Osh7p was shown to determine the interaction with Vps4p. Deletion of *VPS4* or *OSH6-OSH7* double deletion resulted in a defect of sterol ester synthesis, and Osh7p overexpression partially replenished sterol esterification in the *vps4Δ* strain. One interpretation of these findings is that Osh6p and Osh7p act as sterol transfer proteins, and that Vps4p catalyzes their dissociation from membranes as an essential part of their functional cycle. Alternatively, Osh6p and Osh7p could regulate the activity of Vps4p. Since Vps4p dissociates the ESCRT III complex responsible for sorting of cargo proteins to the multivesicular body (Babst et al. 2002a, b), Osh6p and Osh7p could via Vps4p mediate lipid signals to the machinery regulating endosomal sorting/membrane trafficking.

### 2.3.8 *Osh4p Regulates Secretory Vesicle Transport*

The best known example of ORP involvement in intracellular vesicle transport was described in *S. cerevisiae*. Fang et al. (1996) demonstrated that deletion of *OSH4/KES1* leads to by-pass of the temperature-sensitivity of mutants in *SEC14*, a gene encoding a phosphatidylinositol transfer protein (PITP; Sec14p) essential for secretory vesicle biogenesis (Bankaitis et al. 2005). This suggested that yeast Osh4p acts as a negative regulator of Golgi secretory function. Sec14p is thought to maintain a membrane composition permissive to Gcs1p, a GTPase activating protein (GAP) for Arf1, a small GTPase with a central role in transport vesicle formation (Yanagisawa et al. 2002). Also, Osh4p was suggested to exert its effect via regulation of Arf1 activity (Li et al. 2002). Positive membrane curvature plays a distinct role in the Arf GTPase cycle and in transport vesicle formation (Antonny et al. 2005). In addition, Osh4p was shown to contain a specific type of amphipathic helix that targets membranes with a positive curvature (Bigay et al. 2005), consistent with a functional interplay of Osh4p and Arf1p. Intriguingly, a recent study (Fairn et al. 2007) shows that Osh4p reduces both the cellular content of PI(4)P and its availability for recognition by other proteins, which include components involved in transport vesicle formation (D'Angelo et al. 2008). PI(4)P and Arf act in concert to recruit transport factors on Golgi membranes; For example, the PI(4)P adaptor proteins (FAPPs) essential for Golgi to plasma membrane vesicle transport bind both Arf and PI(4)P (Godi et al. 2004). Therefore, the observation of Fairn and co-workers may provide one explanation to the connection of Osh4p function with Arf. As a conclusion, the mechanism by which Osh4p impacts post-Golgi vesicle transport is not completely understood, but recent findings have brought us close to the solution of this dilemma. Analysis of site-specific *osh4* mutants

revealed no clear correlation between sterol binding capacity and the ability to inhibit Golgi-derived vesicular transport (Im et al. 2005). Therefore, the relationship between the functions of Osh4p in post-Golgi membrane trafficking and in sterol transport remains unclear.

Interestingly, a recent study demonstrated that the Osh proteins, including Osh4p, play important roles in yeast cell polarization by maintaining the proper subcellular localization of septins, the Rho GTPases Cdc42p and Rho1p, and the Rab GTPase Sec4p (Kozminski et al. 2006). The mechanisms underlying this observation are as yet poorly understood. However, since a functional secretory pathway is essential for polarized growth, the impact of ORPs on cell polarization may involve a lipid-dependent action in secretory vesicle transport.

### 2.3.9 Mammalian ORPs and Intracellular Vesicle Transport

Even though the most studied case of ORP function in vesicle transport is *S. cerevisiae* Osh4p/Kes1p (see Sect. 2.3.8), there are several reports suggesting the involvement mammalian ORPs in vesicle transport along the secretory or the endocytic pathways of membrane trafficking. Most of these studies employed overexpression of an intact ORP or of a PH domain separated from its context. ORP1L overexpression was shown to interfere with the delivery of endocytosed cargo to late endosomes/lysosomes (Johansson et al. 2005) and excess ORP2 was reported to interfere with endocytotic uptake of markers from the cell surface (Hynynen et al. 2005). Furthermore, the overexpression of human ORP2 in CHO cells and in *S. cerevisiae* was reported to interfere with Golgi vesicle transport (Laitinen et al. 2002; Xu et al. 2001). Similarly, Levine and Munro (1998) showed that overexpression of the OSBP PH domain interferes with Golgi secretory function.

The mechanisms underlying the cellular effects of ORP overexpression are unclear and may not faithfully reflect the physiologic function of the proteins for the following reasons:

1. Overexpression of certain ORPs is known to cause distortion of the structure of the ER and Golgi compartments (Lehto et al. 2005; Wyles and Ridgway 2004), which could lead to artefactual disturbances in membrane trafficking.
2. Association of an excessive amount of an ORP with membrane phosphoinositides may disturb vesicle transport through interference with the trafficking machinery known to interact with these negatively charged phospholipids (see Fairn et al. 2007).

Johansson et al. (2007) employed ORP1L knock-down with siRNA to show that the protein is required for the clustering of late endocytic compartments in the pericentriolar region. However, the actual late endocytic trafficking functions were not assessed in this study. Studies employing gene silencing are now necessary to make reliable observations on ORP function in intracellular membrane trafficking.

### 2.3.10 ORPs – Integrating Lipid Cues with Cell Signaling Cascades

The first convincing evidence for the involvement of ORPs in cell signalling was provided by the study of Sugawara et al. (2001), who identified a *Caenorhabditis elegans* ORP, designated BRAM-interacting protein, BIP, as a modulator of transforming growth factor (TGF)- $\beta$  signalling. The authors carried out a two-hybrid screen using bone morphogenetic protein (BMP) receptor associated protein (BRAM) as a bait, and identified a *Xenopus* ORP (BIP) as a BRAM binding partner. Thereafter they isolated the *C. elegans* homologue of this cDNA and showed that it interacts with the *C. elegans* BRAM homologues BRA-1 and BRA-2. They further demonstrated that inhibition of BIP expression by RNA interference produces a Sma phenotype characteristic of disturbance of the *C. elegans* TGF- $\beta$  pathway that regulates body length. The documented interaction of the ORP (BIP) with the BRA proteins evidences for a direct role as a modulator of the signaling pathway.

Increasing evidence is accumulating for the involvement of ORPs in signaling events in mammals. Wang et al. (2005c) identified OSBP as a sterol-sensing scaffolding factor that regulates the dephosphorylation and thereby the activity of extracellular signal-regulated kinases (ERK), key components of the mitogen activated protein kinase (MAPK) signaling pathways. OSBP was shown to bind both cholesterol and 25-OHC; and the cholesterol-bound state scaffolds a protein phosphatase complex (PP2A serine/threonine phosphatase and HePTP, a PTPBS family tyrosine phosphatase) which dephosphorylates and thereby inactivates ERK. Reduction of the cellular cholesterol content or addition of 25-OHC dissociates the phosphatase complex, leading to hyperphosphorylation of the ERKs. A second signaling function of OSBP was reported by Romeo and Kazlauskas (2008), who found evidence that up-regulation of profilin-1 (an actin-binding protein implicated in endothelial dysfunction and atherosclerosis) by 7-KC is mediated by OSBP. The signal transduction route involves interaction of OSBP-7-KC complex with the tyrosine kinase JAK-2, which phosphorylates Tyr394 on OSBP. This leads to the activation of STAT3, which is responsible for the induction of profilin. An interesting possibility brought up by these findings is that also other members of the ORP family could have lipid-specific scaffolding functions in signaling pathways. Indeed, Lessman et al. (2007) demonstrated that ORP9 contains a phosphoinositide-dependent kinase-2 (PDK-2) phosphorylation site, the phosphorylation of which is dependent on PKC- $\beta$  or mTOR in bone marrow-derived mast cells or HEK293 cells, respectively. The authors also provided RNA interference and immunoprecipitation evidence that ORP9 interacts with these kinases to negatively regulate phosphorylation of the PKD-2 site in Akt/protein kinase B, a major controller of cell survival, cell cycle progression, and glucose metabolism (Hanada et al. 2004).

Interestingly, ORP3 and ORP7 were recently found to interact with R-Ras, a small GTPase that regulates cell adhesion and migration (Goldfinger et al. 2007; Kinbara et al. 2003), implying a functional role of these ORPs in Ras signaling. Consistent with the above findings, Lehto et al. (2008) demonstrated that ORP3 controls cell adhesion and spreading, organization of the actin cytoskeleton,

$\beta$ 1-integrin activity, and macrophage phagocytic function, cellular processes also subject to regulation by R-Ras. ORP3 is present at highest levels in leukocytes (T-cells, B-cells, monocytes, macrophages) and the epithelia of several tissues; and abnormally high expression is detected in certain forms of leukemia and solid tumors, suggesting that the protein may modify the signaling processes and adhesion properties of cells in a manner that facilitates malignant growth.

Even though the role of ORP interactions with lipidous ligands has not been addressed in all of the above studies, it is plausible that liganding of both the PH domain and the ORD is crucial for the signaling functions outlined above. One can thus envision that, in analogy with the sterol-dependent function of OSBP in ERK and JAK-2/STAT3 signaling, the other ORPs may also relay essential lipid cues to cellular signaling cascades.

## 2.4 Future Perspectives

New roles of oxysterols as potent regulators not only of cellular and body lipid metabolism but also of other vital cellular processes, such as signaling cascades, differentiation, and apoptosis, have emerged at an increasing pace. In addition to the suggested roles of oxysterols as potentially harmful substances that accumulate in pathophysiologic states, these compounds have at their normal concentrations at least the potential to function as signaling molecules that maintain cellular and body lipid homeostasis and determine cell fate. The fact that oxysterols act as ligands of the LXRs, transcription factors with potentially antiatherogenic effects, has prompted intense efforts towards the development of synthetic agonists that would selectively induce cardioprotective LXR target genes. In analogy, the discovery of oxysterol interactions with new receptor proteins involved in lipid metabolism, such as the Insig proteins, the estrogen receptors, NPC1 and the OSBP-related proteins, brings up the possibility of developing new oxysterol-related pharmacologic compounds that modify the function of these target proteins, with beneficial effects on lipid metabolism and atherogenesis. Furthermore, modulation of the expression levels or activity of the cytochrome P450 enzymes responsible for generating oxysterols, or inhibition of 2,3-oxidosqualene cyclase could be feasible therapeutic approaches.

Discovery of oxysterols as regulators of Hedgehog signaling uncovered a new role of oxysterols as regulators of embryonic development. Together with the increasing number of reports on the involvement of ORP proteins in cell signaling, this finding has opened new perspectives. We can expect a plethora of novel connections of oxysterols, oxysteroids, and their receptor proteins with differentiation and developmental processes.

The ORPs constitute a highly interesting protein family present ubiquitously in eukaryotic cells, thus representing machinery of fundamental importance in cell physiology. The functions suggested for ORPs in different organisms are summarized in Table 2.2. The bulk of the literature suggests that binding of sterols or other lipids within the ligand binding domain of ORPs serves a regulatory function, but in certain

**Table 2.2** A summary of the suggested ORP functions

Organism	Protein	Suggested function	References
Mammals	OSBP	Sterol-dependent regulation of ERK, JAK-2/STAT3 and sphingomyelin synthesis; modulation of hepatic lipogenesis, ABCA1 stability and processing of the amyloid precursor protein	Wang et al. (2005c), Perry and Ridgway (2006), Yan et al. (2007b), Bowden and Ridgway (2008), Romeo and Kazlauskas (2008), Zerbiniatti et al. (2008)
	ORPIL	Motility and distribution of late endosomes; macrophage lipid metabolism	Johansson et al. (2003, 2005, 2007), Yan et al. (2007a)
	ORPIS	Vesicle transport from Golgi	Xu et al. (2001)
	ORP2	Sterol transport; neutral lipid metabolism; vesicle transport from Golgi	Xu et al. (2001), Laitinen et al. (2002), Hynynen et al. (2005), Käkälä et al. (2005)
	ORP3	Regulation of cell-cell and cell-matrix adhesion	Lehto et al. (2008)
	ORP4	Vimentin-dependent sterol transport and/or signalling	Wang et al. (2002), Wyles et al. (2007)
	ORP8	Regulation of ABCA1 expression and cholesterol efflux	Yan et al. (2008)
	ORP9	Regulation of Akt phosphorylation	Lessmann et al. (2007)
Rainbow trout	OORP-T	Utilization of yolk lipids in oocyte development	Ramachandra et al. (2007)
<i>Drosophila melanogaster</i>	OSBP-Dm (CG6708)	Cell cycle control	Alphey et al. (1998)
<i>Caenorhabditis elegans</i>	BIP (obr-3)	Modulation of TGF- $\beta$ signalling	Sugawara et al. (2001)
<i>Dicystoselium discoideum</i>	OSBPa	Regulation of slug-fruiting body switch	Fukuzawa and Williams (2002)
<i>Cryptosporidium parvum</i>	CpORP1	Lipid transport across the parasitophorous vacuole membrane	Zeng and Zhu (2006)
<i>Solanum tuberosum</i>	StOBP1	Function in a non-specific defence pathway	Avrova et al. (2004)
<i>Petunia inflata</i>	PiORP1	Pollen development	Skirpan et al. (2006)
<i>Glycine max</i>	GmOSBP	Reactions to stress response and cotyledon senescence	Li et al. (2008)
<i>Saccharomyces cerevisiae</i>	Osh1p	Post-synthetic sterol regulation; piecemeal microautophagy of the nucleus; cell polarity establishment	Jiang et al. (1994), Beh et al. (2001), Kvam and Goldfarb (2004), Kozminski et al. (2006)
	Osh2p	Sterol metabolism; cell polarity establishment	Daum et al. (1999), Beh et al. (2001), Kozminski et al. (2006)

Osh3p	Sterol transport; sphingolipid metabolism; regulation of nuclear fusion during mating and pseudohyphal growth	Park et al. (2002), Yano et al. (2004), Hur et al. (2006), Raychaudhuri et al. (2006)
Osh4p/Kes1p	Sterol transport and metabolism; post-Golgi vesicle transport; cell polarity establishment	Jiang et al. (1994), Fang et al. (1996), Li et al. (2002), Kozminski et al. (2006), Raychaudhuri et al. (2006)
Osh5p	Sterol transport and metabolism	Jiang et al. (1994), Raychaudhuri et al. (2006)
Osh6p	Sterol transport; regulation of Vps4p function; cell polarity establishment	Wang et al. (2005a, b), Kozminski et al. (2006)
Osh7p	Sterol transport; regulation of Vps4p function	Wang et al. (2005b)
Osh3	Regulation of pseudohyphal growth	Hur et al. (2006)

*Candida albicans*

cases there is also evidence for a sterol transport function. The initial findings on ORP function (*S. cerevisiae* Osh4p/Kes1p) involved intracellular vesicle transport. However, more recent studies in various organisms have connected a majority of ORP proteins with cellular lipid metabolism or cell signaling events. The interest in this protein family is constantly expanding and the ground-breaking studies published during the past few years have paved the way for creating new functional hypotheses. Testing these hypotheses in cultured cell set-ups and in animal models will in near future enable major progress in our understanding of the physiologic role of ORPs. Most likely, many central functions of the ORPs turn out to involve regulation of cellular and body lipid metabolism. However, lipid homeostasis must be integrated with a number of other regimes. Numerous new functional connections of the ORPs with the control of intracellular vesicle transport, cell differentiation, proliferation, polarity, adhesion, migration, and survival/death, will no doubt be discovered. Furthermore, it will be of major importance to elucidate the role of the ORP proteins and genetic variation within the *OSBPL* genes in human diseases such as cancer, dyslipidemias, and atherosclerosis.

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# Chapter 3

## Cellular Lipid Traffic and Lipid Transporters: Regulation of Efflux and HDL Formation

Yves L. Marcel, Mireille Ouimet, and Ming-Dong Wang

**Abstract** Cellular cholesterol homeostasis integrates multiple pathways of cellular cholesterol movement and the transcriptional regulation of genes implicated in cholesterol metabolism as a function of cholesterol uptake and export. The uptake of lipoprotein-derived cholesterol and the export of cholesterol by lipoprotein secretion and/or efflux to exogenous acceptors are highly regulated processes dependent on intracellular cholesterol transport. Exogenous cholesterol derived from lipoprotein internalization is delivered to LE and lysosomes, where cholesteryl esters are hydrolyzed to generate free cholesterol. Cholesterol from the LE must be re-distributed to the ER, where together with newly synthesized cholesterol, it can be esterified by ACAT and stored in LD. LD-associated cholesterol undergoes multiple cycles of hydrolysis and esterification as a function cellular requirements and can be mobilized for efflux, a process dependent on the presence of exogenous acceptors and on upstream events that remain to be fully elucidated. ABCA1, which is highly regulated by transcriptional and posttranscriptional mechanisms, is the major transporter for phospholipid and cholesterol efflux and, along with mediators of cellular lipid transport, regulates HDL formation.

### 3.1 Introduction

Cellular export of lipids includes the secretion of lipoproteins, the shedding of membranes and the regulated lipid efflux pathway, which occurs through secondary interactions between the core constituents of the secreted lipoproteins, apolipoproteins and multiple cell types in peripheral tissues.

Apolipoprotein A-I (apoA-I)-containing lipoproteins, the major class of circulating HDL particles, are secreted by hepatocytes and intestinal cells, whereas apoE-containing lipoproteins are synthesized and secreted by a number of cells, including

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macrophages, hepatocytes and neuronal cells. Several lipid transporters are known to participate in cellular lipid traffic and eventually in the secretory and efflux pathways, including members of the ATP-binding cassette (ABC) family, ABCA1, ABCA2, ABCA7, ABCG1, ABCG4, as well as the scavenger receptor-BI (SR-BI).

Newly secreted apoA-I-containing lipoproteins released into the circulation by hepatocytes and intestinal epithelial cells, are heterogeneous and not fully lipidated. These nascent lipoproteins acquire additional lipids at the cell surface of hepatocytes and peripheral cells through ABCA1- and ABCG1-mediated pathways. Further modification of the secreted HDL particles by esterification of cholesterol and interactions with postprandial remnant lipoproteins eventually generates the mature circulating HDL<sub>3</sub> and HDL<sub>2</sub> particles, which can be selectively taken up via SR-BI or CETP by hepatocytes. These lipoproteins are further remodeled by hepatic lipase, endothelial lipase, cholesteryl ester transfer protein and phospholipid transfer protein, culminating in the release of pre $\beta$ -apoA-I, a partially phospholipidated apoA-I that is highly active in mediating efflux via ABCA1. However, many potentially important details are lacking in the well-studied HDL metabolism pathway. How much cholesterol does the liver contribute to the secreted HDL and how much is contributed by extrahepatic tissues under normal physiological conditions? Is nascent hepatic HDL similar to the HDL formed by lipid efflux to pre $\beta$ -apoA-I in peripheral cells?

In this review, we compare the specificity of cells involved in lipid export and secretion and focus on differences that are relevant to the physiological assembly of HDL particles. The lipid efflux pathway depends not only on the activity of transporters that mediate lipid export to extracellular acceptors but also on upstream cellular trafficking events required for lipid mobilization. We summarize current understanding of how cellular cholesterol trafficking regulates lipid efflux.

### **3.2 Regulation of apoA-I Synthesis, Lipidation and Secretion in Hepatocytes: Genesis of apoA-I-Containing Lipoproteins and HDL**

The liver synthesizes about 70% of plasma apoA-I, with the remainder being produced by the intestine (Eisenberg 1984; Tall 1990). ApoA-I is part of the apoA-I/apoCIII/apoA-IV gene cluster, which is controlled by a common enhancer upstream of the apoCIII gene. Important transcription factors regulating enhancer activity include SPI1, HNF-4 and other nuclear receptors (Zannis et al. 2001). Hepatic expression of apoA-I is regulated by nutrients and hormones, in particular estradiol (Jin et al. 1998; Hargrove et al. 1999; Lamon-Fava et al. 1999; Mooradian et al. 2006). Early studies demonstrated generation of a spectrum of poorly lipidated apoA-I and nascent HDL by hepatocytes (Dixon and Ginsberg 1992) and HepG2 cells (Chisholm et al. 2002). Elevated expression of ABCA1 in hepatocytes (Wellington et al. 2002) first suggested a role for hepatic ABCA1 activity in the lipidation of HDL. Subsequently, it was shown that ABCA1 mediates the transfer

of both phospholipids and cholesterol to apoA-I. In primary murine hepatocytes infected with an adenovirus for human apoA-I, endogenously synthesized human apoA-I forms a population of apoA-I-containing lipoproteins (LpA-I) similar to those formed by incubation with exogenous apoA-I (Kiss et al. 2003). Using this model, our studies indicate that ABCA1 is responsible for more than 60% of endogenous apoA-I phospholipidation, and the remainder occurs independently of hepatic ABCA1.

Importantly, ABCA1 inactivation in hepatocytes does not affect apoA-I secretion and similarly sized particles are formed, although fewer large particles accumulate. Therefore, apoA-I secretion is not controlled by lipid availability, and the type of particles generated appears to be dictated by apoA-I structure (i.e. its ability to form dimers and tetramers (Kiss et al. 2003), and by ABCA1 oligomerization (Denis et al. 2004a, b). Investigation of the early steps of apoA-I lipidation as it traffics from the endoplasmic reticulum (ER) to the Golgi shows that phospholipidation in the ER is independent of ABCA1 activity (Maric et al. 2005). The bulk of lipidation of apoA-I, however, occurs in the Golgi and at the plasma membrane (Maric et al. 2005), consistent with the cellular distribution of ABCA1 (Neufeld et al. 2001, 2004). In conclusion, apoA-I can be secreted in a lipidated form by hepatocytes, but the observation that a large proportion of apoA-I particles are poorly lipidated suggests that lipid supply and/or ABCA1 expression may be limiting.

### 3.3 Cell Specificity of ABCA1 Expression and HDL Formation in Vivo: Insight from Genetically Modified Mice

Targeted *Abca1* inactivation in mice demonstrated that ABCA1 activity in the liver, intestines and macrophages could contribute 80%, 30% and 20%, respectively, of the apoA-I lipidation necessary to maintain plasma HDL levels (Haghighpassand et al. 2001; Timmins et al. 2005; Brunham et al. 2006). Furthermore, hepatic and intestinal ABCA1 activities are additive and mice deficient in both have only 10% of wild-type HDL levels (Brunham et al. 2006).

Overexpression of hepatic ABCA1 in mice by adenoviral infection or transgenesis (Vaisman et al. 2001 Basso 2003; Joyce et al. 2006) has also shown that hepatic ABCA1 activity is limiting for HDL lipidation. Both approaches increased plasma apoA-I and HDL cholesterol, but since apoA-I lipidation and secretion were not assessed, it is unclear whether the elevated apoA-I-associated cholesterol was due to a decrease in apoA-I catabolism, increased hepatic de novo cholesterol synthesis or both (Joyce et al. 2006). Wellington and colleagues infected mice with increasing doses of ad-ABCA1 (Wellington et al. 2003), which elevated HDL cholesterol to a plateau at relatively low ABCA1 protein levels (1.2-fold compared to control). At higher doses of ad-ABCA1, non-HDL cholesterol increased. Similar results have been obtained by others using an ABCA1 transgene driven by the apoE promoter (Joyce et al. 2006). Mice expressing a BAC ABCA1 transgene demonstrated a pattern of tissue specific expression similar to the endogenous mouse gene

(Cavelier et al. 2001; Singaraja et al. 2001). BAC transgenic mice with low BAC copy number had increased HDL, which increased with ABCA1 expression. Of note, the latter BAC ABCA1 transgenic line showed a 65% increase in HDL cholesterol (Singaraja et al. 2001). Therefore, ABCA1 driven by its natural promoter and ubiquitously expressed increases HDL-cholesterol.

These studies have established that ABCA1 activity is limiting for the lipidation of apoA-I and the maintenance of HDL levels. Together, hepatic and intestinal ABCA1 activities account for the near totality of HDL production, which indicates that apoA-I lipidation by peripheral cells is negligible with the exception of macrophage ABCA1, which normally exports the excess cholesterol accumulating in these cells.

### 3.4 Transcriptional and Posttranscriptional Regulation of ABCA1

ABCA1 expression is highly regulated, both transcriptionally and posttranscriptionally (Schmitz and Langmann 2005). Transcription of ABCA1 is markedly regulated by oxysterols (Lawn et al. 1999) ligands for LXR $\alpha$  and LXR $\beta$ , and by retinoic acid, acting via RXR (Costet et al. 2000; Schwartz et al. 2000). Treatment of cells with oxysterols and/or retinoic acid induces ABCA1 expression and the combined effect is synergistic (Repa and Mangelsdorf 1999; Costet et al. 2000; Schwartz et al. 2000). The activation of ABCA1 transcription by the LXR $\alpha$ /RXR and LXR $\beta$ /RXR heterodimers may be modulated the transcriptional factor SP1, which binds to the proximal ABCA1 promoter, upstream of the LXR/RXR responsive element, and physically interacts with LXR/RXR receptors bound to the promoter (Thymiakou et al. 2007). Two major ABCA1 transcripts, p-ABCA1 and l-ABCA1, have been identified that are mainly expressed in peripheral tissues and the liver, respectively; these transcripts contain different corresponding promoter regions (Tamehiro et al. 2007). Compactin, a member of the class of cholesterol-lowering agents, HMG-CoA reductase inhibitors (statins), increases the liver-type transcript and decreases the peripheral-type transcript. The stimulation of ABCA1 transcription by statins in rat hepatoma McARH7777 cells was associated with the binding of SREBP-2 to the novel liver-type promoter. The same two transcripts were also dominant in human and mouse livers, whereas the intestine contains only the peripheral-type transcript (Tamehiro et al. 2007). This study further documents the complex tissue-specific regulation of ABCA1 expression and offers an explanation for the conflicting results of statin treatment on HDL formation and cholesterol efflux from macrophages and SREBP-2 effects on vascular cells (Wong et al. 2004; Zeng et al. 2004). In addition, the human ABCA1 promoter contains an activator protein (AP)2-binding site between positions -368 and -147, upstream of LXR-binding site, which negatively regulates ABCA1 transcription in CHO cells. HepG2 cells lack AP2 $\alpha$  expression, an additional complexity of the tissue-specific regulation of ABCA1 expression (Iwamoto et al. 2007).

The significant discordance between ABCA1 mRNA and protein expression is consistent with a high level of posttranscriptional regulation (Wellington et al. 2002). Under basal conditions in cultured cells, ABCA1 protein is highly unstable, with a half-life of 1–2 h (Oram et al. 2000; Wang and Oram 2002; Wang et al. 2003). Binding of apoA-I to ABCA1-expressing cells reduces ABCA1 degradation by preventing its proteolysis by calpain (Wang et al. 2003) and thiol proteases (Arakawa and Yokoyama 2002). Pathological conditions such as free cholesterol loading (i.e. ER stress) can also induce ABCA1 degradation by the ubiquitin–proteasome-mediated pathway (Feng and Tabas 2002). Whereas the binding of calpain to the phosphorylated PEST sequence of ABCA1 targets the latter to the degradative pathway, binding of apoA-I to ABCA1 causes the dephosphorylation of the PEST sequence that is followed by a decrease of ABCA1 degradation by calpain, resulting in increased ABCA1 at the cell surface (Martinez et al. 2003). Similarly, ABCA1 with a deleted PEST domain remains at the cell surface, does not traffic to late endosomes (LE) and consequently effluxes cholesterol from the plasma membrane but not from LE (Chen et al. 2005).

ABCA1 stability is also modulated by PKC activity. ApoA-I activates PKC $\alpha$  by PC-PLC-mediated generation of diacylglycerol initiated by the removal of cellular sphingomyelin (Ito et al. 2002) and activated PKC $\alpha$  subsequently phosphorylates and stabilizes ABCA1 (Yamauchi et al. 2003). However, the phosphorylated amino acid residues are yet to be defined. Suppression was also reported for cholesterol efflux, but not for phospholipids, to apoA-I in human monocyte-derived macrophages and human THP-1 macrophages treated with a panPKC inhibitor (Kiss et al. 2005). These results indicate that the regulation of ABCA1-mediated efflux by PKC activity may be more complicated than previously anticipated. Indeed, unsaturated fatty acids that are elevated in diabetes increase ABCA1 phosphorylation but destabilize ABCA1 through a PKC $\delta$  pathway, perhaps contributing to the abnormal HDL metabolism in patients with diabetes (Wang and Oram 2005, 2007).

The incubation of apolipoproteins with ABCA1-expressing cells activates the protein–tyrosine kinase, JAK2, which in turn initiates a process that enhances apolipoprotein interactions with ABCA1 to positively regulate lipid efflux. However, tyrosine kinase-mediated phosphorylation of ABCA1 is undetectable, suggesting that the target of JAK2 is not ABCA1 but rather an associated protein, perhaps autophosphorylation of JAK2 itself (Tang et al. 2004). An active JAK2 is required for apolipoprotein binding to ABCA1 and for lipid efflux, yet apoA-I cellular binding stabilizes ABCA1 independently of JAK2 activity (Tang et al. 2006). This has been interpreted as evidence that apolipoproteins coordinate the activity of ABCA1 through several distinct pathways that are likely to involve numerous and possibly novel molecules. Probable candidates include the syntrophins and their associated proteins as constituents of the ABCA1 protein complex (see below).

Whereas efflux to apoA-I is impaired in NPC1-deficient macrophages, it is increased by up to 8-fold in NPC1-deficient hepatocytes (Wang et al. 2007a). The increased efflux correlates with a marked increase in ABCA1 protein in NPC1-deficient hepatocytes, which contrasts to the observed reduction of ABCA1 in NPC1-deficient macrophages. Elevated ABCA1 expression is largely posttranslational,

since ABCA1 mRNA is only slightly increased in NPC1<sup>-/-</sup> hepatocytes. Moreover, LXR $\alpha$  mRNA in these cells is comparable to wild-type hepatocytes, whereas multiple LXR $\alpha$  target genes are reduced (possibly as a result of mitochondrial dysfunction). Interestingly, the lysosomal enzyme, cathepsin D (CTSD; which is a positive modulator of ABCA1; Haidar et al. 2006), is markedly increased at both mRNA and protein levels by NPC1 inactivation in hepatocytes but not in macrophages (Wang et al. 2007a). Although PPAR $\gamma$  and CREB mRNA levels are upregulated by NPC1 inactivation, the stimulation of hepatocytes with a PPAR $\gamma$  agonist or cAMP does not increase ABCA1 expression. In contrast, administration of the PPAR $\gamma$  agonist, rosiglitazone, and the CREB inducer, cAMP, markedly increase ABCA1 expression in murine macrophages. Therefore, ABCA1 expression is apparently subject to unique regulation in hepatocytes, where NPC1 activity modulates cathepsin D and ABCA1 expression (Wang et al. 2007a).

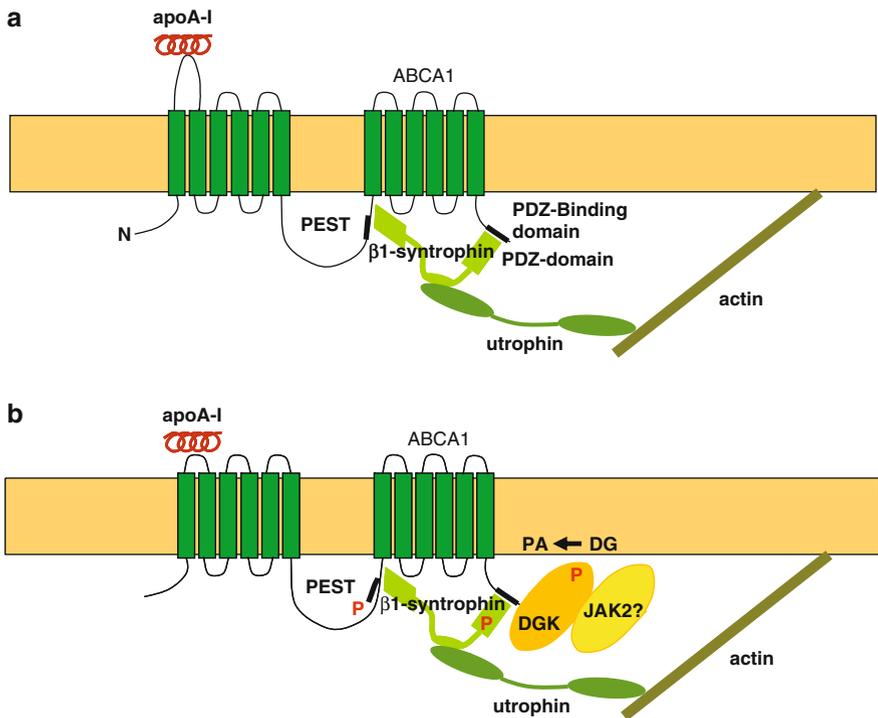
### 3.5 Cellular Traffic of ABCA1

#### 3.5.1 *Syntrophin and the Regulation of Lipid Efflux Activity*

Neufeld and colleagues demonstrated a complex intracellular trafficking route for ABCA1, which was suggested to play a role in modulating lipid efflux and cellular cholesterol homeostasis (Neufeld et al. 2001): ABCA1-GFP was shown to reside on the cell surface and in subsets of early endosomes (EE), LE and lysosomes; and time-lapse microscopy revealed dynamic interactions between these compartments. Subsequent studies established that ABCA1 in LE plays a role in lipid efflux from that organelle and that apoA-I shuttles between the plasma membrane (PM) and LE (Neufeld et al. 2004). LE traffic is defective in Tangier disease cells and LE accumulates high levels of cholesterol, sphingolipids and NPC1 protein (Neufeld et al. 2004). The addition of apoA-I reduces NPC1 content in LE of normal but not Tangier cells (Neufeld et al. 2004). Noteworthy is the impaired secretory vesicular transport from the Golgi to PM in Tangier cells, which shows that ABCA1-mediated efflux of cholesterol also modulates secretory activity and the movement of membranes from the Golgi (Zha et al. 2003). However, the phospholipid flippase activity of ABCA1 may also contribute to this process (Roosbeek et al. 2004). The PEST sequence regulates ABCA1 internalization from the PM and ABCA1- $\delta$ PEST display defective internalization and trafficking to LE, which in turn impairs cholesterol efflux from LE (specifically labeled with acetylated-LDL in SRA expressing cells) but not cholesterol efflux from the PM (labeled with LDL; Chen et al. 2005). However the significance of this pathway for efflux to apoA-I was recently challenged (Denis et al. 2008; Faulkner et al. 2008; see below).

The complex intracellular trafficking of ABCA1 suggests the involvement of additional accessory proteins. A yeast two-hybrid library identified  $\alpha$ 1-syntrophin and Lin7 as ABCA1-interacting proteins and  $\alpha$ 1-syntrophin expression was found

to increase the half-life of ABCA1 and enhance cholesterol efflux (Munehira, Ohnishi et al. 2004). A putative PDZ-binding domain at the C terminal region of ABCA1 has been shown to bind the  $\beta$ 2-syntrophin/utrophin complex (Buechler, Boettcher et al. 2002) and the  $\beta$ 1-syntrophin/utrophin complex (Okuhira, Fitzgerald et al. 2005). The syntrophin family comprises five isoforms ( $\alpha$ 1,  $\beta$ 1,  $\beta$ 2,  $\gamma$ 1,  $\gamma$ 2), each with the same molecular organization including two tandem pleckstrin homology (PH) domains, a PDZ domain and a C-terminal syntrophin unique (SU) domain (Ahn et al. 1996). The C-terminal region, including the PH2 and SU domains, interacts with the dystrophin family of proteins, dystrophin, utrophin, and dystrobrevin (Albrecht and Froehner 2002). Thus, the syntrophins link the dystrophin family of proteins to other proteins that bind to their PDZ domains, providing a scaffold at the cell surface Fig. 3.1.



**Fig. 3.1** Interactions between ABCA1,  $\beta$ 1-syntrophin, utrophin and actin. **a** The binding of  $\beta$ 1-syntrophin PDZ domain to ABCA1 PDZ-binding domain at its C-terminus.  $\beta$ 1-Syntrophin binds to utrophin, which anchors the complex to actin and retains ABCA1 at the cell surface (adapted from Buechler et al. 2002; Okuhira et al. 2005). **b** The putative regulation of the  $\beta$ 1-syntrophin-ABCA1 complex.  $\beta$ 1-Syntrophin binds to a diglyceride kinase (*DGK*), which is proposed to phosphorylate  $\beta$ 1-syntrophin and a residue adjacent to the PEST sequence on ABCA1. JAK2 is a protein-tyrosine kinase, which is activated upon binding of apoA-I to ABCA1 and phosphorylates an unknown target that activates the ABCA1 complex (Tang et al. 2004), independently of JAK2 activity (Tang et al. 2006)

The tissue-specific expression of syntrophins contributes to their specificity: the  $\alpha 1$  isoform is expressed highly in heart, brain and muscle,  $\beta 1$  in the liver and  $\beta 2$  is highest in the intestines. Freeman and colleagues have shown that  $\beta 1$ -syntrophin expression regulates ABCA1 levels and cholesterol efflux. Inhibition of  $\beta 1$ -syntrophin expression accelerates the degradation of newly synthesized ABCA1, and co-expression of  $\beta 1$ -syntrophin and ABCA1 results in the formation of cell surface ABCA1 clusters, which protects it from degradation and increases its concentration at the cell surface and efflux activity (Okuhira et al. 2005). The authors also identified utrophin and  $\beta$ -dystrobrevin as ABCA1-interacting proteins. Since utrophin is an adaptor protein that binds both to syntrophin and actin, it can provide an important bridge between ABCA1 at the plasma membrane and the cytoskeleton, providing motility and controlling its endocytosis and traffic. Another conserved C-terminal motif (VFVNFA) in ABCA1 between residues 41–46, next to the PDZ-binding domain (first four residues) binds a yet unidentified protein (Fitzgerald et al. 2004). Mutation of this motif eliminated both apoA-I binding and efflux without alteration of ABCA1 trafficking. Therefore,  $\beta 1$ -syntrophin and utrophin form complexes with ABCA1 that stabilize this transporter, link it to the cytoskeleton and contribute to the regulation of its activity. Interestingly, we have found significantly decreased  $\beta 1$ -syntrophin (but not utrophin) mRNA expression in a transcriptome analysis of monocyte-derived macrophages from low HDL subjects with efflux defects despite normal ABCA1 sequence (Kiss et al. 2007; Sarov-Blat et al. 2007). The enhanced ABCA1 protein level in NPC1-deficient hepatocytes, which is mainly regulated by a posttranslational mechanism, is also associated with increased  $\beta 1$ -syntrophin expression (Marcel et al., unpublished data). These observations could be compatible with regulation of ABCA1 stability by syntrophins. The mechanism by which syntrophin stabilizes ABCA1 and increases cholesterol efflux remains to be elucidated.

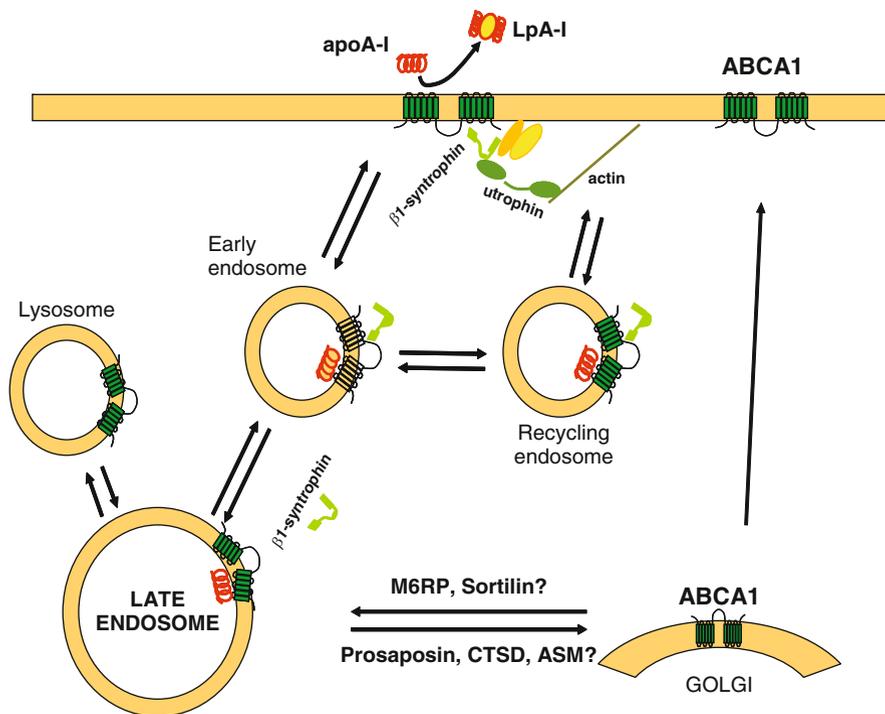
The exocytosis of neurosecretory granules is controlled by a system that presents striking analogies with the mobilization of ABCA1 (Ort et al. 2001). The islet cell autoantigen (ICA) 512, a tyrosine kinase receptor, is regulated by insulin; its cytoplasmic tail binds to  $\beta 2$ -syntrophin, which connects it to utrophin and actin. Its tail also has a PEST sequence and a  $\nu$ -calpain cleavage site adjacent to the PDZ-binding motif. Binding of  $\beta 2$ -syntrophin shields ICA512 from degradation and arrests the secretory granules on the cytomatrix. Dephosphorylation of  $\beta 2$ -syntrophin weakens its binding to ICA512, which is then degraded, triggering the release of the secretory granules to facilitate their exocytosis (such as the release of insulin by islet  $\beta$ -cells; Ort et al. 2001). The subcellular localization of diacylglycerol kinase (DGK) $\zeta$  in skeletal muscle, which converts DAG into PA, follows a similar mechanism: DGK $\zeta$ , GDP-bound Rac1 and syntrophin form a cytosolic complex, which upon PKC-mediated phosphorylation of a MARCKS domain of DGK $\zeta$ , induces translocation of the complex to the plasma membrane, where Rac1 is activated by GTP hydrolysis and Rac1 dissociates from DGK $\zeta$  and syntrophins (Hogan et al. 2001; Abramovici et al. 2003; Yakubchuk et al. 2005). One can thus

propose: (1) a similar model may apply to ABCA1, (2) syntrophins contribute to its retention in subcellular reticulated structures, large intracellular puncta or close to the cell surface (Okuhira et al. 2005 and (3) syntrophins are recruited to these sites in response to increased cellular cholesterol load, vesicular cholesterol traffic and/or apoA-I binding.

Tamehiro et al. recently identified an ABCA1-binding protein that is a subunit of the serine palmitoyltransferase enzyme, SPTLC1, which negatively regulates ABCA1 stability and cholesterol efflux (Tamehiro et al. 2008). Pharmacologic inhibition of SPTLC1 with myriocin (which resulted in the disruption of the SPTLC1–ABCA1 complex) and siRNA knockdown of SPTLC1 expression both stimulated ABCA1 efflux by nearly 60%. In 293 cells, SPTLC1 inhibition of ABCA1 activity led to the blockade of ABCA1 exit from the ER. In contrast, myriocin treatment of macrophages increased the level of cell surface ABCA1. These results indicate that the physical interaction of ABCA1 and SPTLC1 results in intracellular retention of ABCA1 protein, in opposition to the effect of syntrophins (Tamehiro et al. 2008).

### ***3.5.2 Sorting of ABCA1 Between Golgi, Plasma Membrane and LE-Lysosomes: Contribution of Sortilin***

The short half-life of ABCA1 requires ongoing synthesis and selective trafficking to replenish the active pools. Little is known about ABCA1 secretion and sorting from the Golgi. ABCA1 activity affects or is affected by the transport of cholesterol and PL, particularly sphingolipids. Secretory vesicular transport, including both raft-rich (defined by GPI-anchored proteins) and raft-poor (defined by VSVG) vesicles, is accelerated by lipid efflux to apoA-I, an effect not observed in cells with inactive ABCA1 (Zha et al. 2003). This suggests that ABCA1 activity in the Golgi alters membrane composition and stimulates vesicular movement. Recent observations indicate that the transport of prosaposin by LRP1 and GULP regulates ABCA1 in the LE compartment (Kiss et al. 2006). Prosaposin and acid sphingomyelinase (ASM) are first transported from the Golgi to lysosomes by mannose-6-phosphate receptors (M6PR) and sortilin (Ni and Morales 2006). Sortilin is an alternative receptor capable of sorting soluble lysosomal enzymes from the Golgi to lysosomes. CTSD expression increases prosaposin and saposins and upregulates ABCA1, whereas inhibition of CTSD decreases ABCA1 levels (Haidar et al. 2006). These observations suggest that the ABCA1 pathway, which is linked to the metabolism and transport of sphingolipids through LE and lysosomes, is contingent on the sorting and transport of both sphingolipids and lysosomal enzymes from the Golgi. Preliminary data suggest that sortilin but not M6PR is regulated by NPC1 (unpublished results) and may contribute to the sorting of ABCA1 and ASM, either directly or indirectly via effects on sphingolipids and cholesterol Fig. 3.2.



**Fig. 3.2** ABCA1 cellular traffic. Newly synthesized ABCA1 is delivered at the cell surface by the secretory pathway; and ABCA1 activity also modulates secretory activity and the movement of membranes from the Golgi (Zha et al. 2003). It is proposed that the ABCA1–β1-syntrophin complex illustrated in Fig. 3.1 may be released from its interaction with the utrophin–actin scaffold at the plasma membrane to enter the endosomal pathway as described by Neufeld and colleagues (2001, 2004). P6RP and sortilin have been proposed to contribute to traffic between Golgi and LE. This traffic may also include prosaposin, CTSD and ASM, which contribute to the regulation of ABCA1 activity through unknown mechanisms

### 3.6 Integrated Models of Lipid Efflux and Lipoprotein Assembly: Nascent HDL Formation

As discussed above, ABCA1 presentation at the cell surface of hepatocytes, intestinal cells, macrophages and other cell types is essential for the formation and maturation of HDL. Lipid free and/or lipid poor apoA-I are the seed molecules in the initiation of HDL formation, first intracellularly during secretion in hepatocytes and epithelial intestinal cells and second by interaction with ABCA1 at cell surface. Relevant to this process, several questions remain:

1. How does apoA-I interact with cell surface ABCA1 protein and what are the consequences?

2. Does endocytosis of the apoA-I/ABCA1 complex contribute to HDL formation?

### ***3.6.1 Interaction of apoA-I with Cell Surface ABCA1***

Earlier observations showed that optimal cholesterol efflux in macrophages requires binding of the C-terminal domain of apoA-I to a cell surface-binding site and the subsequent translocation of intracellular cholesterol to an efflux-competent pool (Burgess et al. 1999). Vedhachalam and colleagues, investigating the mechanisms of cholesterol and phospholipid efflux in macrophages, showed by cross-linking that only about 10% of cell surface bound apoA-I directly interacting with ABCA1 and the major band migrated as monomeric apoA-I. This study indicated that two apoA-I cell surface-binding sites as well as the apoA-I C-terminal domain are required for the acquisition of lipids. ABCA1 activity thus creates two types of high affinity apoA-I-binding sites at the cell surface. The low capacity site formed by direct interaction of apoA-I and ABCA1 has a regulatory role and contributes to the genesis of a second site, which has a very high binding capacity for apoA-I C-terminal-dependent binding and lipidation (Vedhachalam et al. 2007a).

In further studies, a three step model for apoA-I binding and lipidation was formulated (Vedhachalam et al. 2007b). First, the binding of apoA-I to ABCA1 at the cell surface generates a net phospholipid translocation to the plasma membrane exofacial leaflet, causing the asymmetric packing of the two leaflets and inducing a strain in the membrane. Second, the membrane strain is released by membrane bending and the creation of exovesiculated lipid domains (protrusion curvature). Third, this lipid domain allows apoA-I molecules to insert and remove phospholipids and cholesterol by C-terminal dependent binding, which results in the release of discoidal particles containing two, three or four molecules of apoA-I (Vedhachalam et al. 2007b). Here, other questions arise:

1. Does the apoA-I that first interacts with ABCA1 immediately acquire phospholipids upon interaction or does this occur while the membrane is bending?
2. Does it migrate laterally to the protrusion curvature and acquire phospholipids and cholesterol?
3. If this is the case, does this model reconcile with the two-step lipidation theory (Fielding et al. 2000)?
4. Given that the last and productive step in apoA-I binding and lipidation is dependent on an unstable phospholipid bilayer and does not involve ABCA1 interaction, should it be dependent on the oligomerization of ABCA1 (Denis, Haidar et al. 2004a)?

ApoA-I normally self-associates to form oligomers. Its three-dimensional structure is that of a helical bundle structure in which the N- and C-termini are in close proximity (Silva et al. 2005) and their interaction mediates the self-association of lipid-free apoA-I (Silva et al. 2005; Zhu and Atkinson 2007). This suggests that

oligomerization during efflux may be largely dependent on apoA-I itself and occurs when apoA-I concentration increases at the site of its recruitment for efflux. Kinetic studies of the formation of apoA-I lipidated particles by ABCA1 in J774 macrophages and HEK-293 cells showed concurrent formation of particles with two, three and four apoA-I molecules with no evidence of a precursor–product relationship, therefore compatible with a model of nascent apoA-I oligomers being lipidated at the plasma membrane.

### **3.6.2 Contribution of Retroendocytosis**

The contribution of retroendocytosis of ABCA1 and apoA-I to the ABCA1-mediated lipidation of apoA-I was initially proposed based on the observation that ABCA1 traffic is triggered by the addition of apoA-I (Neufeld et al. 2001, 2004). This issue has recently been revisited. Cell surface biotinylation experiments revealed that two-thirds of apoA-I was bound at the cell surface and one-third in intracellular compartments in an ABCA1 and apoA-I C-terminal domain-dependent manner. Quantification of the turnover of apoA-I at these sites demonstrated a four-fold faster dissociation of apoA-I from the internal compartment compared to the plasma membrane, suggesting a greater contribution of the internal ABCA1 compartment to apoA-I lipidation (Hassan et al. 2008). This conclusion was not consistent with the studies of the other two groups. Faulkner and colleagues determined the functional significance of ABCA1-mediated endocytosis of bacterially expressed apoA-I in which cysteines were introduced at strategic positions to allow either fluorescent labeling or <sup>35</sup>S-cysteine labeling in murine macrophage cell line RAW264.7. The majority of internalized <sup>35</sup>S-apoA-I was re-secreted as a degraded protein. Furthermore, specific inhibitors were able to uncouple apoA-I lipidation from apoA-I internalization (Faulkner et al. 2008), casting a doubt on the significance of the endocytic pathway in lipid efflux or HDL formation. Denis and colleagues, who also analyzed apoA-I internalization and lipidation as a function of ABCA1 expression by confocal microscopy and efflux assays, concluded that the majority of HDL formation occurs at the cell surface, whereas the ABCA1-dependent internalization of apoA-I leads to its targeting to lysosomes and degradation (Denis et al. 2008). The apparent contradictions concerning the role of apoA-I endocytosis in ABCA1-mediated lipidation may be related to the duration of apoA-I interaction with the cells or to the cholesterol loading status and cell types. Hassan et al. (2008) have suggested that the half-time for apoA-I and ABCA1 interaction, leading to the resecretion of lipidated apoA-I was shorter than that which culminated in lysosomal targeting and degradation (Denis et al. 2008).

Further studies will be required to identify and validate the endocytic pathways that lead to apoA-I lipidation rather than to lysosomal targeting and degradation. It is still unclear whether the targeting of apoA-I and ABCA1 to lysosomes and their degradation is a function of the length of physical interactions or possibly a function of cellular cholesterol levels. Cells with excess cholesterol should increase ABCA1

presentation at the cell surface in order to bind apoA-I for its productive lipidation, whereas cells with low cholesterol levels should express little cell surface ABCA1 and target apoA-I that binds to ABCA1 for degradation in lysosomes.

### **3.7 Complementarities of ABCA1, ABCG1 and SR-BI in Lipid Efflux and HDL Formation and Their Combined Role in Reverse Cholesterol Transport in Vivo**

Many lipid transporters have been identified that display tissue specific expression and regulation and exhibit different functions. For example, ABCA1 and ABCG1/ABCG4 exist in almost all tissues, while ABCG5/ABCG8 is exclusively expressed on the apical (bile canalicular) surface of hepatocytes where it mediates cholesterol excretion via bile. ABCA7 is highly homologous to ABCA1 and mediates cellular cholesterol and phospholipid release to apolipoproteins when transfected in vitro. Intriguingly, in murine fibroblasts, ABCA7 expression is lowered by increased cellular cholesterol whereas ABCA1, in turn, is upregulated. However, silencing ABCA7 does not reduce efflux to apoA-I but decreases phagocytic activity (Iwamoto et al. 2006), suggesting a role distinct from that of ABCA1. SR-BI is an HDL receptor and a multifunctional cholesterol transporter (Trigatti et al. 2000), which mediates selective uptake of HDL cholesteryl esters in hepatocytes and adrenergic cells. It also contributes to bidirectional cholesterol flux to and from lipoproteins in various cell types, including macrophages.

#### **3.7.1 HDL Genesis in Various Types of Cells**

The predominant pathway for efflux to apoA-I and the formation of pre- $\beta$ -migrating HDL is an ABCA1-dependent mechanism. It is still unclear whether ABCA1 alone operates in all cell types for mature HDL formation when the incubation time is extended, or when HDL is remodeled in the circulation. Also unclear are the conditions and factors that modulate the maturation of pre- $\beta$ -HDL to  $\alpha$ -HDL. When human fibroblasts, HepG2 or Caco2 cells are treated with LXR and RXR agonists (22-OH, 9-cis RA) or when macrophages are activated with cAMP and then incubated with apoA-I for 24 h,  $\alpha$ -migrating HDL are formed (Krimbou et al. 2005; Duong et al. 2006). In contrast, pre- $\beta$ -migrating HDL particles are observed after 16 h incubation of non-stimulated cells, including human hepatoma cells (HepG2), human intestinal transformed cells (Caco2) and CHO cells stably expressing full-length human apoA-I (Chau et al. 2006). Similarly, HEK293 cells overexpressing ABCA1, but not expressing ABCG1, SR-BI, LCAT, PLTP or apoM, form pre- $\beta$ -HDL particles of various sizes, but not  $\alpha$ -HDL (Mulya et al. 2007). This study, designed to eliminate the contribution of the known HDL-modifying factors, showed that overexpression of ABCA1 alone did not generate  $\alpha$ -migrating HDL. Most

importantly, the minimally lipidated pre- $\beta$ -HDL particles formed by ABCA1 in these cells had reduced ability to again interact with ABCA1. This observation suggested that ABCA1 could depend on other transporters for further lipidation of the various pre- $\beta$ -HDL particles formed. ABCG1 is the best candidate for this process. ABCG1 is a half transporter, which may homodimerize or heterodimerize with ABCG4 to form a full transporter that functions at the cell surface (Cserepes et al. 2004). The cholesterol efflux activity of ABCG1 and ABCG4 to both smaller HDL<sub>3</sub> and larger HDL<sub>2</sub>, but not to lipid free apoA-I, was first described in 293 cells transfected with both transporters (Wang et al. 2004). The complementarities between ABCA1 and either the ABCG1/ABCG4 heterodimer or potentially the homodimers of ABCG1 or ABCG4 were extended and demonstrated in several studies (Gelissen et al. 2006; Vaughan and Oram 2006), whereas SR-BI activity could not complement ABCA1-mediated efflux (Lorenzi et al. 2008). However, ABCG1 $-/-$  mice display normal levels of normal plasma HDL, indicating that a compensatory mechanism may exist or that ABCG1 is redundant for HDL formation. Further investigations are needed to clarify the physiological complementarities of these lipid transporters. We also need to identify the factors controlling the transformation of pre- $\beta$ - to  $\alpha$ -HDL, particularly in hepatocytes, which account for up to 80% of total circulating HDL, but which mainly form pre- $\beta$ -HDL in vitro (Kiss et al. 2003).

### 3.7.2 Cholesterol Efflux to apoA-I in Macrophages

Lipid efflux to apoA-I is solely dependent on ABCA1 in cholesterol loaded macrophages, but to a lesser extent in unloaded macrophages (Wang et al. 2007b). ABCG1 inactivation does not affect circulating HDL, however *Abcg1* $-/-$  mice challenged with a high fat diet show significant cellular accumulation of neutral lipids and phospholipids in hepatocytes, macrophages and other cells (Kennedy et al. 2005), pointing to a very different role for ABCG1 in cellular lipid transport. ABCG1 might also provide an additional mechanism for cholesterol removal under stress conditions. Of interest, macrophages of patients with type 2 diabetes patients have no detectable ABCG1 but normal levels of ABCA1 (Mauldin et al. 2008). ABCG1 can efflux cholesterol to lipoprotein particles arising from ABCA1-mediated efflux to apoA-I, suggesting that the two transporters act in concert to maximize the efflux of excess cholesterol from macrophages (Gelissen et al. 2006; Vaughan and Oram 2006), although this process and its functional significance are not fully resolved (Cavelier et al. 2006; Jessup et al. 2006). Adorni et al. (2007) quantified the relative contribution of different transporters to cholesterol efflux to human sera in loaded or unloaded macrophages, both in vitro and in vivo. In normal unloaded macrophages, ABCA1 ablation has no effect on total efflux whereas in cholesterol loaded macrophages ABCA1 accounted for 37% of the net efflux, ABCG1 for 21% and SR-BI for 9%, with the remainder attributable to diffusional efflux, consistent with similar results for the relative contributions of these transporters to in vivo reverse cholesterol transport (Adorni et al. 2007).

### 3.7.3 *In Vivo Cholesterol Efflux from Macrophages and Reverse Cholesterol Transport*

Ablation of macrophage *Abca1* (Wang et al. 2007c) and ABCG1 (Wang et al. 2007b) significantly decrease in vivo reverse cholesterol transport in wild-type mice. In contrast, total body ablation of *Abca1* does not affect hepatobiliary metabolism, a result linked to the low circulating HDL and plasma cholesterol levels in these mice (Groen et al. 2001). Interestingly, experiments in mice with either *Abca1* ablation, liver specific *Abca1* overexpression or total *Abca1* expression showed that hepatic ABCA1 levels contributed to HDL catabolism and HDL-associated CE selective uptake in hepatocytes (Singaraja et al. 2006), suggesting that ABCA1 in hepatocytes might also be involved in reverse cholesterol transport. Although the contribution of SR-BI to efflux from cholesterol loaded macrophages may be minimal since SR-BI expression in these cells is almost completely suppressed (Yu et al. 2004; Wang et al. 2007b), bone marrow transplantation experiments have demonstrated that macrophage SR-BI deficiency leads to increased atherosclerotic lesion size (Zhang et al. 2003). Increased liver specific expression of SR-BI was also shown to significantly increase reverse cholesterol transport from peripheral tissues (Zhang et al. 2005), further corroborating the critical role of liver SR-BI in HDL uptake.

Macrophage ABCG1 is a mediator of cholesterol efflux to HDL in vitro and a significant contributor to in vivo reverse cholesterol transport from cholesterol loaded macrophages, but its effects on atherosclerosis in mouse models are complex. Studies of bone marrow transfer of *Abcg1*<sup>-/-</sup> macrophages to *Ldlr*<sup>-/-</sup> or *ApoE*<sup>-/-</sup> mice by several investigators showed unexpectedly that arterial atherosclerotic lesions were not increased, but rather decreased (Baldan et al. 2006; Ranalletta et al. 2006). In contrast, Out et al. (2007) reported that whole body *Abcg1* deficiency conferred a significant 1.9-fold increase in atherosclerotic lesion size in mice fed with atherogenic diet for 12 weeks as compared to wild-type mice (Out et al. 2007). Further experiments by Yvan-Charvet et al. (2008) showed that bone marrow transfer from mice with combined *Abcg1* and ABCA1 deficiency to *Ldlr*<sup>-/-</sup> mice fed a high fat diet resulted in a 2.5-fold increase in atherosclerosis as compared to control or single *Abca1*<sup>-/-</sup> bone marrow recipients. The isolated *Abca1*<sup>-/-</sup> and *Abca1*<sup>-/-</sup> *Abcg1*<sup>-/-</sup> macrophages also had impaired efflux to apoA-I and HDL and increased susceptibility to apoptosis compared to *Abcg1*<sup>-/-</sup> macrophages (Yvan-Charvet et al. 2007). A follow up study by Out et al. (2008) demonstrated that *Ldlr*<sup>-/-</sup> mice transplanted with bone marrow from *Abca1*<sup>-/-</sup> *Abcg1*<sup>-/-</sup> mice and fed a Western diet for 6 weeks developed atherosclerotic lesions smaller than those of *Ldlr*<sup>-/-</sup> mice receiving ABCA1<sup>-/-</sup> macrophages, but larger than those receiving *Abcg1*<sup>-/-</sup> macrophages. A striking observation was the massive accumulation of foam cells in mice receiving *Abca1*<sup>-/-</sup> *Abcg1*<sup>-/-</sup> bone marrow, despite their very low plasma cholesterol (Yvan-Charvet et al. 2007; Out et al. 2008), suggesting a purely cellular phenotype. Interestingly, the *Abca1*<sup>-/-</sup> *Abcg1*<sup>-/-</sup> macrophages displayed marked lipid accumulation, both in vitro and in vivo, but were not recruited to the arterial wall (Out et al. 2008), presumably because the low

circulating cholesterol level in these mice did not create the arterial wall inflammation required for monocyte recruitment. Together these experiments suggest that the activation of ABCA1 and ABCG1 has both additive and distinct effects on lipid accumulation that affect macrophage susceptibility to apoptosis.

## 3.8 Cellular Lipid Traffic Through the Late Endosomes

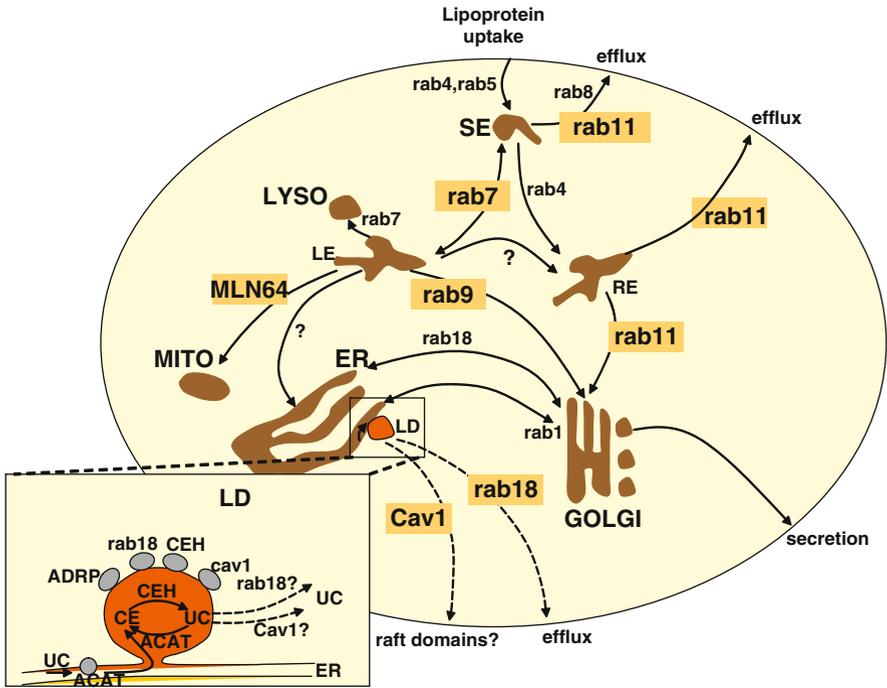
### 3.8.1 *Egress of Cholesterol from LE*

Cellular lipids originating from either exogenous lipoproteins internalized via endocytosis or phagocytosis of cell debris, as well as de novo synthesized lipids, need to be sorted and directed to the proper cellular organelle. Circulating plasma lipoproteins (i.e. LDL) are taken up by the endocytic route, which fuses with early endosomes and rapidly traffics to sorting endosomes, where some constituents are recycled to the cell surface. The recycling endosome membranes are rich in cholesterol and henceforth in phospholipids with cholesterol affinity (Ikonen 2008), which account for the high proportion of LDL-derived cholesterol that is transferred to this compartment (Wang et al. 2007c). Receptor-bound LDL particles are then delivered to the late endosome/lysosome compartments. The hydrolysis of cholesteryl ester in the LDL particle is initiated in early endosomes, but mainly occurs in late endosomes/lysosomes (Brown and Goldstein 1986; Sugii et al. 2003). Normally, cholesterol released from the late endosomes transfers to other membranes, including recycling endosomes and the plasma membrane for efflux, ER for esterification, LD for storage and to mitochondria. This transport is assumed to involve the segregation of endosomal membranes, but its rapidity has so far prevented the identification of the primary intermediate membranes. Molecular transfer by cholesterol binding proteins also takes place, but it is unclear whether it mediates the bulk transport. Sterol-carrier protein 2 (SCP-2) and its homologue SCP-x, were first considered but appeared to traffic toward peroxisomes and to be involved in the oxidation of branched fatty acids (Gallegos et al. 2001). Furthermore, overexpression of SCP-2 in hepatocytes minimally altered cholesterol traffic to mitochondria (Ren et al. 2004). MLN64 is a well characterized cholesterol binding protein. Its N-terminal domain binds to late endosomes, leaving the C-terminal cholesterol binding domain START exposed to the cytosolic side (Alpy and Tomasetto 2006) where it participates in actin-mediated dynamics of late endosomes (Holtta-Vuori et al. 2005). MLN64 presumably transports cholesterol to cytosolic acceptors or membranes. Cellular overexpression of MLN64 contributes to the mobilization of lysosomal cholesterol and its transfer to mitochondria in a START domain-dependent fashion (Zhang et al. 2002), supporting a role for MLN64 in lipid transport from the LE. However, targeted mutation of the MLN64 cholesterol-binding domain, the StAR-related lipid transfer (START) domain, caused only moderate alterations in cellular sterol metabolism

(Kishida et al. 2004), perhaps reflecting the functional redundancy of the 15 murine proteins containing a START domain (Alpy and Tomasetto 2006). The START member, StARD1, appears essential for cholesterol transfer to the inner mitochondrial membrane in steroidogenic tissues for the regulated synthesis of steroid hormone (Alpy and Tomasetto 2005). StarD1 overexpression in hepatocytes increased bile acid biosynthesis by 5.7-fold, as compared to a 1.2-fold increase with overexpression of MLN64 (Ren et al. 2004). The members of StarD4 subfamily of START proteins (StarD4, StarD5, StarD6) are newly identified cytosolic cholesterol binding proteins of the START-domain protein family. Like StarD1, StarD4 only binds cholesterol (Rodriguez-Agudo et al. 2008), and its expression in mouse liver is negatively regulated by cholesterol loading and positively regulated by SREBP-2 (Soccio et al. 2002). StarD4 transfection in COS-1 cells significantly stimulated LXRE reporter activity as did transfection with StarD1, MLN64 and STARD5 (Soccio et al. 2005). In contrast, StarD5, which binds both cholesterol and 25-hydroxycholesterol, is regulated by ER stress (Soccio et al. 2005). StarD5 is highly expressed in macrophages (Rodriguez-Agudo et al. 2006) and further upregulated in free cholesterol loaded macrophages (Soccio et al. 2002, 2005).

### ***3.8.2 Regulation of Cholesterol Traffic in LE***

Cholesterol export from LE is regulated by the sequential and independent actions of late endosome luminal soluble NPC2 and membrane-bound NPC1 (Liscum and Sturley 2004), as demonstrated by the arrest of cholesterol transport at that stage by functional mutations in the genes encoding for these two proteins. NPC2 protein has a unique cholesterol binding pocket, whereas the 240-amino-acid soluble luminal loop of the late endosome membrane bound NPC1 can bind cholesterol and oxysterols and is regulated by cellular cholesterol levels (Infante et al. 2008a, b). In addition to cholesterol, other lipids, particularly sphingolipids, accumulate in the late endosomes, where together with lyso-bis phosphatidic acid (LBPA), which induces membrane invagination, they form luminal membranes that are typical of the multi-vesicular bodies (MVB; Matsuo et al. 2004). These densely packed membrane structures account for the high cholesterol storage capacity of LE and MVB (Russell et al. 2006). Glycosphingolipid storage disorders show that the storage of sphingolipids in LE contributes to a secondary accumulation of cholesterol (Pagano 2003), suggesting that the enrichment of LE in both sphingolipids and cholesterol impairs endosomal traffic. Incubating cells with exogenous sphingolipids results in their intracellular accumulation, which in turn increases LDL internalization by the LDL receptor and consequently cholesterol uptake (Puri et al. 2003). This suggests a model in which sphingolipids in endocytic compartments stimulate cholesterol retention in membranes, thus serving as “a molecular trap”. Hence, overload and storage of either sphingolipids or cholesterol can impair late endosomal functions Fig. 3.3.



**Fig. 3.3** Cellular cholesterol transport pathways. Various Rab GTPases have been shown to regulate the vesicular traffic of cholesterol-rich membrane and cargo from the sorting endosome (SE), the recycling endosome (RE), the late endosome (LE) to the Golgi and the endoplasmic reticulum (ER) and the lipid droplet (LD). *Highlighted Rab proteins* have been shown to enhance transport of cholesterol to the plasma membrane. LD cholesterol undergoes cycles of esterification by ACAT and hydrolysis by cholesteryl ester hydrolase that allows its export from the cell by a number of putative pathways, including the secretory pathway, vesicular transport via rab18, caveolar transport and others

The exact mechanism by which cholesterol and sphingomyelin are mobilized from the LE is not known, but several lines of evidence show that cholesterol traffic can be rescued by Rab-dependent membrane transport. Overexpression of Rab7 (which mediates cargo progression from early to late endosomes) and Rab9 (which characterizes LE to lysosome conversion) but not Rab11 (which is associated with recycling endosomes that recycle to the plasma membrane (Zerial and McBride 2001), reduced LE cholesterol accumulation. This was accompanied by an increase in cholesteryl ester formation and by normalization of glycosphingolipid transport (Choudhury et al. 2002; Narita et al. 2005). Interestingly, TIP47, a cargo adaptor for MPR, was reported to traffic from LE to the Golgi together with MPR (Diaz and Pfeffer 1998; Carroll et al. 2001). TIP47 binds to a cytosolic domain of MPR and activated (GTP-bound) Rab9 (Carroll et al. 2001). Upon

reaching the Golgi, TIP47 may be released to its stable cytosolic form and/or transferred to the lipid droplet (LD). In cells exposed to fatty acids and accumulating triacylglycerides, TIP47 association with LD increases (Wolins et al. 2001) and stabilizes the forming LD (Wolins et al. 2006). Interestingly, TIP47 was shown to be an inhibitor of retinyl ester hydrolysis in keratinocytes (Gao and Simon 2006), suggesting that it could also regulate the mobilization of cholesteryl esters from LD.

In summary, traffic of lipids and specially cholesterol out of LE clearly depends on functional NPC1 and NPC2 proteins, on molecular transporters such as MLN64 and on membrane-mediated transport that are either inhibited by accumulation of sphingolipids and/or cholesterol or stimulated by vesicular mechanisms that depend on specific Rab GTPases. Exit of cholesterol and sphingolipids from LE allows transport to a various cellular sites, including movement of excess cholesterol to the ER for storage in lipid droplets and efflux to exogenous acceptors.

### 3.9 Cholesterol Traffic Through the Lipid Droplet

All cells can assemble lipid droplets, which are dynamic organelles that serve as the sites of accumulation and storage of excess fatty acids and cholesterol in the form of neutral triacylglycerides and cholesteryl esters. Newly synthesized cholesterol as well as lipoprotein-derived cholesterol can be incorporated into lipid droplets in the ER where the ER-resident protein acyl-CoA:cholesterol acyl transferase (ACAT) catalyzes the esterification of excess cholesterol for storage in LD. In this organelle, cycles of hydrolysis and re-esterification control cholesterol availability for cell membranes and for efflux to cholesterol acceptors. The LD is recognized as a metabolically active but atypical intracellular organelle (Brasaemle 2007) constituted of a hydrophobic core of triacylglycerides (TG) and cholesteryl esters (CE) which are surrounded by a phospholipid monolayer coated with specific proteins. Several proteomic studies of lipid droplet proteins have identified signature coat proteins, including the PAT family of proteins [perilipin, adipophilin/adipocyte differentiation-related protein (ADRP), TIP47, S3-12, OXPAT, LSD1, LSD2]; for a review, see Brasaemle (2007). Many of these proteins are exchangeable and stable either in lipid bound form or free in the cytosol, a property which may be structurally linked to plasma exchangeable apolipoproteins. TIP47 for example contains 11-mer repeats which can form amphipathic  $\alpha$ -helices organized in a domain similar to the N-terminal  $\alpha$ -helical bundle of apoE, which could explain its stability in both lipid bound and soluble forms (Wolins 2006). In keeping with the postulated ER origin of the LD, several enzymes of the neutral lipid synthetic pathways that reside in the ER have also been found to be associated with this organelle (Coleman and Lee 2004), including ACAT1 (Lin et al. 1999). SNARE proteins, including SNAP23, syntaxin-5, VAMP4, NSF and  $\alpha$ -SNAP, are associated with LD and represent an essential mechanism to mediate their fusion and growth (Bostrom et al. 2007).

### ***3.9.1 Regulation of Cholesterol Traffic in the Adipocyte LD***

The cholesterol content and nature of LD differ between cells, reflecting the specialized nature of lipid storage. The adipocyte LD is primarily enriched in TG, but its cholesterol store is unique because of its high free cholesterol localized to the surface of the LD and very low cholesteryl ester content. It is remarkable that this LD-associated free cholesterol store represents up to 30% of adipocyte cellular cholesterol (Prattes et al. 2000). Since the adipocyte synthesizes very little cholesterol (Angel 1970), cellular cholesterol homeostasis is maintained through a balance between cholesterol export and import. Early studies demonstrated cholesterol efflux to HDL by adipocytes (Barbaras et al. 1986, 1987), but it is not known whether this efflux is mediated by ABCG1 or SR-BI. Insulin and angiotensin II induce the translocation of SR-BI from intracellular sites to the plasma membrane in adipocytes, a process associated with increased HDL cholesterol uptake and increased adipocyte cholesterol content (Tondu et al. 2005; Yvan-Charvet et al. 2007). In these adipogenic conditions, selective uptake of cholesteryl esters by SR-BI is stimulated, leading to cholesterol accumulation. In human adipocytes, SR-BI, CETP and LRP1 make similar contributions to selective uptake of HDL-CE (Vassiliou and McPherson 2004). Upon  $\beta$ -adrenergic stimulation of lipolysis, cholesterol efflux to HDL increases, likely related to liberation of free cholesterol on the surface of the LD. Under these conditions, SR-BI expression is unaffected (Verghese et al. 2007). There are no data on ABCG1-mediated efflux to HDL in adipocytes; however ablation of ABCG1 in mice results in lower adipose tissue mass and decreased adipocyte size (Buchmann et al. 2007) but no changes in plasma HDL cholesterol (Kennedy et al. 2005).

Cholesterol ester transfer protein (CETP) is expressed in adipocytes (Jiang et al. 1991) and in preadipocytes (Gauthier et al. 1999), where its expression is regulated by cellular cholesterol (Radeau et al. 1995) and where it mediates selective uptake of HDL cholesteryl esters (Benoist et al. 1997). Adipocyte-specific overexpression of CETP in mice results in decreased HDL plasma levels (Zhou et al. 2006), likely due to both secretion of CETP into plasma and selective uptake of HDL-derived cholesterol.

### ***3.9.2 Regulation of Cholesterol Traffic in the Macrophage LD***

Primary monocyte-derived macrophages and macrophage cell lines primarily accumulate CE in lipid droplets upon incubation with acetylated or aggregated LDL, whereas they accumulate TG upon exposure to TG-rich lipoproteins, such as VLDL and remnant lipoproteins. This raises the question as to whether neutral lipid storage occurs in separate pure CE and TG droplets or rather in mixed CE and TG droplets. The incubation of acetylated LDL-loaded macrophages with the acyl-CoA synthetase inhibitor triacsin D, which depletes cells of accumulated TG, has been used to vary the cellular TG/CE ratio. This results in a progressive change in lipid

droplet anisotropy, indicating that TG and CE originally accumulated in mixed lipid droplets (Lada et al. 2002). Additionally, this study showed that the CE in mixed droplets in the liquid or isotropic state is effluxed more effectively than that in the liquid crystalline or anisotropic droplets resulting from the TG depletion. Interestingly, independently of the nature of the neutral lipid accumulating in the macrophage LD, lipoprotein loading leads to increased adipophilin (human ortholog of ADRP) expression in differentiated THP-1 cells concomitant with decreased perilipin content (Persson et al. 2007). This is reminiscent of the adipocyte LD maturation program, whereby as the lipid droplets acquire more lipids and enlarge, the composition of the coat proteins changes from ADRP to exclusively perilipin in the mature adipocyte (Brasaemle 2007).

### ***3.9.3 Regulation of Cholesterol Traffic in the Hepatocyte LD***

The hepatocyte LD participates in high throughput and turnover of triglycerides and cholesteryl esters, the latter either transiting through the ER secretory pathway or trafficking to the plasma membrane and other organelles. The LD of a human hepatic cell line, HuH7, apparently includes adipophilin, acyl-CoA synthetase 3 and 17 $\beta$ -hydroxy steroid desaturase 11 as its major coat proteins and contains significantly more CE than triglycerides (Fujimoto 2004). However, the latter observation may have been a result of de-differentiation of the cell line as observed for HepG2 hepatoma cells, where the impairment of triglyceride mobilization for LD storage impairs VLDL secretion (Wu et al. 1996; Tsai et al. 2007). Hepatocytes accumulate LD in both the cytosol and in the lumen of the ER, which arise from a microsomal fraction and function to provide lipids for VLDL assembly. Proteomic and lipid analyses of murine ER luminal LD showed a unique composition with the absence of coat proteins found on cytosolic lipid droplets (ADRP, TIP-47). Rather, luminal LD are characterized by the presence of proteins not found in cytosolic LD, including ER luminal carboxylesterase Ces3, commonly referred to as triacylglycerol hydrolase (TGH), the microsomal triglyceride transfer protein (MTP) and apoE (Wang et al. 2007). Intraluminal LD isolated from liver homogenates of mice fasted overnight was shown to contain significantly more TG than CE. The composition of hepatic LD, their enrichment in TG or CE, can thus be manipulated as a function of culture conditions. However, exceptions to this observation can occur. Notably, incubation of HepG2 in lipoprotein-deficient serum (LPDS)-containing medium supplemented or not with oleic acid does not appreciably change the CE/TG ratio (10%) and the CE/TC ratio (27%) (Borradaile et al. 2002).

The LD of hepatocytes as well as fibroblasts and adipocytes are associated with specific Rab GTPases, including Rab5c, Rab7, Rab11 and Rab18, supporting the dynamic nature of LD. Of these, Rab18 exclusively labels the LD surface (Martin et al. 2005; Ozeki et al. 2005). This seemingly LD-specific Rab18 is particularly interesting because its localization to the LD in adipocytes is dynamically regulated by lipolytic stimulation (Martin et al. 2005). In response

to lipolytic stimuli, Rab18 is recruited to LD and, intriguingly, Rab18-labeled LD are frequently found in association with ER (Ozeki et al. 2005) as well as with non-identified tubulovesicular elements (Martin et al. 2005). It is tempting to speculate that Rab18 functions in the displacement of lipids from metabolically active LD to supply lipids as required elsewhere in the cell. Preliminary results indicate that Rab18 overexpression in macrophages stimulates cholesterol efflux, suggesting a role for Rab18 in the mobilization of cholesterol from lipid droplets (R.S. Kiss and Y.L. Marcel, unpublished data). In hepatocytes, Rab18 localizes to LD displacing ADRP and promoting the close apposition of LD to ER-derived membranes (Ozeki et al. 2005).

### 3.10 Caveolin and Cellular Cholesterol Transport

The present discussion is limited to the contribution of caveolins to lipid traffic through LD and to the efflux pathway. The facts that caveolin-1 associates with cholesterol in a 1:1 stoichiometry (Murata et al. 1995) and is the major cellular protein associated with photoactivatable-cholesterol (Thiele et al. 2000) represent the most compelling arguments for the structural association of caveolin with cholesterol. However, the role of caveolin as an essential cholesterol carrier remains controversial. Caveolin-1 associates with LD in a regulated and transient manner (Martin and Parton 2005). Caveolin-1 and -2 traffic to LD upon addition of fatty acids and leave the LD as lipids are mobilized (Pol et al. 2004). Liver regeneration is strikingly dependent on caveolin-1, because the formation of LD is essential to this regenerative process (Fernandez et al. 2006). The function of caveolin-1 in LD regulation is only emerging. Clear evidence that caveolin is involved in the maintenance of LD integrity and cholesterol homeostasis is provided by the effect of the truncated mutant, caveolin-3DGV, which blocks LD motility and prevents re-distribution of cholesterol to the cell surface, resulting in the accumulation of neutral lipids in LD and perturbation of cell surface lipid rafts (Pol et al. 2004). Additionally, this mutant has been associated with increased free cholesterol retention in late endosomes that impairs their motility, similarly to the well-characterized phenotype of NPC1<sup>-/-</sup> cells (Pol et al. 2001). Recent work in our laboratory reveals that NPC1<sup>-/-</sup> hepatocytes themselves display a 3-fold increase in caveolin-1 levels compared to the wild type, which parallels the increase in cholesterol storage in these cells (Wang et al., unpublished data). Interestingly, in these studies caveolin-1 and cholesterol co-localize in perinuclear regions of hepatocytes and accumulate in the same subcellular density fractions, but in hepatocytes cultured without oleate, caveolin-1 and cholesterol do not co-localize with LD.

Plasma membrane caveolae have been reported to mediate the efflux of cellular free cholesterol, including newly synthesized cholesterol (Fielding and Fielding 1995). Caveolin-1 expression was also shown to increase the efflux of newly synthesized cholesterol in hepatocytes (Fu et al. 2004), an effect which we also observed in HepG2 cells transfected with adeno caveolin-1 (Marcel, unpublished

data). Moreover, the Cav<sup>DGV</sup> mutant has been shown to cause a reduction in both cholesterol efflux and cholesterol biosynthesis (Pol et al. 2001). However, rat hepatoma McA-RH7777 cells stably transfected with caveolin-1, which formed more caveolae and synthesized higher levels of cholesterol, showed no change in cholesterol efflux to either HDL or apoA-I (A. McKenzie, P. Links et al., unpublished data). In conclusion, there is consensus that caveolin-1 plays a role in the transport of newly synthesized cholesterol to the cell surface for efflux but the contribution of caveolin-1 to the transport of LD-derived cholesterol remains to be further investigated.

### 3.11 Mobilization of LD Lipids for Efflux

#### 3.11.1 *The LD is the Major Source of Cholesterol for Efflux*

In macrophages, cholesteryl esters derived from endocytosed LDL or modified LDL are delivered to LE/lysosomes, where they are hydrolyzed. Excess lipoprotein-derived cholesterol is esterified by the ER-resident protein, ACAT, and stored as CE in cytoplasmic LD. Cholesterol in the LD undergoes constitutive cycles of esterification–hydrolysis (Brown et al. 1979; McGookey and Anderson 1983), whereby unesterified cholesterol released from the LD by CE hydrolysis can be effluxed to a cholesterol acceptor or re-esterified by ACAT. Although the LD represents the major source of cholesterol available to the efflux pathway, efflux from the LD has been ignored because of the emphasis placed on the mobilization of LE cholesterol. An early study concluded that acetylated LDL-derived cholesterol deposited in late endosomes/lysosomes was the preferential source of cholesterol for ABCA1-mediated efflux in murine macrophages, whereas this pathway was defective in NPC1 mutant macrophages (Chen et al. 2001). In fibroblasts, ABCA1 traffics to LE, and its overexpression stimulates efflux and corrects the Tangier phenotype (Neufeld et al. 2004). This was interpreted as evidence that the pool of ABCA1 in LE was important for efflux and could normalize the elevated cholesterol levels characteristic of Tangier cells. In parallel, deletion of the ABCA1 PEST sequence was shown to impair the internalization of cell surface ABCA1 and its transport to the LE, resulting in decreased efflux of acetylated LDL-derived cholesterol to apoA-I (Chen et al. 2005). However, with the exception of acid lipase and NPC1 deficiency, cholesterol accumulates as cholesteryl esters in LD, and not in LE in normal cells, including macrophages (Brown and Goldstein 1986). Upon addition of apoA-I, cellular LD CE stores are rapidly reduced. Thus, the maintenance of normal cellular cholesterol homeostasis requires mechanisms for the rapid mobilization and re-distribution of cholesterol from LD to efflux competent compartments. The routes of cholesterol traffic from LD to cholesterol acceptors, such as HDL and apoA-I, are not well established. It is unclear whether this process primarily occurs via vesicular or non-vesicular transport, or both. Potential contributors in

this process include Rab18 and caveolin-1, as discussed above. Whether LD-associated CE hydrolysis in macrophages is a constitutive process that results in efflux when cholesterol acceptors are present, or whether this process can be triggered by a signaling cascade initiated by the binding of cholesterol acceptors to lipid transporters at the plasma membrane is an exciting prospect to be explored.

### ***3.11.2 Hydrolysis and Mobilization of LD Cholesteryl Esters for Efflux***

Although mechanisms for cholesterol traffic from LD to an efflux compartment remain to be fully elucidated, it is becoming clear that LD-associated CE hydrolysis can be a limiting factor in cholesterol efflux. In macrophage foam cells, addition of a LXR ligand, epoxycholesterol, markedly enhanced ABCA1 and ABCG1 expression but failed to increase cholesterol efflux to HDL and/or apoA-I (Ouimet et al. 2008). Epoxycholesterol impaired the mobilization of cholesteryl esters from LD in cholesterol-loaded macrophages, indicating that hydrolysis of CE in LD is a prerequisite for cholesterol efflux. Dysregulation of hydrolysis decreases efflux, even when essential cholesterol transporters are overexpressed and cholesterol acceptors are present in excess. Additional evidence is provided by studies demonstrating that overexpression of CE hydrolases markedly increases cholesterol efflux from macrophages, whereas impaired CE hydrolase activity contributes to lipid accumulation, characteristic of foam cells in atherosclerotic lesions.

Neutral cholesteryl ester hydrolase belongs to the multigene family of carboxylesterases that are involved in drug metabolism, detoxification and these enzymes hydrolyze ester, thioester and amide bonds (for a review, see Dolinsky et al. 2004). The murine carboxylesterase 3 (Ces3), commonly referred to as triglyceride hydrolase (TGH), is expressed predominantly in the liver with lower levels in adipose tissue, macrophages, intestine, heart and kidney; and it hydrolyzes triglycerides and to a lesser extent cholesteryl esters. The human homolog of TGH, CES1, cloned from human peripheral blood macrophages, THP-1 macrophages (Ghosh 2000) and from human livers (Zhao et al. 2005), was shown to have CE hydrolase activity and has been called neutral CEH. Interestingly, nCEH overexpression in THP1 cells reduces CE accumulation and enhances ABCA1- and ABCG1-mediated cholesterol efflux (Zhao et al. 2007b), while macrophage-specific CEH expression *in vivo* reduces atherosclerosis in *Ldlr(-/-)/Ceh* transgenic mice fed an atherogenic diet (Zhao et al. 2007a). Enhanced reverse cholesterol transport from CEH overexpressing macrophages was characteristic of these *Ldlr(-/-)/Ceh* transgenic mice, demonstrating the physiological importance of the CE hydrolase and its limiting activity in murine macrophages (Zhao et al. 2007a).

Hormone sensitive lipase (HSL), a triacylglycerol lipase, also has a rate-limiting cholesteryl ester hydrolase activity in adipose tissue (Kraemer and Shen 2006) and contributes significantly to CE hydrolysis in steroidogenic tissues (Ali et al. 2005). HSL overexpression was shown to increase CE hydrolysis in macrophage foam

cells and lowered total cholesterol cellular content, although the effect of enhanced CE hydrolysis on lipid efflux in this study was not documented (Escary et al. 1998). Macrophage-specific transgenic expression of rat HSL and apoA-IV in mice challenged with an atherogenic diet has also been shown to reduce the size of aortic lesions as compared to wild-type C57BL/6J littermates (Choy et al. 2003). Taken together, these studies support the view that enhanced LD CE hydrolysis is antiatherogenic.

### ***3.11.3 Is ABCA1 Involved in the Mobilization and Traffic of LD Cholesterol for Efflux?***

The ABCA1 transporter functions both in cellular cholesterol trafficking and in plasma membrane cholesterol efflux. ABCA1 expression re-distributes cholesterol in the plasma membrane, resulting in expansion of non-raft domains with which apoA-I preferentially associates (Zha et al. 2001; Landry et al. 2006). In addition, secretory vesicular transport from the Golgi is enhanced by stimulation of cholesterol efflux by apoA-I in normal cells, but not in Tangier cells with deficient cholesterol efflux due to inactive ABCA1 (Zha et al. 2003). Structural and functional abnormalities in caveolar processing and in the *trans*-Golgi secretory network have been reported in cells lacking functional ABCA1, which indicates that the activity of this transporter affects the vesicular budding between the Golgi and the plasma membrane (Orsó et al. 2000). Together, these findings indicate that ABCA1 functions to promote vesicular trafficking of membranes in the secretory pathway and contributes to the flow of lipids from intracellular storage sites to the plasma membrane via the Golgi (Zha et al. 2003). These observations may link ABCA1 activity with the mobilization of lipids from LD to the ER and subsequent trafficking via the Golgi to the plasma membrane for lipid efflux. It is tempting to speculate that Rab18, which tethers the LD to the ER, may be involved in regulating the delivery of LD-associated cholesterol to the ER for its subsequent ensuing trafficking through the Golgi to the plasma membrane for efflux.

## **3.12 Conclusions**

Cellular lipid traffic and homeostasis integrates the regulation of cellular lipid metabolism with cellular lipid uptake and with cellular lipid export. We emphasize here that lipid and particularly cholesterol traffic are intimately linked to lipid secretion and efflux. The coordinated regulation of cholesterol efflux pathways starts with de novo synthesis of cholesterol and lipoprotein uptake and with their cellular traffic by vesicular and molecular transport mechanisms, which together with extracellular lipid acceptors control the transporters involved in cellular export.

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# Chapter 4

## Bile Acids and Their Role in Cholesterol Homeostasis

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**Abstract** Bile acids are synthesized from cholesterol and have long been thought to be just a degradation product with an additional function in food digestion. During the past decade many new functions of bile acids emerged and, instead of functioning at the interphase of the outside world and the body, bile acids turned out to be extremely important signal transduction molecules which play an important role in balancing flux through diverse metabolic pathways. In this chapter we focus on the function of bile acids in regulation of cholesterol homeostasis at both the cellular and organismal level.

### 4.1 Introduction

The word cholesterol has a negative connotation due to its association with cardiovascular disease. However, cholesterol in itself is a molecule of undisputed biological importance and has a variety of functions in higher eukaryotes. Cholesterol is first of all an essential structural component of cell membranes. Owing to its bipolar structure, it is located inside the lipid bilayer, generates a semi-permeable barrier between cellular compartments and regulates membrane fluidity. As a component of HDL and LDL, it travels through the blood as part of a system that regulates the distribution of cholesterol in various tissues. Cholesterol is also a precursor for steroid hormones and, although this process is quantitatively not very important, it has a major physiological impact. The six different steroid hormones in humans function as lipophilic signalling molecules during metabolism, growth and reproduction. The human body is capable of producing the daily need of cholesterol and therefore does not need cholesterol from food as an additional source. However, it does take up cholesterol from food highly efficiently and, when the food supply of cholesterol is high, the excess cholesterol can be stored for short-term buffering as

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cholesterol esters in the liver. Cholesterol in itself cannot be degraded but high levels of cholesterol provide a negative feedback signal that stops *de novo* synthesis, preventing cholesterol overload (Engelking et al. 2005). The biosynthesis of bile represents the prime pathway of cholesterol catabolism. Approximately 90% of the cholesterol that is taken up by food or that is produced *de novo* is eventually converted into bile acids. In this manner superfluous cholesterol can be eliminated from the body. Cholesterol can also be directly excreted via a pathway involving direct transintestinal excretion (Kruit et al. 2005; van der Velde et al. 2007) although the contribution of this pathway differs strongly between different species.

Under normal conditions, depending on the *species*, about 50% of cholesterol is absorbed (Bhattacharyya and Eggen 1980; Crouse and Grundy 1978; Wang et al. 2001). Bile acids play a role in cholesterol absorption by emulsifying lipids and allowing them to travel from the aqueous luminal milieu to the brush border membrane of enterocytes. Here, cholesterol is taken up by specific receptors as discussed in this chapter. There appears to be a relationship between circulating levels of bile acids and cholesterol (Bays and Goldberg 2007). This association is now the subject of extensive research and it is clear that bile acids do not only serve as physiological detergents in the intestine. By acting as a nuclear hormone receptor activator, they regulate the expression of important genes in homeostasis of lipid, glucose and cholesterol as well as their own synthesis (Scotti et al. 2007; Thomas et al. 2008; Zimmer and Gespach 2008). Furthermore, bile acids have been described to regulate energy homeostasis at least in mice (Houten et al. 2006).

A vision emerges of a complex interplay between bile acids and cholesterol, whereby bile acids control cholesterol homeostasis by regulation of synthesis, catabolism and uptake, and where the supply of cholesterol is needed for *de novo* bile acid synthesis. This interplay involves both physical interactions and control at the level of gene transcription. In this chapter we discuss the homeostasis of bile acids and cholesterol, how these two are related and how they influence each other.

## 4.2 Bile Acid Synthesis

Bile is formed by the liver and consists of bile acids, cholesterol, phospholipids and waste products. After synthesis, bile acids are transported across the canalicular membrane into bile canaliculi and, in species such as mice and humans, are stored in the gallbladder (Hofmann 1990; Hofmann and Hagey 2008). When food is ingested they stream into the small intestine, where they help with the digestion. At the end of the ileum, they are re-absorbed by active transport and returned to the liver. This enterohepatic cycling of bile acids is highly efficient and can take place two to three times during a meal. However, about 5% escapes re-absorption in the intestine and is lost in the faeces (Hofmann 1990; Hofmann and Hagey 2008). This loss is compensated for by neosynthesis from cholesterol in the liver. The biosynthesis involves a variety of enzymes in the endoplasmic reticulum, mitochondria, cytosol and peroxisomes.

During the process of bile acid synthesis, the conformation of the cholesterol molecule changes from *trans* to *cis*. As a consequence, all hydrophilic groups move to one side of the molecule, making it strongly amphiphatic and providing it with the ability to form micelles (for reviews, see Hofmann 1999; Hofmann and Hagey 2008).

The most abundant human bile acids are the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA). After synthesis the amino acids glycine or taurine can be added to carbon 24 via an amide bond; and formally we should then speak in terms of bile salts. This final conjugation takes place before secretion into bile and provides the bile with stronger detergent properties to facilitate lipid and vitamin absorption in the intestine. Mice have a different bile acid pool with mostly muricholic acid conjugated to taurine. These bile acids can then be modified by the intestinal flora, giving rise to secondary bile acids, lithocholic acid and deoxycholic acid. These secondary bile acids are highly toxic, especially lithocholic acid (Hofmann and Hagey 2008; Russell 2003).

According to the current concept, two different routes exist for bile acid synthesis: the “classic or neutral” pathway and the “alternative or acidic” pathway. The classic route is also called the neutral route because the intermediates in this pathway are neutral up to the last steps of the synthesis route. This pathway consists of various steps in which first the sterol nucleus is hydroxylated by the microsomal enzyme cholesterol 7 $\alpha$ -hydroxylase (CYP7A1). This is also the step that is highly regulated, as discussed in the next paragraph. Other modifications of the sterol nucleus include saturation of the double bond, epimerization of the 3 $\beta$ -hydroxyl group and hydroxylations. This is followed by shortening of the side-chain to 3 C-atoms and, finally, carboxylation of the last C-atom of the side-chain (for an extensive description of BA biosynthesis, see the reviews by Chiang 2004; Russell 2003). The alternative pathway starts with side-chain modification by the enzyme sterol 27 hydroxylase (CYP27A1). In subsequent steps in the pathway the steroid ring structure of the formed oxysterols are modified by 7 $\alpha$ -hydroxylation. This step is not catalysed by CYP7A1, but by CYP7B1. The enzyme CYP7B1 is structurally similar to CYP7A1, but has different and broader substrate specificity (Norlin and Wikvall 2007). The relative contribution of these pathways to total bile acid synthesis varies between species and with various physiological and pathological conditions. The neutral pathway is considered quantitatively the most important because its contribution to total bile salt synthesis is ~90% in humans and ~75% in mice (Chiang 2004; Russell 2003). Both CA and CDCA are formed by this pathway in roughly equal amounts in humans.

#### ***4.2.1 Regulation of Synthesis by Nuclear Receptors***

Maintaining a balance between bile acid synthesis, secretion and intestinal re-absorption is vital since every aspect of their homeostasis is linked to various important physiological processes. Under normal conditions, accumulation of bile acids in hepatocytes is avoided through a tight control of uptake, synthesis and

secretion; and this control is organized by a series of feedback and feed-forward autoregulatory processes. These mechanisms involve the participation of a series of nuclear receptors which function as ligand inducible transcription factors.

#### ***4.2.2 Oxysterol Feed-Forward Regulation of Bile Synthesis***

In mammals, cholesterol homeostasis is maintained by the control of uptake, de novo synthesis, storage as cholesterol esters and catabolism. When the cholesterol supply from food is high, a feed-forward pathway is activated that leads to the catabolic elimination of cholesterol as bile acids. The rate of bile acid synthesis parallels the activity of CYP7A1, which is the probably the main rate-controlling enzyme of the bile acid biosynthetic pathway (Russell 2003). Activation of CYP7A1 expression is mediated by LXR $\alpha$ , a nuclear receptor that binds oxysterols formed during the de novo synthesis of cholesterol (Chen et al. 2007). LXR belongs to the family of nuclear receptors; it is expressed in liver, spleen, adipose tissue, lung and pituitary and requires heterodimerization with RXR to become functionally active (Goodwin et al. 2008). The generation of LXR knockout mice provided insight in the action of LXR. LXR knockout mice accumulate large amounts of cholesterol in the liver on a cholesterol-rich diet, but cannot respond to this with up-regulation of CYP7A1 (Peet et al. 1998a, b). Furthermore, these mice have changes in various genes such as SREBP1 and 2, HMGCR, HMGCS and SCD, suggesting a wide range of functions for this nuclear receptor (Peet et al. 1998b; Quinet et al. 2006). LXR is highly conserved between humans and rodents. However, LXR has much less effect on hamster and human CYP7A1, which lack a LXR binding motif. It therefore seems that rat and mouse are unique in the ability to convert excess cholesterol to bile acids by activation of LXR and subsequent stimulation of CYP7A1 (Peet et al. 1998a).

#### ***4.2.3 Bile Acid Feedback Regulation of Bile Synthesis***

Initial studies showed that interruption of the enterohepatic circulation of bile acids by biliary diversion or treatment with bile acid binding resins increases the rate of bile acid synthesis and the activity of CYP7A1 by about 3- to 4-fold (Dueland et al. 1991; Gustafsson 1978). Conversely, expansion of the bile acid pool by intraduodenal infusion of bile acids suppresses CYP7A1 expression and reduces the rate of bile acid synthesis (Nagano et al. 2004). No direct bile acid binding site was detected in the promoter of CYP7A1 and the mechanism behind the feedback regulation was unknown for a long time. This changed in 1999, when it was discovered that bile acids are the endogenous ligands for FXR (NR1H4; Makishima et al. 1999). It was known by that time that oxysterols positively induce bile synthesis via LXR and it was postulated that the feedback mechanism also involves the activation of a nuclear receptor. The nuclear receptor FXR was a candidate because: (i) it is specifically expressed in tissues where bile acids function (such as the liver, intestine, kidney),

(ii) it is evolutionarily related to LXR and (iii) it also functions as a heterodimer with the retinoid X receptor (RXR). FXR belongs to a family of transcription factors [the nuclear receptor (NR) superfamily] that is involved in diverse physiological functions such as reproduction, development and metabolism (Kuipers et al. 2007; Rader 2007). Various studies have now shown that FXR regulates a network of genes involved in synthesis, metabolism and transport of bile acids.

The suppression of CYP7A1 promoter activity through the activated FXR-RXR complex is mediated by an indirect mechanism involving interaction with other transcription proteins. Binding of bile acids to the FXR-RXR complex induces the transcription of SHP (small heterodimer partner). SHP is a receptor that binds to and inhibits a third receptor, the liver receptor homologue 1 (LRH-1 or NR5A2). LRH-1 is an orphan receptor that positively regulates CYP7A1 by binding to BARE-II in the CYP7A1 promoter (del Castillo-Olivares and Gil 2000; Lee and Moore 2002). The interaction between SHP and LRH-1 blocks the transcriptional activity of LRH-1; and CYP7A1 expression is stopped leading to a drastic decrease in bile acid output (Goodwin et al. 2008). Studies in FXR knockout mice show that indeed these mice no longer react to bile acids by down-regulation of CYP7A1 (Kuipers et al. 2007; Lambert et al. 2003).

Bile acids also regulate the expression of other genes via FXR. Cyp8b1, the enzyme that controls the ratio in which the primary bile acid species cholate and chenodeoxycholate are formed, seems to be under the same negative feedback control as CYP7A1 (Sinal et al. 2000; not reproduced by Kok et al. 2003; Box 4.1).

#### ***4.2.4 FGF-Regulated Feedback of Bile Synthesis***

Although most studies initially focused at the liver to unravel the mechanism underlying regulation of bile acid synthesis, it was clear that this could not be the only organ involved and another pathway must exist. This idea originated from the observation

##### **Box 4.1**

FXR is called the bile acid sensor and plays a major role in regulation of bile acid homeostasis. After binding to DNA as a heterodimer with the retinoid X receptor, FXR controls the synthesis, conjugation, secretion, detoxification, excretion and uptake of bile acids. In the liver, FXR controls bile acid biosynthesis (CYP7A1; sterol 12 $\alpha$ -hydroxylase, CYP8B1), simusoidal uptake (NTCP), and canalicular secretion (BSEP). In the intestine, FXR controls almost all genes involved in bile acid detoxification. In the enterocyte, FXR controls bile acid absorption (ASBT), intracellular trafficking (IBABP) and basolateral efflux (OST $\alpha$ , OST $\beta$ ). The development of specific FXR agonists (GW4064, fexaramine, AGN34, 6 $\alpha$ -ethyl-chenodeoxycholic acid) and the generation of FXR  $-/-$  mice have provided powerful tools to study the pathways that are in part controlled by FXR (Hubbert et al. 2007).

that, in rodents, blocking the flow of bile acids into the intestine by bile duct ligation increased the expression of CYP7A1 and activity in the liver (Dueland et al. 1991; Gustafsson 1978). Hepatic concentrations of bile acids increase under these conditions but the expected down-regulation of CYP7A1 does not occur. Furthermore, studies in rat showed that the intraduodenal administration of taurocholic acid inhibited CYP7A1 expression in the liver, whereas direct intravenous or portal administration did not (Nagano et al. 2004; Pandak et al. 1991). It was therefore suggested as early as 1991 that the intestine must be involved by secreting a factor in response to bile acids, which either changes bile acid composition or in itself signals back to the liver. It is now clear that this factor exists and that it is a member of the fibroblast growth factor family, namely FGF19, or the mouse orthologue FGF15 (Box 4.2).

The first evidence that FGF19 was involved came from studies that aimed to find FXR target genes. Treatment of human hepatocytes with the FXR agonist GW4064 strongly up-regulated the mRNA expression of FGF19 (Inagaki et al. 2005). In the search for a ligand, FGF19 was reported to bind to the FGFR4 in vitro (Xie et al. 1999). The expression of this receptor is found mainly in liver, in large hepatocytes adjacent to the central vein and in smaller hepatocytes throughout the liver; and it is the sole FGFR expressed significantly in mature liver hepatocytes. FGFR4 null mice exhibit depleted gallbladders, an elevated bile acid pool, reduced activity of

#### **Box 4.2**

FGFs constitute a large family of growth factors that influence a wide variety of biological processes, such as angiogenesis, embryogenesis, differentiation (Galzie et al. 1997; Goldfarb 1996). FGFs induce their biological effects by binding to and activating FGFRs (for a review, see Schlessinger et al. 2000). This occurs by dimerization of the *trans*-membrane receptors upon binding of the FGF, followed by autophosphorylation of a number of tyrosine residues and recruitment of downstream effectors, such as the FGF receptor substrate protein 2 alpha (FRS2a). The FRS2a is a membrane-linked docking protein that contains myristyl anchors, phosphotyrosine binding domains (PTB) and multiple tyrosine phosphorylation sites at its C-terminus. These are docking sites for Grb2 and Shp2 linking the FGFR with the Ras and MAPK cascade (Wiedlocha and Sorensen 2004). Within the family of human FGFs, there are seven phylogenetic subfamilies based on amino acid sequence identities (Bottcher and Niehrs 2005; Wiedlocha and Sorensen 2004). In general, FGFs within the same family tend to share functional activity, but this does not hold true for the subfamily consisting of FGF19, FGF21 and FGF23. The core sequences of these three FGFs are very diverse and so are their functions. The mouse orthologue of human FGF19 is FGF15; and although they share only 53% total amino acid identity, their role in regulation of bile synthesis is very similar.

JNK and elevated excretion of bile acids, due to elevated levels of Cyp7a1 (Yu et al. 2000). Conversely, transgenic mice expressing a constitutively active FGFR4 have increased JNK activity, decreased CYP7A1 expression and a reduced bile acid pool size (Yu et al. 2005).

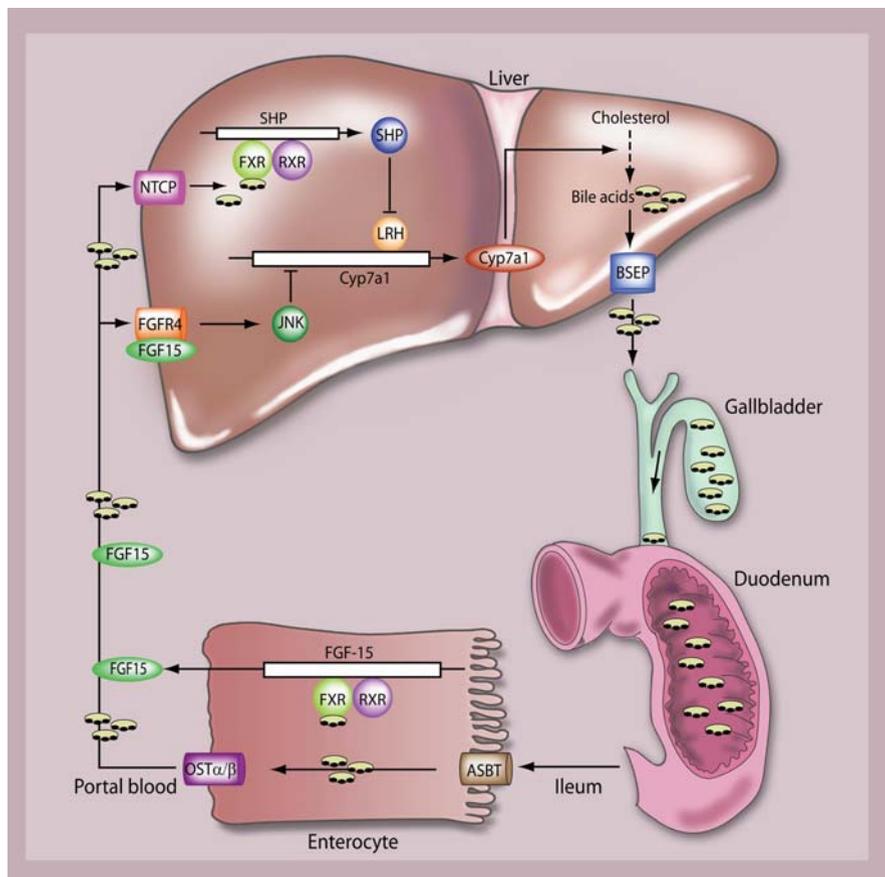
The data from FGFR4 null mice, together with the observation that FGF19 is a target gene for FXR and that it specifically binds to FGFR4 *in vitro*, prompted researchers to test the ability of FGF19 to down-regulate CYP7A1 expression. Indeed it was found to decrease transcription of CYP7A1 in a dose-dependent manner in human hepatocytes. However, no expression was found in human or mouse liver (Inagaki et al. 2005). The solution came from studies in mice, where it was shown that FGF15 is predominantly expressed in the small intestine following administration of GW4064 or cholic acid. FGF15 mRNA is highly expressed in the ileum and only at low levels in other enterohepatic organs. FGFR4 shows the reverse expression pattern, with high expression in the liver and with little or no expression in the intestine (Inagaki et al. 2005).

It is now clear that FGF15/19 is predominantly responsible for the feedback inhibition triggered by bile acids in the intestine. In the intestine, bile acids activate the nuclear transcription factor FXR, which in turn activates the transcription of FGF15/19. This results in the secretion of FGF15/19 to either the lymph or blood from where it reaches the liver to provide a feedback signal for bile synthesis (Kim et al. 2007; Fig. 4.1).

Activation of FGFR4 by FGF *in vivo* is dependent on the presence of beta-klotho. The function of this protein was initially unknown and beta-klotho null mice appeared normal upon examination (Ito et al. 2005). However, they had pronounced alterations in bile acid metabolism similar to that observed in FGFR4 null mice or FGF15 null mice, including increased expression of CYP7A1 and bile excretion in faeces (Ito et al. 2005). Beta-klotho was found to be expressed in liver, pancreas and fat (Ito et al. 2000). Beta-klotho is a membrane protein that contains two regions in the extracellular domain with homology to those in family 1 glycosidases, which hydrolyse glycosidic bonds (Ito et al. 2002). The function of beta-klotho remains elusive, although it has been suggested that it regulates the concentration of cofactors for FGFR4 (glycosaminoglycans). The reliance on beta-klotho adds another level of selectivity to the signalling capacity of FGF15/19.

#### **4.2.5 Other Pathways**

The nuclear receptor regulation of bile acid synthesis involving FXR, SHP, LXR and LRH-1 explains a major part of the regulatory responses in bile acid biosynthesis. However, experiments in SHP knockout mice have indicated the existence of SHP-independent mechanisms for the suppression of CYP7A1. SHP knockout mice increase the synthesis and accumulation of bile acids and produce more cholic acid compared to their controls (Wang et al. 2003). These increases are caused as expected by a decreased down-regulation of CYP7A1 gene expression (Boulias et al. 2005). However, this effect is not as dramatic as in FXR knockout mice, suggesting



**Fig. 4.1** Feedback regulation of bile synthesis is mediated by pathways involving both FGF15 and bile acids. Cholesterol in the liver is converted to bile acids through 7 $\alpha$ -hydroxylation by the enzyme *Cyp7a1*. The produced bile acids are secreted via *BSEP* and stored in the gall bladder. After ingestion of a meal, they flow into the duodenum where they help with food digestion. In the distal ileum they are reabsorbed via *ASBT*. In the enterocyte, the bile acids activate *FXR* which leads to induction of the *FGF-15* gene. Both bile acids and FGF-15 are secreted to either the lymph or blood from where they reach the liver. FGF-15 binds the *FGFR4* and this result in suppression of *Cyp7a1* expression through a *JNK*-dependent signaling cascade. Bile acids are taken up from the portal blood via *NTCP*. Activation of *FXR* in the liver by bile acids leads to induction of the *SHP* gene, which can directly suppress the *Cyp7a1* gene through an interaction with *LRH-1*

the existence of *SHP* independent mechanisms for the down-regulation of *CYP7A1* (Watanabe et al. 2004). *SHP* knockout mice fail to repress *CYP7A1* in response to the *FXR* agonist GW4064 as expected (Inagaki et al. 2005; Wang et al. 2003). Remarkably, these mice remain responsive to bile acid feeding by down-regulation of *CYP7A1* (Wang et al. 2003). This pathway may be secondary to liver injury from high bile acid levels. Bile acids are known to induce inflammatory cytokines,

TNF $\alpha$  and IL-1 $\beta$ , in hepatic macrophages (Kupffer cells; Li et al. 2008). These cytokines may cross the sinusoid and inhibit CYP7A1 gene expression in hepatocytes via activation of JNK. Bile acids can also signal via JNK by the protein kinase C pathway. This may involve phosphorylation and inactivation of transcription factors such as HNF4 $\alpha$ , which is a crucial factor stimulating the expression of CYP7a1 (Li et al. 2008). In addition, the pregnane X receptor (PXR) and the vitamin D receptor (VDR; Jiang et al. 2006) may suppress CYP7A1 by SHP-independent mechanisms (Chiang 2004).

### 4.3 Regulation of the Enterohepatic Circulation

During their function as emulsifiers of dietary lipids and fat-soluble vitamins, bile acids cycle through several organs that are part of the enterohepatic circulation, including the liver, the bile ducts, the gallbladder and the intestine; and they return to the liver via the portal vein. During this journey they have to pass different organs; and to do this they need to cross cell membranes various times. The majority of bile acids are conjugated to taurine or glycine amino acids and exist in the form of membrane impermeable anions. Specialized transporters located in the membranes of organs in the enterohepatic cycle allow the proper transport of bile acids (for reviews, see Meier and Stieger 2000; Oude Elferink et al. 2006). These transporters influence bile acid concentrations in different compartments of the intestinal tract and it is not surprising that bile acids have a regulatory role in the expression of these transporters. The following paragraphs discuss the various bile acid transporters and how they are regulated.

#### 4.3.1 Liver

Bile acids returning from the intestine via portal blood are taken up by the hepatocytes, transported through the hepatocyte and re-secreted at the other side to continue cycling between the liver and the intestine. The large pool of bile acids that are fluxed in this way through the hepatocytes with a high transport rate provide the main force for bile flow. This flux through the hepatocytes occurs against a steep concentration gradient and therefore requires distinct active transport systems expressed in a polarized fashion. Large pores (fenestrae) allow bile acids to enter the space of Disse. The process of bile acid extraction is efficient: 75–90% from the first pass of portal blood (for reviews, see Meier and Stieger 2000; Oude Elferink et al. 2006). Sodium-dependent and sodium-independent transport pathways have been identified to play a key role in hepatic uptake of bile acids from sinusoidal blood. The sodium-dependent process is represented by the sodium taurocholate cotransporter polypeptide (Ntcp, Slc10a1; for a review, see Hagenbuch and Dawson 2004), the substrate specificity of which is essentially limited to

conjugated bile acids and certain sulfated steroids. NTCP accounts for more than 80% of conjugated (i.e., taurocholate and glycocholate) but less than 50% of unconjugated (i.e., cholate) bile acid uptake. In contrast, the sodium-independent pathway is represented by different members of the superfamily of organic anion-transporting polypeptides (OATP/SLCO; for a review, see Hagenbuch and Meier 2004). In human liver, the highest expressions are found for OATP1B1 (SLCO1B1) and its 80% sequence homologue OATP1B3 (SLCO1B3), both of which are predominantly if not exclusively expressed in the liver (Stieger et al. 2007).

The expression of NTCP and OATP1B1 (SLC2A1) is repressed by high levels of bile acids, as can be observed in cholestasis. Under these conditions, FXR induces SHP; and this blocks the stimulating effect of retinoic acid receptor and RXR heterodimer on the NTCP promoter (in rats; Denson et al. 2001). Similarly, activation of SHP leads to repression of hepatocyte nuclear factor 1, which is the major transcriptional activator of OATP1B1. In mice this regulatory mechanism is different or at least another pathway exists, since the repression in gene expression under high bile acid conditions still takes place in SHP  $-/-$  mice. It is of interest to note that cholestasis leads to activation of OATP1B3. It is speculated that the up-regulation of this transporter might constitute an escape mechanism promoting the hepatocellular clearance of xenobiotics during cholestasis.

After uptake of bile acids at the basolateral membrane, they are transported to the canalicular membrane. The movement is still not very well understood and might be mediated by vesicle transport or by transport proteins. Evidence for the latter comes from studies demonstrating rapid cytosolic diffusion of fluorescent derivatives of bile acid before their canalicular secretion (Bahar and Stolz 1999). At the canalicular membrane the bile acids are effluxed against a steep concentration gradient into the bile. Whereas the influx at the basolateral membrane is primarily driven by a net influx of  $\text{Na}^+$ , the efflux ability of the canalicular pump depends on the availability of ATP. Canalicular transport is mediated by the bile acid export pump (BSEP or ABCB11). BSEP has a broad specificity for bile acids and pumps conjugated bile acids such as taurine- or glycine-conjugated cholate, chenodeoxycholate and deoxycholate into bile (Meier and Stieger 2000; Stieger et al. 2007). A minor bile acid export pump is the ABC transporter MRP2 which mediates the export of bilirubin conjugates and a wide variety of organic substrates, such as glutathione, glucuronide and sulfate-conjugated drugs. MRP2 also mediates the transport of divalent bile acids such as sulfated tauro- and glycolithocholate. MRP2 is the main driving force for bile acid-independent bile flow through canalicular excretion of reduced glutathione (Meier and Stieger 2000; Oude Elferink et al. 2006). MDR3 (ABCB4) was shown to function as an ATP-dependent phospholipid flippase, translocating phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane (Oude Elferink et al. 2006). Canalicular phospholipids are then solubilized by canalicular bile acids to form mixed micelles, thereby protecting cholangiocytes from the detergent properties of bile acids. The expression of BSEP, MRP2 and MDR3 is regulated by FXR and leads to an increase in bile efflux when intracellular bile acid levels rise (Kuipers et al. 2007).

### 4.3.2 *Intestine*

Apical sodium dependent bile acid transporter (ASBT) is the ileal counterpart of the hepatic NTCP. It efficiently transports conjugated and unconjugated bile acids with a preference for the taurine and glycine conjugates over the unconjugated form (Dawson and Oelkers 1995). The essential role of ASBT in intestinal bile acid absorption is evident from studies in ASBT null mice that show intestinal bile acid malabsorption and consequent disturbance of the enterohepatic circulation. Recent studies showed that ASBT is under control of 25-hydroxycholesterol and the presence of this sterol inhibits ASBT activity in human intestinal epithelial cells (Alrefai et al. 2005). The negative feedback is mediated by the induction of SHP via FXR activation. SHP in its turn represses LXR-1-dependent gene activation.

After uptake by the enterocyte, bile acids are bound to the intestinal bile acid binding protein (IBABP). IBABP is a small soluble protein, expressed exclusively in the terminal ileum. IBABP gene is positively expressed by bile acids via FXR activation (Nakahara et al. 2005). The concerted action of FXR activation (positive regulation of bile binding and negative regulation of uptake) helps to reduce the cytotoxic effects of bile acids in the ileum.

Although the proteins responsible for uptake and intracellular transport of bile acids have been known for many years, the mechanism of transport across the basolateral membrane was unknown until recently. Two proteins have been identified that function as a heterodimer to transport bile acids: Ost $\alpha$  and Ost $\beta$  (Dawson et al. 2005). The regulation of expression seems to be under positive control of bile acids via activation of FXR (Dawson et al. 2005). The discovery of Osta/b also further supported the central role for FGF15 signalling in control of bile synthesis. Osta/b null mice show increased expression of FGF15 and down-regulation of CYP7A1 in the liver (Dawson et al. 2008). This is likely due to an increase in enterocyte bile acid concentration. Conversely, mice lacking the bile acid uptake transporter ASBT show decreased FGF expression and an increase in bile acid synthesis (Jung et al. 2007).

## 4.4 Cholesterol in the Enterohepatic Circulation

Body cholesterol derives from two sources, i.e., de novo biosynthesis and diet. Since, in humans, total body sterol output almost always exceeds dietary intake, continuous synthesis is essential. The liver is the predominant organ for cholesterol synthesis but the intestine also plays an important role, particularly in rodents (Dietschy and Turley 2002). Cholesterol is synthesized from two-carbon acetyl-CoA moieties. The rate-limiting enzyme in the synthetic pathway is HMG-CoA reductase, a highly regulated enzyme that catalyses the conversion of HMG-CoA into mevalonate. Cholesterol itself regulates feedback inhibition of HMG-CoA reductase activity, as accumulation of (oxy)sterols in the endoplasmic reticulum (ER) membrane triggers HMG-CoA reductase to bind to Insig proteins, which leads to ubiquitination and degradation of HMG-CoA reductase (Gong et al.

2006). In addition, cholesterol regulates the gene expression of HMG-CoA reductase indirectly by blocking the activation of the transcription factor, sterol regulatory element-binding protein 2 (SREBP2). Under low-cholesterol conditions, SREBP2 in the ER is escorted by the SREBP cleavage activating protein (SCAP) to the Golgi. In the Golgi, SREBP2 is cleaved to generate its transcriptionally active form, which activates transcription of the HMG-CoA reductase encoding gene (Brown and Goldstein 1999). Conversely, when sterols accumulate in the ER membrane, cholesterol binding to the sterol-sensing domain of SCAP causes a conformation change, which induces binding of SCAP to the ER anchor protein Insig, preventing the exit of SCAP-SREBP2 complexes to the Golgi and thereby preventing activation of SREBP2. This effect is transduced by oxysterols which bind to Insigs (Gong et al. 2006; Radhakrishnan et al. 2007), causing Insigs to bind to SCAP. Mutational analysis of the six transmembrane helices of Insigs reveals that the third and fourth are important when Insigs are binding to oxysterols and Scap (Gong et al. 2006). The interaction of oxysterols with Insigs finally explains the long-known ability of oxysterols to inhibit cholesterol synthesis in animal cells.

#### ***4.4.1 Cholesterol Absorption in the Intestine***

In an aqueous milieu the solubility of unesterified cholesterol is about 1  $\mu\text{M}$ . Depending on the amount of cholesterol taken in via food, the concentration in the intestinal lumen is at least three orders of magnitude higher. Cholesterol in food stuffs is mostly present in an oily phase, whereas cholesterol coming into the intestine via the bile is present either as a mixed micelle together with bile acid and phospholipids or as a vesicle with phospholipids only (Hernell et al. 1990). Until relatively recently, cholesterol was generally assumed to be absorbed via passive diffusion. In model systems the sterol can flip-flop rapidly through lipid bilayers, so in principle no proteins seemed necessary to assist in cholesterol absorption. However, this situation changed drastically with the discovery of the cholesterol absorption inhibitor ezetimibe (Rosenblum et al. 1998). Ezetimibe and analogues comprise a new class of sterol absorption inhibitors that reduce diet-induced hypercholesterolemia. Using a bioinformatics approach, the NPC1L1 protein was identified as a putative cholesterol transporter in intestinal cells and target for ezetimibe (Altmann et al. 2004). Indeed the NPC1L1 knockout mice show a 69% reduction in cholesterol absorption which cannot be further reduced by ezetimibe treatment (Altmann et al. 2004). The NPC1L1 protein contains 13 putative transmembrane domains: the third to seventh transmembrane helices are thought to constitute a sterol sensing domain also present in NPC1, SCAP and HMG-CoA reductase (Alrefai et al. 2007; Altmann et al. 2004). Recent studies show that interaction of this domain with cholesterol induces a conformational change in the protein; this in turn induces endocytosis, taking a cholesterol-rich domain with it into the cell (Ge et al. 2008). Thus cholesterol regulates its own absorption in the intestine via a

feed-forward mechanism. The mechanism does not account for down-regulation in the presence of a cholesterol overload. Perhaps an excess of cholesterol in the plasma membrane may hamper the formation of endocytotic vesicles. The form in which cholesterol is transported to the brush border membrane of the enterocytes has not been studied. It has always been assumed that micelization via bile acids is an essential step. In principle, with the discovery of the NPC1L1-mediated mechanism, uptake via diffusion could be possible. Yet, a number of studies have shown bile acids to be essential for cholesterol absorption. Almost no cholesterol is taken up in the absence of bile acids (Wang 2007; Wang and Lee 2008). Whether the bile acids are just necessary for donating the cholesterol to the membrane or whether they accelerate NPC1L1-mediated cholesterol uptake directly has not yet been studied.

In addition to NPC1L1 several other proteins have been suggested to play a role in cholesterol absorption. In *in vitro* experiments the scavenger receptors SR-B1 and CD 36 have been shown to mediate the uptake of unesterified cholesterol (Knopfel et al. 2007). Aminopeptidase N has also been suggested to be actively involved in cholesterol absorption (Kramer et al. 2000, 2005). Evidence for a substantial contribution of these three proteins to cholesterol uptake *in vivo* is, however, lacking.

#### **4.4.2 Intestinal Cholesterol Secretion**

In addition to protein mediated uptake also a mechanism for protein mediated sterol efflux has been identified. In 2000 Berge et al. reported that mutations in the ABC transporter heterodimer ABCG5/ABCG8 were responsible for the excessive accumulation of plant sterols in patients with the disease sitosterolemia (Berge et al. 2000). Subsequently, the same group showed these proteins to be present also on the canalicular membrane of hepatocytes where they mediate transport of cholesterol and plant sterols into the bile (Yu et al. 2002a). Overexpression of ABCG5/ABCG8 in transgenic mice leads to an increase in biliary cholesterol secretion and a reduced intestinal absorption of dietary cholesterol, providing strong evidence for ABCG5/ABCG8 being involved in hepatocellular secretion and intestinal efflux of cholesterol (Yu et al. 2002b). The identification of these proteins has been a major step forward in the elucidation of the mechanism of biliary lipid secretion. Until the discovery of ABCG5 and ABCG8, biliary cholesterol secretion was supposed to be a largely passive process driven by the transport of bile acids and phospholipids. Because of the above-mentioned rapid flip-flop of cholesterol across membranes, no transporter was deemed necessary. Small explained this enigma by assuming that the heterodimer Abcg5/Abcg8 is a “liftase” instead of a floppase (Small 2003). Assuming that cholesterol easily flops through the membrane, the proteins lift cholesterol out of the plane of the membrane so that it is more accessible, reducing the activation energy for uptake in bile acid/phospholipids mixed micelles. Recently it was shown that this hypothesis may not hold true. Abcg8 knockout mice showed a 50% decrease in the cholesterol content of the

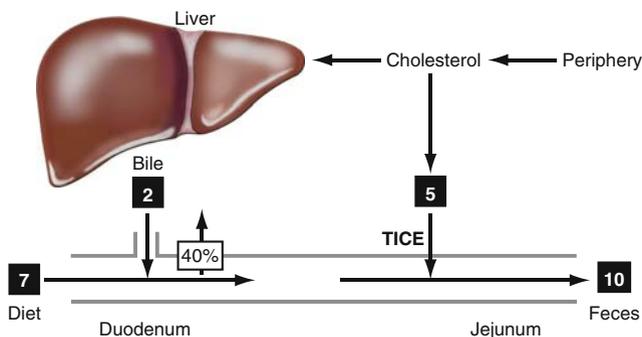
canalicular membrane, which is more compatible with floppase activity of the Abcg5/g8 heterodimer (Kosters et al. 2006). Yet, in vitro experiments with cells overexpressing the proteins also demonstrated an absolute requirement for the presence of bile acid micelles, suggesting that Abcg5/g8 donate cholesterol directly to bile acid micelles (Vrins et al. 2007). Taken together these results demonstrate restricted diffusion of cholesterol through the canalicular membrane. This may also explain why a protein such as NPC1L1 is required for cholesterol transport into the enterocyte. Restricted diffusion of cholesterol across the brush border membrane necessitates the presence of proteins to facilitate cholesterol import into the enterocyte.

The functional coupling of ABCG5/G8 activity to bile acids not only affects the kinetics of biliary cholesterol secretion but also influences plant sterol absorption in the intestine. It is generally assumed that efflux of plant sterols is a primary function of intestinal ABCG5/G8. In patients with defects in bile acid synthesis one might therefore expect to find accumulation of plant sterol in the body. This has indeed been observed. Whether intestinal ABCG5 and G8 also play an important role in cholesterol secretion is not yet clear. The group of Hobbs constructed mice overexpressing human ABCG5/G8 in liver and intestine (Yu et al. 2002b). These mice showed strongly increased faecal neutral sterol output. However, when these mice were crossbred with Abcb4 knockout mice that have both abrogated biliary phospholipid and cholesterol secretion, faecal sterol secretion normalized despite the overexpression of intestinal ABCG5/G8, indicating a minor role for the intestinal proteins in cholesterol homeostasis (Langheim et al. 2005). Experiments with tissue-specific knockout models are required to substantiate these findings.

#### ***4.4.3 Novel Pathways for Cholesterol Excretion***

It is generally accepted that the only important route for cholesterol to leave the body is the above-described hepatobiliary excretion followed by intestinal passage into the faeces. Probably because the design of this pathway seems so logical and its dynamics have been investigated in many species there has been very little research on alternative pathways. Yet very early work has hinted at the existence of non-hepatobiliary pathways for cholesterol excretion. As early as 1927 Sperry concluded from studies with bile-diverted dogs that these animals continue to excrete cholesterol and he concluded that “that under some conditions the cholesterol of the faeces comes from neither food nor the bile which may be secretion through the intestinal wall, desquamated epithelium, or bacteria” (Sperry 1927). The data from Sperry’s work have been largely ignored and it took almost 50 years before Pertsemlidis et al. (1973) confirmed the data of Sperry, also in studies with dogs. Likewise, faecal sterols of non-dietary origin are present in the faeces of patients with biliary obstruction (Cheng and Stanley 1959b) or rats with long-term bile diversion. A major drawback in such studies is the lack of biliary components in the enterohepatic cycle under these conditions. Particularly, the absence of bile

acids compromises cholesterol absorption, and consequently affect intestinal cholesterol synthesis, as well as lipid absorption, with unknown side-effects. This is probably the reason that these and similar studies have gone largely unnoticed in the literature. With time, experimental set-ups improved and, in the early 1980s, Miettinen et al. (1981) investigated the origins of faecal neutral steroids in normal rats, using an isotopic balance method developed in their laboratory (Miettinen 1970; Miettinen et al. 1990) and the isotopic steady-state balance procedure (Chevallier 1967; Wilson 1964), they established that the specific activity of faecal cholesterol was consistently lower than that of plasma cholesterol or the faecal bile acids. An observation consistent with earlier reports (Chevallier 1960; Danielsson 1960; Peng et al. 1974), this result indicated that a considerable portion of the faecal neutral steroids was derived from cholesterol not in equilibrium with the rapidly exchangeable pool of body cholesterol. The study of Miettinen et al. (1981) showed that approximately 40–50% of faecal neutral sterols in rats fed a sterol-free diet arise from a source of non-exchanging cholesterol. They concluded that these sterols have at least two origins: fur-licking and sterols originating directly from the intestine. Under conditions that prevent fur-licking, either by acetone-washing of the animals or by physical restraint, the contribution of non-exchanging cholesterol to total faecal neutral sterol output was still approximately 33%. A similar phenomenon has been shown also to occur in humans. In 1967 Simmonds et al. performed elegant intestinal perfusion studies in humans and observed significant direct secretion of cholesterol in the small intestine. Until recently the origin of this cholesterol has remained an enigma. The development of transgenic mouse models that have abrogated biliary lipid secretion has made it possible to study the role of the intestine in cholesterol excretion, isolated from the biliary system. A case in point is the *Abcb4* knockout mouse. In this model biliary phospholipid and cholesterol secretion is completely absent. Yet bile acid secretion and bile flow are not affected. Neutral sterol excretion is completely normal in these mice proving that an alternative pathway for cholesterol output is present or can be activated. Kruit et al. demonstrated that intravenously administered radiolabelled cholesterol finds its way to the faeces demonstrating a direct pathway for cholesterol from the blood compartment to the intestinal lumen (Kruit et al. 2005). Activation of LXR target genes with the agonist T0901317 stimulated the pathway indicating active transport. To obtain more insight in the underlying mechanism cholesterol secretion was studied in an isolated intestinal perfusion set-up. By cannulating intestinal segments *in situ*, keeping the blood supply intact, the intestinal lumen can be perfused with buffers to which cholesterol acceptors can be added. Measurement of cholesterol in the perfusate allows assessment of cholesterol transport across the intestinal wall. Surprisingly, cholesterol secretion was highest in the proximal 10 cm of mouse small intestine encompassing duodenum and the first part of jejunum (van der Velde et al. 2007). Output gradually decreased to reach very low levels in the colon (Fig. 4.2). The presence of bile acids and particularly phospholipid was necessary to induce the process. The molecular mechanism by which cholesterol is transported from blood through intestinal wall is still incompletely understood. Surprisingly, *Abcg5/g8* seem not to be involved because the rate of transintestinal



**Fig. 4.2** A simplified scheme illustrating the different sources of cholesterol in the intestine. In wild-type mice that have free access to normal chow diet, the cholesterol input into the intestine from both diet and bile (per 100 g bodyweight) is approximately 9  $\mu\text{mol/day}$ . Approximately 40% of this cholesterol is reabsorbed from the intestine and this should result in an output in the faeces of 4–5  $\mu\text{mol/day}$ . Twice as much (10  $\mu\text{mol/day}$ ) is found, indicating that a significant amount (5  $\mu\text{mol/day}$ ) of the cholesterol output in faeces cannot be explained by input via diet and bile alone. Intestinal perfusion studies demonstrated that direct transintestinal cholesterol excretion (*TICE*) exists and that this output is highest in the proximal 10 cm of mouse small intestine. *TICE* gradually decreases to reach very low levels in the colon

cholesterol secretion (*TICE*) was unaltered in *Abcg8* knockout mice (van der Velde et al. 2007). Also *Srb1* does not mediate *TICE*, in contrast the rate of *TICE* was 2-fold higher in *Srb1* knockout mice. In addition, feeding mice ezetimibe failed to influence *TICE*, indicating that also *NPC1L1* plays no role in this process. Yet, in addition to bile acids and phospholipids *TICE* could be influenced via diet and an agonist of the nuclear receptor *PPAR- $\delta$*  (Vrins, unpublished data). High-fat diet doubled neutral sterol output in wildtype mice and also doubled the capacity of *TICE*. Interestingly, a diet high in cholesterol failed to exert a similar activity (van der Velde et al. 2008). The effects of high-fat diet could be mimicked by giving the mice *GW742X*, an agonist of the nuclear receptor *PPAR- $\delta$* , suggesting that the effects are mediated through this nuclear receptor.

Which lipoprotein serves as donor of cholesterol for *TICE* is not yet clear. Literature data obtained with *Abca1* null mice that have no HDL suggest that this lipoprotein may not be important because these mice have completely unaltered neutral sterol excretion. Triglyceride-rich lipoproteins could be involved. In a recent report, Brown et al. (2008) demonstrated increased *TICE* in mice in which the enzyme acylCoA: cholesterol acyltransferase-2 was knocked down by treating the mice with antisense oligonucleotide. Surprisingly, although cholesterol-esterifying capacity in the liver was almost gone, these mice responded not by increasing biliary cholesterol secretion but instead by enhancing output of triglyceride-rich VLDL. In elegant experiments, Brown et al. (2008) isolated radiolabelled particles in an isolated liver perfusion set-up of both wild-type and liver *Acat-2* knockdown mice. Subsequently the particles were infused in a cross-over design in both wild-type and *Acat-2* knockdown recipient mice and the fate of the radiolabelled cholesterol

was determined. No change in biliary output was observed yet labelling of lumen and cells of the proximal 10 cm intestine increased when mice were infused with isolated perfusate of Acat-2 knockdown mice, suggesting that the substrate for TICE was increased in these samples (Brown et al. 2008). Clearly more work is required to isolate the active component. As indicated above, the heterodimer Abcg5/g8 does not contribute to TICE as measured in isolated intestinal perfusion studies. Additional factors may be required in the perfusate to induce Abcg5/g8 activity. Recent *in vivo* studies by van der Veen et al. determined the contribution of TICE in mice that were treated with the LXR agonist T0901317. This compound has been shown to increase neutral faecal sterol excretion 2- to 3-fold and also to directly stimulate faecal secretion of macrophage-derived cholesterol. Van der Veen et al. determined whole-body cholesterol fluxes by using elaborate stable isotope methodology. TICE strongly increased after treating wild-type mice with T0901317. In addition they demonstrated significantly decreased TICE in Abcg5 knockout mice. Taken together these results indicate a role for Abcg5/g8 in TICE *in vivo*. Use of similar methodology may be employed to quantify TICE in humans. The pathway does exist in man as well. Faecal sterols of non-dietary origin are present in the faeces of patients with biliary obstruction (Cheng and Stanley 1959a); and Simmonds et al. (1967) demonstrated direct intestinal cholesterol secretion in intestinal perfusion studies in man. However, the contribution of TICE to faecal sterol excretion is probably lower in humans than in mice. On average, humans secrete about 1 g/day of neutral sterols (Grundy 1983; Grundy and Ahrens 1969) Dietary cholesterol intake is about 400 mg (Samuel and McNamara 1983) and biliary cholesterol secretion amounts to 1000 mg (Hernell et al. 1990; Phillips 1960). Cholesterol absorption has been estimated to be about 50% (Grundy 1983; Grundy and Ahrens 1969; Miettinen 1970; Miettinen et al. 1990). Hence, the average TICE in humans can be estimated to be around 300 mg/day for 70 kg body weight, which is about one-third of the amount secreted into bile. The much higher biliary cholesterol secretion in man is probably the reason for the lower contribution of TICE to sterol excretion compared to mice.

#### **4.5 Role of the Enterohepatic Cycle in the Control of Cholesterol Homeostasis**

Secretion of bile is generally thought to be necessary for adequate digestion and handling of lipids in the food. Bile acids are the primary component; phospholipids are assumed to be added to prevent detergent bile acid action in the biliary tree. Since there seems to be no other pathway for cholesterol excretion from the body, the biliary route seems to be designed to accomplish this function. The identification of TICE forces re-evaluation of these paradigms. Particularly in mice, TICE is the predominant pathway for removing cholesterol from the body. In addition, the simple function of bile acids as emulsifiers of dietary lipids has been challenged. The past decade revealed multiple functions of bile acids in the fine transcriptional

control of lipid metabolism and even energy homeostasis (Scotti et al. 2007; Thomas et al. 2008; Zimmer and Gespach 2008). When bile acids only serve to help in digesting food, one would expect biliary secretion to strongly decrease during prolonged starvation. In mice the opposite has been observed. Bile formation increases in mice starved for up to 48 h (Kok et al. 2003; Scotti et al. 2007; Thomas et al. 2008; Zimmer and Gespach 2008). This is mainly due to an increase in bile acid secretion; yet bile acid synthesis progressively decreases, indicating that the animal increases the rate of energetically costly enterohepatic cycling during prolonged starvation. Apparently, the enterohepatic cycle serves an important role in maintaining lipid homeostasis. It is not clear which biliary component is most important in this homeostatic mechanism. It will be interesting to carry out prolonged starvation in an animal model with a (partially) disrupted enterohepatic cycle.

## 4.6 Concluding Remarks

During the past two decades, progress in the field of bile acid and cholesterol research has been enormous. The role of both steroids in controlling intricate transcription networks has emerged. Particularly, bile acids have lost their role as relatively non-specific detergents, to become important connectors of metabolic pathways. The importance of both bile acid and cholesterol for lipid homeostasis in mammals is exemplified by the extremely complex networks involved in regulation of expression and activity of the key enzymes in the pathways, i.e., HMG-CoA reductase and 7- $\alpha$ -hydroxylase. The liver is the most important site at which the activity of these enzymes is regulated but intensive cross-talk between liver and intestine plays a major role.

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# Chapter 5

## Cholesterol Trafficking in the Brain

Dieter Lütjohann, Tim Vanmierlo, and Monique Mulder

**Abstract** After it became clear that aberrations in cerebral cholesterol metabolism could lead to severe neurological diseases the interest in the regulation of brain cholesterol homeostasis increased. In particular when evidence was obtained for an important role of cholesterol in the still largely unknown molecular mechanisms underlying Alzheimer's Disease. Many proteins involved in peripheral cholesterol metabolism are also present in the brain. Yet, brain cholesterol metabolism is very different from that in the remainder of the body. The present chapter first addresses the overall cholesterol turnover in the brain; where cholesterol is synthesized, where it resides and how it is secreted from the brain. Subsequently, the focus is on mechanisms related to intercellular cholesterol trafficking between astrocytes and neurons.

### 5.1 Introduction

Brain cholesterol metabolism must be strictly regulated for optimal brain functioning. This is evident from the fact that disturbances herein can lead to severe neurological diseases, such as Smith–Lemli Opitz syndrome (Bjorkhem et al. 2001), Niemann–Pick type C1 (Wiegand et al. 2003) and cerebrotendinous xanthomatosis (Moghadasian et al. (2002). Disturbed brain cholesterol metabolism may also play a role in the development and progression of AD (Marx 2001; Puglielli et al. 2004).

Many proteins involved in cholesterol transport are expressed within the brain and lipoprotein-like particles are thought to mediate the intercellular trafficking of

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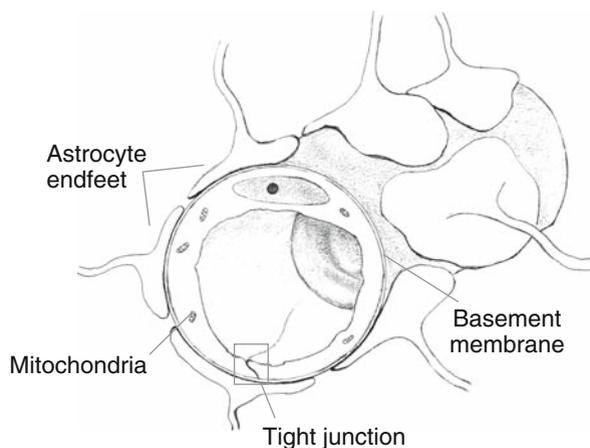
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cholesterol. Yet, brain cholesterol metabolism is very different from that in the remainder of the body. Cholesterol in the body can be derived from the diet or from de novo synthesis. The brain in contrast fully relies on de novo synthesis (Dietschy and Turley 2001) and cannot retrieve cholesterol from the circulation (Bjorkhem and Meaney 2004). A major site of cholesterol synthesis in the brain is the astrocytes, which secrete it in the form of apoE-containing HDL-like lipoproteins. Neurons can synthesize cholesterol, but after birth and full differentiation of astrocytes they are thought to shut down their cholesterol synthesis and rely on astrocytes for their cholesterol supply. How neurons regulate their cholesterol supply remains to be determined in detail. Evidence has been obtained indicating a role for the brain-specific cholesterol metabolite, 24(S)-hydroxycholesterol.

## 5.2 Cholesterol Turnover in the Brain

The brain is the most cholesterol-rich organ in the body. It contains about 25% of all free cholesterol present in the body, while it represents only 2% of the total body weight. Essentially all cholesterol in the brain is unesterified. Surprisingly relatively little is still known about the maintenance of cholesterol homeostasis and about its specific roles within the central nervous system (Snipes and Suter U1997).

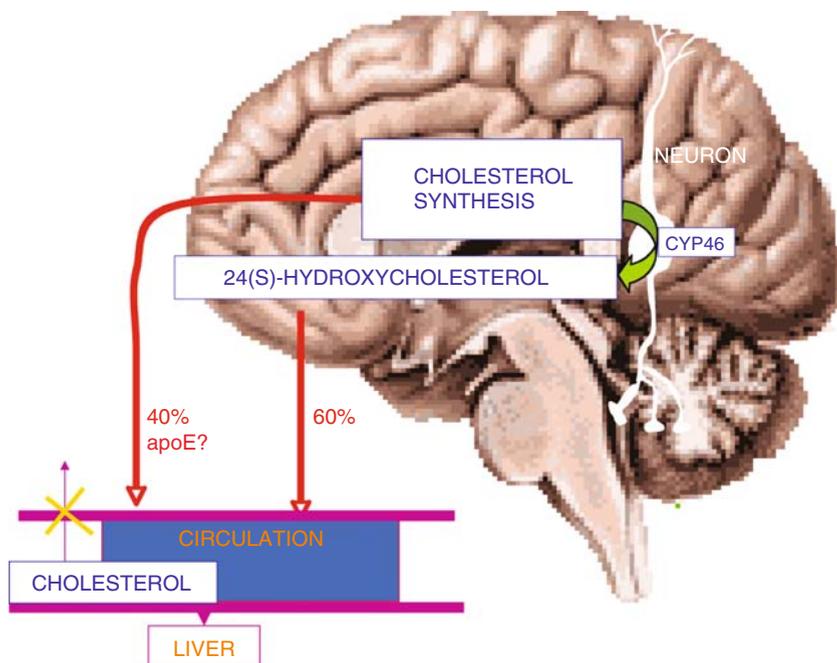
Almost all of the cholesterol present in the brain is synthesized locally. In spite of the high cholesterol content, cholesterol turnover in the brain was found to be much slower than that in the rest of the body (20- to 80-fold lower than in the liver; Spady and Dietschy 1983) and its concentration is kept remarkably stable. This may explain why, until about 15 years ago, little attention had been paid to cholesterol metabolism in the brain. This changed when it turned out that cerebral cholesterol homeostasis is much more dynamic than initially thought. It is generally assumed that, under non-diseased conditions, cholesterol from plasma lipoproteins does not enter the brain, since it cannot cross the endothelial cells of the BBB. The cerebral capillary endothelium is the anatomical substrate of the BBB, isolating the brain neuropil from the systemic circulation. The cerebral endothelium lining the blood vessel lumen consists of a single layer of cells joined together by tight intercellular junctions. This layer of cells is supported by a basement membrane, which is the laminar structure formed by the fusion of the endothelial and glial vascular basement membrane. The end feet of astrocytes make up a discontinuous sheath at the abluminal surface of the basement membrane (Fig. 5.1). This has been confirmed by studies in mice, sheep and rabbits with use of either labeled lipoproteins or labeled cholesterol (for a review, see Dietschy and Turley 2001). For example, apoE-deficient mice display dramatically increased plasma cholesterol levels, but no alterations in brain cholesterol levels (Lomnitski et al. 1999). However, a recent study with guinea-pigs suggested that minor amounts (~1%) of cholesterol might cross the BBB (Lütjohann et al. 2004).



**Fig. 5.1** Schematic representation of the blood–brain barrier. The blood–brain barrier consists of endothelial cells lining the vessel wall, connected via tight junctions. These are surrounded by a thick basement membrane which is covered by astrocytic endfeet

Cholesterol, or better its steroid ring structure, cannot be degraded in the human body and high concentrations of free cholesterol can lead to the formation of crystals which are toxic to cells, in particular neurons (Lemaire-Ewing et al. 2005; Travert et al. 2006). Therefore, excess cholesterol is secreted from the brain into the circulation (Brown and Goldstein 1997) and finally released from the body via the liver where it is converted into bile acids (Lutjohann et al. 1996). Within the brain, cholesterol can be modified into its major brain metabolite 24(*S*)-hydroxycholesterol by the enzyme CYP46A1. Based on experiments with mice deficient for the enzyme CYP46A1, it was calculated that about 64% is being secreted in the form of this polar cholesterol metabolite (Fig. 5.2; Bjorkhem et al. 1997; Xie et al. 2003). The remaining 36% of cholesterol is secreted from the brain via another, yet unknown pathway that may involve apoE (Lund et al. 2003).

24(*S*)-Hydroxycholesterol is, in contrast with cholesterol, able to traverse the BBB (Bjorkhem et al. 1997, 1998; Lutjohann et al. 1996). At first it looks quite controversially that a compound more polar than cholesterol is able to pass a lipophilic barrier. As with other plasma membranes, the membranes of the endothelial cells are freely permeable to water (Panzenboeck et al. 2002). Introduction of an hydroxyl group in the side-chain of the cholesterol molecule leads to a local reordering of membrane phospholipids such that it is energetically more favourable to expel the oxysterol (Kessel et al. 2001). When a sterol like cholesterol is hydroxylated, there is an increase in the maximal aqueous activity that can be achieved, but a reduction in the passive permeability coefficient. However, the increase in solubility is proportionately greater than the reduction in the permeability coefficient, so that the net effect of hydroxylation is to greatly increase the maximal rate of passive diffusion of the molecule across the BBB.



**Fig. 5.2** Schematic representation of the overall cholesterol turnover in the brain. Cholesterol is synthesized endogenously predominantly by astrocytes and is converted by the enzyme CYP46A1 that resides in a subset of neurons, whereafter it is released from the brain into the circulation. This pathway is thought to be responsible for about 60% of the secretion of cholesterol from the brain, while the remaining 40% is secreted via a yet unknown pathway that may involve apoE

The enzyme CYP46A1 has been characterized at the molecular level. Its gene contains 15 exons and is located on human chromosome 14q32.1. CYP46A1 is predominantly found in the brain, mainly located in a subset of specific neurons. Deficiency for CYP46A1 in mice results in suppression of cholesterol synthesis in brain by about 25% (Lund et al. 2003; Xie et al. 2003), probably to compensate for the decreased efflux of cholesterol from the brain. Evidence has been obtained indicating a role for CYP46A1 in neurosteroid metabolism (Mast et al. 2003). Besides converting cholesterol into 24(S)-hydroxycholesterol, CYP46A1 can also inactivate neurosteroids that may either be synthesized endogenously or derived from the circulation.

Cholesterol in the brain resides in three major compartments with different turnover rates. The largest pool (70–80%, or 260 mg kg<sup>-1</sup> of the total 330 mg kg<sup>-1</sup>) has the slowest turnover (>1%) and is present in myelin membranes. Of the remaining 70 mg kg<sup>-1</sup>, about 10% reside in neurons, which represent about 10% of the brain cells, and therefore contain about 7 mg kg<sup>-1</sup> of cholesterol. The remaining 63 mg kg<sup>-1</sup> are present in glial cells (Davison 1965; Muse et al 2001; Xie et al. 2003).

As mentioned, the CYP46A1 enzyme is expressed in a subset of metabolically active neurons such as pyramidal cells of the cortex and Purkinje cells of the cerebellum (Lund et al. 1999). The cholesterol turnover in these neurons must be very high, since they are estimated to contain about  $4 \text{ mg kg}^{-1}$  of cholesterol while their turnover of 24-hydroxycholesterol is about  $0.9 \text{ mg kg}^{-1} \text{ day}^{-1}$ , which is more than  $20\% \text{ day}^{-1}$ . The overall turnover of cholesterol in the body is about  $0.8\% \text{ day}^{-1}$ , i.e. a value similar to the CYP46A1-independent cholesterol turnover in brain, which is about  $0.5 \text{ mg kg}^{-1} \text{ day}^{-1}$  of a pool of about  $63 \text{ mg kg}^{-1} \text{ day}^{-1}$ .

### 5.3 Release of 24(S)-Hydroxycholesterol from the Brain into the Circulation

24(S)-Hydroxycholesterol release from the brain into the circulation represents the major pathway for the brain to get rid of its cholesterol. From measurements in human tissue, the brain was estimated to contain approximately 80% of the total 24(S)-hydroxycholesterol content in the body. Brain samples also contained other oxysterols, but 24(S)-hydroxycholesterol was always dominating (Lutjohann et al. 1996). The concentrations of 24(S)-hydroxycholesterol in the brain and adrenals were 30- to 1500-fold higher than in any other organ. As judged from the arteriovenous difference over the brain and the levels in cerebrospinal fluid (CSF), 24(S)-hydroxycholesterol was the only oxysterol that was transported from the human brain into the circulation. From the concentrations of 24(S)-hydroxycholesterol in the CSF and the flux of cerebrospinal fluid into the jugular vein it can be calculated that  $<1\%$  of the total flux of 24(S)-hydroxycholesterol from the brain occurs via the cerebrospinal fluid. Thus, 99% must occur through the BBB. The pool size of 24(S)-hydroxycholesterol in rat brain was found to be about 0.3% of that of cholesterol, while the estimated half-life of 24(S)-hydroxycholesterol appears to be about 11 h versus about 2–6 months for cholesterol. The high cholesterol turnover in a subset of neurons that produce 24(S)-hydroxycholesterol seems to be controversial to the idea that after birth neurons shut down their cholesterol synthesis and rely on astrocytes for their cholesterol supply. Therefore, the source of cholesterol remains to be established: *de novo* synthesis by neurons or uptake from astrocyte-released cholesterol. Alternatively, a specific subset of metabolically active neurons may display a very high cholesterol turnover rate, while the other metabolically less active neurons largely shut down their cholesterol synthesis.

An alternative pathway for cholesterol release from the brain has been reported. Meaney et al. (2007) have shown that 27-hydroxycholesterol is metabolized into the known C(27) steroidal acid 7 $\alpha$ -hydroxy-3-oxo-4-cholestenoic acid by neuronal cell models only and can be transferred across the BBB. The contribution of this pathway to the daily release of cholesterol from the brain most likely is restricted and requires further examination (Meaney et al. 2007), particularly since 27-hydroxycholesterol is thought to originate from extracerebral sources.

## 5.4 Lipoproteins in the Cerebrospinal Fluid

The cerebrospinal fluid (CSF) is a particular compartment of the CNS. Functions of the CSF are to protect the brain from mechanical injury, to provide the brain with nutrients and maintain a constant external milieu for neurons, to secrete waste products via the CSF into the blood and to help the trafficking of hormones. The CSF flows via the ventricles into the subarachnoid space surrounding the brain, where it is reabsorbed into the venous sinuses into the circulation. The CSF is produced by the choroid plexus which consists of many capillaries, separated from the ventricles by specific choroid epithelial cells. These are closely connected with each other via tight junctions similar to the endothelial cells of the BBB. Choroid capillary networks are found predominantly in the lateral, the third and the fourth ventricle in the brain. The choroid plexus filters and actively secretes compounds from the plasma into the CSF. Therefore, the BBB at this site predominantly determines the composition of the CSF. Additionally, the CSF compartment is in close anatomical contact with the brain interstitial fluid. Therefore, the composition of the interstitial fluid affects the CSF composition since the two fluids are more or less in equilibrium (Skipor and Thiery 2008).

The presence of spherical lipoprotein-like particles in CSF was first described by Roheim et al. (1979) and later by Pitas et al. (1987). ApoE and apoA-I are the predominant apolipoproteins in human CSF, whereas apoB could not be detected (Borghini et al. 1995; de Vries et al. 1995; Pitas et al. (1987a). Originally, apoE was thought to originate predominantly from astrocytes and possibly also from microglial cells (LaDu et al. 1998; Nakai et al. 1996). ApoA-I present in CSF may be retrieved from the circulation or may be derived from endothelial cells lining the cerebral vessels (de Vries et al. 1995; Elshourbagy et al. 1985; Guyton et al. 1998). Based on an affinity chromatography assay, Koch et al. (2001) described four major classes of CSF-lipoproteins (Lps), all between HDL (10–13 nm) and LDL (22–29 nm) in size, namely: the ones containing apoE, apoA-I, both or none (Koch et al. 2001). All four CSF-Lps groups contained comparable amounts of apoJ. The apoE-containing lipoproteins also contained apoA-IV and ApoD. Lipoproteins containing both apoE and apoA-I resembled those containing apoE exclusively but had an additional presence of apoH. Lipoproteins with apoA-I also contained apoA-II, apo-IV and apoD. Finally, the apoE- and apoA-I-free group was hallmarked by apoD as major apolipoprotein, but additionally contained apo-A-IV, apoH and apoJ. ApoD, H and J are structurally and functionally different from apoE and apoAs. ApoD and apoJ have been suggested to be of neuronal origin (Bassett et al. 2000), while apoAIV is thought to derive from the intestine.

Based on cholesterol content and the protein:lipid ratio, all four lipoprotein classes detected in CSF correspond best with plasma HDL. ApoE is relatively the most abundant apolipoprotein in the CSF. Furthermore, apoE-containing lipoproteins are larger than the other CSF-lipoproteins not containing apoE (Koch et al. 2001; Pitas et al. 1987a). Based on the assumption that the composition of CSF and brain interstitial fluid is very much alike, it can be assumed that similar lipoprotein-like particles are present in interstitial fluid.

## 5.5 Astrocytes Supply Neurons with Cholesterol

After birth, neurons shut down their own cholesterol synthesis and rely on astrocytes for their cholesterol supply since they require their energy for other processes, such as electrical and chemical signaling and generation of action potentials (Argmann et al. 2005).

Astrocytes are the most abundant cell type in the human brain and are intimately associated with all parts of neurons as well as with cells of the BBB. They provide structural, trophic and metabolic support to neurons. They contribute to brain the BBB, releasing growth factors, buffering of extracellular  $K^+$  and regulating the brain immune response.

Cholesterol in neurons is implicated in a number of processes and a well regulated cholesterol supply therefore of utmost importance. Sufficient amounts of cholesterol may be required for vesicle transport and for maintenance of proper signal transduction pathways and optimal neurotransmitter release. Moreover, it serves as a precursor for neurosteroids (Mauch et al. 2001).

One of the key functions of cholesterol is to regulate membrane fluidity. Cholesterol decreases membrane fluidity, which affects the biophysical properties of the membrane, resulting in a reduced permeability for polar molecules and allowing for maintenance of relevant concentration gradients of ions and other molecules (Ikonen 2008). Neurons, for example, require energy for generation of action potentials, which depends upon the permeability characteristics of the plasma membrane surrounding the axon. These are determined in part by cholesterol and other lipid components of the membrane. Surrounding of axons by myelin, containing large amounts of cholesterol, allows neurons to send electrical signals over long distances with high velocity (Puglielli et al. 2004). Changes in the cholesterol levels dynamically alter the micro-environment of the plasma membrane and thereby the functioning of membrane-bound proteins, such as for example the functions of ion channels. Alterations in cholesterol levels of rat hippocampal neurons has been shown to affect their excitability (Guo et al. 2008).

Notably, neurons require cholesterol for generation of new membranes during regeneration after CNS injury and for the formation of new synapses during a process called synaptic plasticity, a process that is thought to be essential for learning and memory. Synapses of neuronal axons contact the dendrites or the somata of other neurons and upon activation of the neurons send signals electrically or chemically to this neuron. The dynamic and continuous reorganisation of these synaptic contacts is called synaptic plasticity.

Cholesterol delivery may at least in part participate in regulating the number of synapses formed (Slezak and Pfrieder 2003). A continuous turnover of cholesterol in neurons may facilitate the cells' ability for efficient and quick adaptation of cholesterol homeostasis required for dynamic structural changes of neurons, their extensions, and their synapses during synaptic plasticity (Pfrieder 2003).

Indeed, apoE in combination with cholesterol was found to induce the outgrowth of neurites in an isoform-specific manner in neuronal cultures. Cholesterol alone also had such a effect but to a lesser extent (Bellosta et al. 1995; Nathan et al. 1994, 1995,

2002; Pfrieger 2003). There are three common human apoE-isoforms (E2, E3, E4) that differ from each other in only one amino acid (Utermann et al. 1979). However, the apoE-isoforms differentially modulate neurite outgrowth. ApoE3 was found to enhance the outgrowth of neurites from cultured embryonic and adult mouse cortical neurons, while apoE4 decreased neurite outgrowth from these cells (Nathan et al. 2002). In addition, an apoE isoform-specific effect on dendritic spines in the dentate gyrus of brains of transgenic mice and humans was observed (Ji et al. 2003).

As described, astrocytes are considered to be the predominant source of brain cholesterol. Astrocytes secrete cholesterol together with phospholipids and apoE in the form of small, HDL-like particles (Gong et al. 2002). HDL are the smallest of the different lipoprotein classes in the circulation where also larger lipoproteins circulate, i.e., chylomicrons, VLDL, LDL and IDL. ApoE is a major apolipoprotein in the brain and, in lipid trafficking, is thought to exert roles here similar to those in the periphery. ApoE secreted by astrocytes constitutes 1–3% of total secreted protein by these cells (Pitas et al. 1987b). Using *in situ* atomic force microscopy, it was found that cultured astrocytes secrete lipoproteins with apoE or apoJ that are a little different from HDL in plasma with respect to their size, shape and aggregation properties. They are significantly flatter and smaller than plasma HDL and, in contrast with plasma HDL, do not form ordered arrays on a silica surface at high concentrations, but rather form amorphous aggregates (Legleiter et al. 2004).

ApoE seems to be required for lipoprotein secretion by astrocytes. Fagan et al. (1999) reported that cultured astrocytes from apoE-deficient mice secrete little phospholipids and free cholesterol in comparison with astrocytes obtained from wild-type mice, despite an unaffected apoJ expression. These authors found that apoE and apoJ normally reside on distinct HDL particles, with apoJ being present on the smallest particles. However, curiously, Karten et al. (2006) found cultured astrocytes obtained from apoE-deficient mice to secrete lipoproteins with a size similar to those secreted by astrocytes obtained from wild-type mice. It is suggested that astrocytes secrete partially lipidated apoE as a major extracellular acceptor of cholesterol released from the cells by an efflux process, mediated by the ABC-transporter (ABC)G1. In contrast with wild-type astrocytes, cholesterol loading of the apoE-deficient astrocytes does not enhance the efflux to apoA-I, but apparently shunts cholesterol into the esterification pathway. Moreover, expression of ABCA1, another cholesterol efflux transporter, was not increased upon cholesterol loading of apoE-deficient astrocytes, unlike that in wild-type astrocytes. Using apoE-deficient mice, evidence was obtained that apoE is not only involved in the delivery of lipids to neurons, but also in the removal of cell debris or membrane fragments, since a decreased clearance of degeneration products was found in the brain of apoE-deficient mice after entorhinal cortex lesioning in comparison with wild-type mice (Fagan et al. 1998). In the presence of apoE these lipids are thought to be re-utilized. In line with this, it was reported that apoE-deficient mice display impaired compensatory sprouting in injured denervated skin (Maysinger et al. 2008).

Since apoE-deficiency does not affect the sterol composition in the murine brain (Jansen et al. 2006), there must be compensatory mechanisms to maintain brain cholesterol homeostasis. In the absence of apoE increased levels of apoD have been

found in murine brain. ApoD is a secreted lipocalin that is present in astrocytes, oligodendrocytes and in some scattered neurons; and it has also been suggested to be involved in re-innervation and regeneration (Navarro et al. 2004).

The fact that astrocytes are of major importance for neuronal cholesterol homeostasis is supported by an experiment with *npc1*<sup>-/-</sup> mice, a model for Niemann–Pick type C disease, that develop neurodegeneration associated with cholesterol accumulation in neurons due to a defect in cholesterol trafficking. As a consequence of mutations in NPC1, cholesterol does not exit the endocytic pathway, leading to a massive accumulation of unesterified cholesterol in late endosomes and lysosomes after uptake of lipoproteins through the clathrin-coated pit pathway (Xie et al. 2000). Replacement of *npc1* in *npc1*<sup>-/-</sup> mice, specifically in astrocytes, results in enhanced survival and decreased neuronal storage of cholesterol associated with less accumulation of axonal spheroids and restoration of myelin tracts (Zhang et al. 2008).

## 5.6 How do Neurons Regulate Their Cholesterol Supply?

If neurons for their cholesterol supply predominantly rely on astrocytes, it can be questioned how neurons communicate with these cells in order to request sufficient amounts of cholesterol for optimal functioning. 24(S)-Hydroxycholesterol is specifically formed in neurons and is a natural ligand for the liver X receptors (LXRs), so-called master regulators of cholesterol homeostasis.

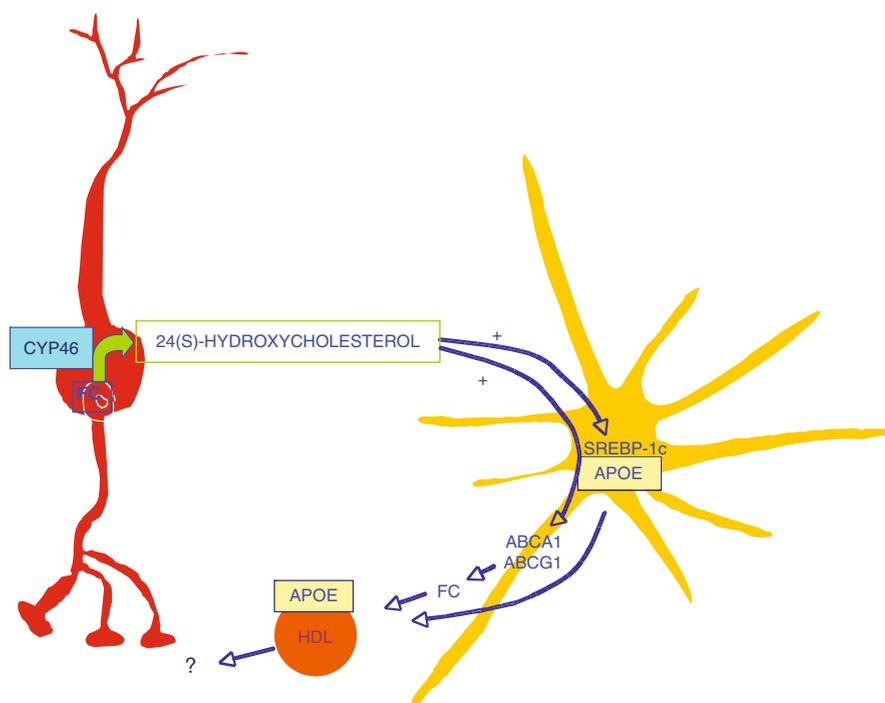
In astrocytes cholesterol synthesis is thought to be regulated by two main transcription regulation systems, i.e., sterol regulatory element binding proteins (SREBPs) and LXRs. SREBP activation increases the transcription of genes involved in increasing cellular cholesterol levels (Goldstein et al. 2006), while LXR activation facilitates the disposition of cholesterol from cells (Eckert et al. 2007).

SREBP residing in the ER is activated in sterol-poor conditions and transported to the Golgi complex where it undergoes proteolytic processing. Subsequently, this fragment is imported into the nucleus, where it switches on the transcription of HMG-CoAR and other sterol-regulated genes. Binding of sterols to the two chaperones SCAP and INSIG induces conformational changes resulting in the inhibition of the transport of SREBP from the ER to the Golgi. Binding of cholesterol to SCAP and binding of oxysterols, such as 24-hydroxycholesterol in the brain, to INSIG causes these chaperones to bind one another thereby preventing SCAP from escorting SREBP to the Golgi by making it inaccessible to COPII and preventing the increase in transcription of gene products (Ikonen 2008).

LXRs belong to the nuclear hormone receptor superfamily. Two forms have been identified: LXR $\alpha$  and LXR $\beta$ . Both are present in the brain and are thought to be involved in the regulation of brain cholesterol homeostasis (Whitney et al. 2002). This is supported by the observation that aged LXR $\alpha/\beta$ -deficient mice display several defects in their central nervous system. These include closed ventricles, lipid accumulation in astrocytes and around blood vessels, proliferation of astrocytes and dysorganisation of myelin sheaths (Wang et al. 2002).

Alternatively, cholesterol homeostasis can be regulated at the level of the proteosomal degradation of HMG-CoAR, such as for example by lanosterol (Song et al. 2005).

Evidence now indicates that 24(*S*)-hydroxycholesterol released by neurons signals to astrocytes and induces the secretion of apoE-containing lipoprotein-like particles via the LXR-pathway (Fig. 5.3; Abildayeva et al. 2006). 24(*S*)-Hydroxycholesterol was found to induce apoE transcription, protein synthesis and secretion in a dose- and time-dependent manner in cells of astrocytic but not of neuronal origin. Moreover, 24(*S*)-hydroxycholesterol primes astrocytoma, but not neuroblastoma cells, to mediate cholesterol efflux to apoE. The synthetic LXR agonist GW683965A exerts similar effects, suggesting involvement of an LXR-controlled signaling pathway. The differential effects observed in astrocytoma and



**Fig. 5.3** Schematic presentation of the differential effects in astrocytes and neurons of 24(*S*)-hydroxycholesterol on the expression of apoE and ABC transporters expression as well as apoE-mediated cholesterol efflux. 24(*S*)-Hydroxycholesterol may act as a signaling molecule that induces the apoE-mediated cholesterol efflux from astrocytes but not from neurons. ABCA1 and ABCG1 may play a role in mediating cholesterol efflux from astrocytes. Thus, in the intact brain, 24(*S*)-hydroxycholesterol derived from neurons may signal astrocytes to increase production of lipidated apoE particles in order to supply neurons with additional cholesterol during synaptogenesis or neuritic remodeling. Moreover alterations in the transcriptional regulation role of 24(*S*)-hydroxycholesterol on apoE-mediated cholesterol efflux may affect the progression of neurodegenerative diseases, including AD

neuroblastoma cells may be explained by a 10- to 20-fold higher basal LXR $\alpha$  and - $\beta$  expression level in astrocytoma cells. LXR isoforms differ in their pattern of expression (Steffensen et al. 2003). In the brain LXR $\beta$  levels are 2- to 5-fold higher than in the liver, whereas LXR $\alpha$  levels are 3.5- to 14.0-fold lower than in the liver (Apfel et al. 1994; Song et al. 1994). However, 24(*S*)-hydroxycholesterol and GW683965A were found to up-regulate LXR $\alpha$  but not LXR $\beta$  expression in astrocytoma cells (data not shown), similar to reports for macrophages and adipocytes, but not liver and muscle (Ulven et al. 2004; Whitney et al. 2001). Therefore, autoregulation of LXR $\alpha$  that has been suggested to occur in adipocytes to coordinate expression of target genes such as APOE (Ulven et al. 2004) may also occur in brain.

The apoE-mediated cholesterol efflux from astrocytoma cells may be controlled by the ATP binding cassette transporters ABCA1 and ABCG1, since their expression was also up-regulated by 24(*S*)-hydroxycholesterol and GW683965A. ABCG4 is most likely not involved, because its expression was induced by LXR-activation only in neuronal cells.

A common feature that astrocytes share with macrophages and adipocytes is their large content of free cholesterol (Krause and Hartman 1984; Vance et al. 2005). In agreement, in macrophages (Argmann et al. 2005), activation of LXR stimulates cellular cholesterol efflux through the co-ordinated induction of ABCA1, ABCG1, and apoE expression. Some aspects of these pathways may be cell-type specific. For example, apoD, which is also synthesized and secreted by astrocytes in lipid-bound form and may compensate functions of apoE in its absence, was unresponsive to LXR agonists in astrocytes, whereas it was induced in adipocytes (Hummasti et al. 2004). In contrast, Patel et al. (1995) found apoD expression in astrocytes to be regulated by 25-hydroxycholesterol and by progesterone.

GW683965A up-regulated the expression of HMG-CoA reductase, LDLR and SREBP2 in astrocytoma cells, supposedly to maintain cellular cholesterol homeostasis during excessive loss by efflux. However, 24(*S*)-hydroxycholesterol down-regulated the expression of these genes. It is known since long that oxysterols reduce the activity of HMG-CoA reductase and are more potent inhibitors of cholesterol synthesis than cholesterol (Kandutsch et al. 1974). However, the mechanisms by which cholesterol and oxysterols reduce cholesterol synthesis differ (Adams et al. 2004). Thus, 24(*S*)-hydroxycholesterol may not exert its effect exclusively via the LXR pathway. Accordingly, 24(*S*)-hydroxycholesterol, but not GW683965A, enhanced the expression of ABCG4 in neurons. Apparently, cholesterol efflux from astrocytes is not driven solely by the rate of cholesterol biosynthesis because an enhanced cholesterol efflux, up-regulation of ABCA1 and ABCG1 and impaired synthesis of cholesterol is observed in astrocytes as a consequence of 24(*S*)-hydroxycholesterol exposure (Abildayeva et al. 2006) and also in macrophages after statin treatment (Argmann et al. 2005).

Numerous studies have demonstrated that ABCA1 is necessary for the efflux of cellular cholesterol to lipid-poor apoA-I (Oram and Lawn 2001). Recently, ABCA1 was found to facilitate the efflux of CNS cholesterol to apoE as the absence of

ABCA1 compromised apoE secretion from both astrocytes and microglia. In addition, apoE that is present in the CSF of ABCA1-deficient animals is poorly lipidated (Hirsch-Reinshagen et al. 2004; Wahrle et al. 2004). A relationship between ABCG1 and the secretion of apoE was suggested by the observation that treatment of macrophages with antisense oligonucleotides to ABCG1 decreased the efflux of cholesterol and phospholipids to HDL and, surprisingly, also the secretion of apoE (Klucken et al. 2000; Wang et al. 2004). Karten et al. (2006) reported that only expression of ABCG1, but not of ABCA1, correlates with cholesterol release by astrocytes. In contrast with ABCA1, ABCG1 and ABCG4 are thought to facilitate the efflux of cholesterol to HDL rather than to lipid-poor apolipoproteins (Klucken et al. 2000; Wang et al. 2004). Although ABCG1 and ABCG4 may function both as homodimers and heterodimers (Oldfield et al. 2002; Rebeck 2004), expression of ABCG1 and ABCG4 overlaps in some but not all tissues assayed (Wang et al. 2004), which may indicate different functions in different tissues. The expression of ABCG4 appears to be largely restricted to nervous tissue (Rebeck 2004). ABCG1 and ABCG4 are thought to regulate cholesterol transport in the brain (Yvan-Charvet et al. 2008). Recently, *in vivo* evidence from transgenic mouse experiments has been provided for a role for *Abcg4* in sterol efflux in the brain and that *Abcg1* and *Abcg4* have overlapping functions in astrocytes, promoting efflux of cholesterol, desmosterol and possibly other sterol biosynthetic intermediates to HDL (Wang et al. 2008). Moreover, they seem to mediate intracellular vesicular transport of cholesterol/sterols within both neurons and astrocytes (Yvan-Charvet et al. 2008).

Neurons are thought to dispose their cholesterol by conversion into 24(*S*)-hydroxycholesterol (Bjorkhem and Meaney 2004). However, it is not yet known exactly how oxysterols are transported across membranes and through the intracellular water phase. The selective up-regulation of ABCG4 in neuroblastoma cells by 24(*S*)-hydroxycholesterol suggests a possible role for this transporter in oxysterol transport. Alternatively, ABCA1 may be involved. Tam et al. have shown that high-affinity uptake of 25-hydroxycholesterol by membrane vesicles can be mediated by ABCA1, besides mediating its efflux from intact cells (Tam et al. 2006).

## **5.7 Alternative Pathway for Cholesterol Release from Neurons?**

Both 24(*S*)-hydroxycholesterol and also GW683865A enhanced, although to a limited extent, apoA-I-mediated cholesterol efflux from neuronal cells, suggesting this is another neuronal pathway to dispose of cholesterol. However, GW683965A had only a limited effect on ABCA1 expression in these cells. Rebeck et al. (2004) recently reported up-regulation of neuronal ABCA1 expression by the synthetic LXR ligand T0901317. A role for apoA-I in the disposal of cholesterol from neurons is in line with its well known role in so called “reverse cholesterol transport”.

ApoA-I is present in brain and in CSF and has been detected in senile plaques in AD brains (Harr et al. 1996; Panin et al. 2000). So far, apoA-I synthesis within the brain has only been ascribed to endothelial cells of the BBB (Mockel et al. 1994). Neurons are always found in close proximity to blood vessels. Considering the total length of the BBB (about 600 km in the human brain), it is assumed that every neuron has its own microvessel. Therefore, the possibility remains that endothelium derived apoA-I participates in the removal of cholesterol and phospholipids from neurons. Evidence supporting the transcytosis of apoA-I across the BBB via the SR-BI was obtained using an in vitro model for the BBB (Kratzer et al. 2007). Upregulation of ABCA1 with oxysterols increased apoA-I binding and internalization. ApoA-I binding, internalization, and transcytosis were reduced by at least 50% after silencing ABCA1 but not after knocking down SR-BI. Thus, it was concluded that ABCA1, but not SR-BI, modulates the transcytosis of apoA-I through endothelial cells (Cavelier et al. 2006). Interestingly, decreased brain levels of total cholesterol have been measured in apoA-I-deficient mice (Fagan et al. 2004).

## **5.8 Role for cAMP Responsive Element Binding Protein in the Regulation of Neuronal Cholesterol Homeostasis**

Besides 24(*S*)-hydroxycholesterol, cAMP responsive element binding protein (CREB) may play a role in the regulation of neuronal cholesterol homeostasis (Lemberger et al. 2008). CREB was found to regulate the induction of specific gene expression patterns in neurons in response to activity. Interestingly, mice deficient for CREB and cAMP responsive element modulator (CREM) displayed altered expression of genes involved in cholesterol metabolism and accumulation of cholesterol in neurons.

How the conversion of cholesterol into 24(*S*)-hydroxycholesterol is regulated remains to be clarified. It was recently reported that the expression of CYP46A1 is regulated by Sp transcription factors (Milagre et al. 2008); and a specific ER-resident ORP8 was also suggested to play a role in oxysterol generation (Yan et al. 2008).

## **5.9 Internalization of Cholesterol by Neurons**

It remains to be established via which pathways neurons internalize cholesterol. Receptors amongst others that have been suggested to be involved are: LDLR, LRP1, LRP8, LR11, apoER2 and VLDLR (Bu et al. 1998; Jaeger and Pietrzik 2008). The LDL-receptor is best known for mediating the internalization of cholesterol in lipoproteins via binding apoE or apoB100. In the brain, the expression of the LDL-receptor is lowest in neurons, where it is expressed in particular in

cell bodies and proximal axons, and is highest in astrocytes (Pitas et al. 1987a). LRP is detected predominantly in neurons and in activated astrocytes (Bu et al. 1994; Rebeck et al. 1993, 1995). A number of *in vitro* and *in vivo* studies indicate a role for the LDLR-related protein (LRP) in the internalization of cholesterol by neurons (for reviews, see Nathan et al. 2002; Zerbinatti and Bu 2005; Zerbinatti et al. 2004, 2006). Besides the LDLR and LRP neurons express apoER2 mostly in distal axons and LR11 in cell bodies/proximal axons and also SR-BI (Offe et al. 2006; Posse De Chaves et al. 2000). Part of the mitochondrial ATP synthase complex has been identified as a hepatocyte receptor for the uptake of HDL particles (Lyly et al. 2008).

Although the absence of LDLR or apoE does not affect the total levels of cholesterol in the brain or its rate of synthesis, it does affect the transbilayer distribution of cholesterol in synaptic membranes (Igbavboa et al. 1997). Consequently, this may affect neuronal signalling functions. ApoE-deficient mice display severely impaired learning and memory functions (Oitzl et al. 1997). Deficiency for LDLR in mice results in impaired spatial memory, decreased synaptic density in the hippocampus and decreased cell proliferation (Mulder et al. 2004, 2007).

## 5.10 The Choroid Plexus as an Alternative Source of HDL

Astrocytes are not the only source of HDL-like lipoproteins in the brain. Epithelial cells of the choroid plexus also release cholesterol and thereby play an important role in the regulation of CSF cholesterol homeostasis (Segal 2000). The total surface of the choroid plexus epithelial cells is said to be comparable to the total surface of the BBB. Similar to its effect in astrocytes, 24(*S*)-hydroxycholesterol was found to induce cholesterol release from the apical membrane of choroid plexus epithelial cells in apoE-isoform-dependent manner concomitantly with the induction of ABCA1 and ABCG1 (Fujiyoshi et al. 2007). Using mice expressing apoE coupled to enhanced green fluorescent protein, Xu et al. (2006) have shown that apoE is expressed in cells of the choroid plexus. In line with a role for apoE, cholesterol granulomas have been detected in the choroid plexus in apoE-deficient mice (Owiny and Strandberg 2000). Tachikawa et al. (2005) reported high ABCA1 mRNA levels in choroid plexus, and absence of ABCA1 in mice results in reduced levels of apoE in CSF (Wahrle et al. 2004).

ApoE is also detectable in smooth muscle cells of large blood vessels and in cells surrounding small micro-vessels as well as in oligodendrocytes and ependymal cells, suggesting an involvement in cholesterol efflux from these cells (Boyles et al. 1985; Poirier et al. 1991; Xu et al. 2006). ApoE is normally not detected in neurons except after injury.

Additionally, microglia have also been reported to secrete apoJ-rich and apoE-poor, spherical lipoproteins that are more LDL-like and different from those secreted by astrocytes (Xu et al. 2000).

## 5.11 Disturbances in Cholesterol Trafficking Between Astrocytes and Neurons in Alzheimer's Disease?

In line with the hypothesis that in AD patients the trafficking of cholesterol from astrocytes to neurons may be compromised, a number of genes that have been associated with AD encode proteins that are involved in this process (for a review, see Carter 2007). APOE4 is the strongest genetic risk factor known for sporadic AD (Corder et al. 1993; Saunders et al. 1993); and it has been hypothesized that apoE may affect the pathogenesis of AD by isoform-specific effects on lipid trafficking between astrocytes and neurons (Michikawa et al. 2000). Moreover, CYP46A1 and ABCA1 have been associated with an enhanced risk of AD. Several studies have suggested a role for 24(*S*)-hydroxycholesterol in the pathogenesis of AD (Reiss 2005; Reiss et al. 2004; Wolozin et al. 2004). Polymorphisms of CYP46A1 have been linked to AD, with the exception of one study which did not find such a relationship (Desai et al. (2002). The expression of this enzyme appeared to have shifted from neurons to glia in AD patients (Bogdanovic et al. 2001). In the early stages of AD, plasma and CSF levels of 24(*S*)-hydroxycholesterol were found to be elevated, but they were reduced in the late stage, possibly due to a loss of CYP46A1-expressing metabolically very active neurons (Heverin et al. 2004; Lutjohann et al. 2000; Papassotiropoulos et al. 2000, 2002). Postmortem cerebrospinal fluid of AD patients contained lower concentrations of cholesterol, phospholipids and fatty acids, while the levels of apoE were only slightly reduced (Bretillon et al. 2000; Heverin et al. 2004; Lutjohann et al. 1996; Mulder et al. 1998; Papassotiropoulos et al. 2002; Schonknecht et al. 2002).

Since the composition of CSF is very similar to that of brain interstitial fluid, it is likely that also in interstitial fluid the levels of HDL-like particles are reduced. This may affect the deposition of A $\beta$ , if these lipoproteins play a role in maintaining it soluble and decreasing its levels by removing it from the brain into the circulation and/or into the CSF.

Although the link between APOE genotypes and AD was established 15 years ago, it remains to be established exactly how apoE4 enhances the risk. ApoE has been suggested to play a role in the still largely unknown, transport and secretion of sterols, other than 24(*S*)-hydroxycholesterol, from the brain across the BBB into the plasma (Mulder and Terwel 1998). Bell et al. (2007) found that lipid-poor apoE is cleared via the BBB, but that lipidated apoE is cleared predominantly via the cerebrospinal fluid and barely via the BBB.

Another indication for a link between cholesterol and AD came from epidemiological studies indicating that cholesterol-lowering drugs, i.e., statins that are widely used for the treatment of hypercholesterolemia, reduced the risk of AD (Wolozin 2004). Although the literature on this topic is rather controversial. Statins were found to reduce the deposition of A $\beta$  in plaques in the brain of AD-mouse models (Eckert et al. 2001). These effects were ascribed to the cholesterol-lowering effects of statins. In agreement, high plasma cholesterol levels and high fat intake were found to be associated with an increased risk of AD (Grant et al. 2002). Ten years ago Sparks et al. (1997) discovered plaques-like structures in brains of patients that died of

cardiovascular diseases and not in brain of patients with other causes of death. Via yet unknown mechanisms, high plasma cholesterol concentrations result in an increased deposit of A $\beta$  in the brains of AD-mouse models (Levin-Allerhand et al. 2002; Oksman et al. 2006). Cholesterol itself was found to be present in plaques (Mori et al. 2001). In a mouse model of AD, chronically elevated plasma cholesterol induced increased APOE mRNA in the brain, while these were reduced by administration of statins (Petanceska et al. 2003).

However, no effects of even very high plasma cholesterol on brain A $\beta$  levels were observed in LDLR-deficient mice (Elder et al. 2007).

Since the brain fully relies on de novo synthesis of cholesterol and is thought to be unable to compensate by enhancing the uptake of dietary derived cholesterol from the circulation, it may be very sensitive to cholesterol synthesis reducing agents. High doses of simvastatin were found to reduce cholesterol synthesis in the brain (Thelen et al. 2006a). Therefore, it was initially suggested that the beneficial effects of statins on the development of AD may be the result of its suppressive effects on cholesterol synthesis (Li et al. 2006). However, it is questionable whether this is an advantage. The use of statins has also been associated with memory complaints (Wagstaff et al. 2003). Furthermore, it was found that statins directly inhibit long-term potentiation, which is regarded as a marker for synaptic plasticity (Kotti et al. 2006). In line with this, Kotti et al. (2006) reported that cholesterol synthesis in the brain is essential for learning processes. It may not be cholesterol itself that is required for learning but a non-sterol by-product of the mevalonate pathway, the isoprenoid "geranylgeraniol". The continuous production of small amounts of geranylgeraniol and consequently, a continuous production of cholesterol, in a subgroup of neurons is supposed to be required for spatial, associative and motor learning. Interesting is the notification that the reported decrease in the cholesterol synthesis rate during aging may be associated with an increase in loss of memory functions (Thelen et al. 2006b).

In contrast with expectations, lovastatin appeared to induce the deposition of amyloid in brain of an AD-mouse model; and George et al. (2004) found that diet-induced hypercholesterolemia reduced brain levels of amyloid in aged mice.

The beneficial effects of statins on the progression of AD may, therefore, not be ascribed to their cholesterol-lowering effect, but to their anti-inflammatory properties or their modulating effects on the cerebral vessel walls.

The observation of an altered processing of cholesterol in fibroblasts from AD patients, suggest that the changes do not remain restricted to the central nervous system (Murphy et al. 2006).

There is evidence to suggest that it is not simply the level of cholesterol in the brain affects the production and deposition of A $\beta$ , but rather its intracellular distribution. In vitro studies show that the cellular amount of cholesterol or the distribution across membranes directly affects the splicing of amyloid from its precursor protein and on its aggregation (Frears et al. 1999; Puglielli et al. 2001; Simons et al. 1998). Cholesterol-depleted neurons produce less amyloid than cholesterol-rich neurons.

Therefore, alterations in brain cholesterol metabolism seem to affect the production and deposition of A $\beta$ . Alternatively, A $\beta$  also seems to directly affect

cholesterol synthesis (Hartmann 2006). Liu et al. (1998) reported that A $\beta$  alters intracellular vesicle trafficking and decreases cholesterol esterification and the distribution of cholesterol in neurons. In AD brains, A $\beta$  selectively accumulates in the perikaryon of pyramidal cells as discrete granules that appear to be cathepsin D-positive (Nixon et al. 2001). Cathepsin D has been suggested to play a role in the regulation of composition, trafficking and/or recycling of membrane components. The A $\beta$ -containing granules are thought to be of endosomal/lysosomal origin (Gomez-Ramos et al. 2007).

Interestingly, apoE has been suggested to be involved in the degradation and clearance of deposited A $\beta$  by astrocytes (Koistinaho et al. (2004). It was found that astrocytes can degrade A $\beta$ , but not if: (i) they are deficient for apoE, or (ii) RAP, an antagonist of the LDLR family, is present or is anti-apoE. It can be speculated that the lipoprotein-like particles secreted by astrocytes that are present in the interstitial fluid pick up A $\beta$  from neurons and direct it to astrocytes which internalize the particles via the LDLR or another member of the LDLR family. The latter is supported by the observation that the absence of the LDLR from PDAPP mice, a model for AD, does not affect amyloid levels nor its deposition (Zerbinatti et al. 2006). However, absence of apoE unexpectedly decreased amyloid load in the brain of these PDAPP mice.

Variations in the expression of ABCA1 and ABCG1 have been found to affect amyloid production and/or its deposition. Overexpression of ABCA1 in PDAPP mice results in a decrease in amyloid deposition in the brain (Wahrle et al. 2008), but apparently not by affecting its transport from the brain into the circulation (Akanuma et al. 2008). In vitro evidence was obtained indicating an impact of ABCG1 on amyloid production, but in vivo such an effect was not detectable in PDAPP mice overexpressing ABCG1 (Burgess et al. 2008). Novel evidence showed that ABCA7, a close homolog of ABCA1, belonging to the same ABCA subfamily of full-length ABC transporters, can regulate APP processing (Chan et al. 2008).

LXR agonists may be promising tools in the treatment of AD. Activation of the LXR pathway via synthetic agonists was found to reduce the production of amyloid in cultured neurons (Sun et al. 2003). It was suggested that this resulted from an upregulated expression of ABCA1. Whether or not this needs to be accompanied by an enhanced neuronal cholesterol efflux remains controversial (Burns et al. 2006; Rebeck 2004; Sun et al. 2003). LXR activation was found to reduce amyloid levels in the brain and its deposition in a AD-mouse model (Koldamova et al. 2005).

## **5.12 Do Alterations in Systemic Sterol Metabolism Alter Brain Sterol Metabolism?**

It remains to be established whether or not variations in extracerebral/systemic cholesterol levels affect brain cholesterol metabolism. Total levels of cholesterol in brain remain unaltered even if a plasma cholesterol are reduced by 84% due to administration

of either simvastatin or pravastatin in guinea-pigs (Lütjohann et al. 2004). In line with a strictly and independently regulated brain cholesterol metabolism, dramatic increases in circulating cholesterol levels in apoE-deficient mice due to administration of a high-fat diet did not affect levels of sterols in the brain with the exception of elevated levels of 27-hydroxycholesterol (Paula J. Jansen, Dieter Lütjohann, Karin M. Thelen, Klaus von Bergmann, Fred van Leuven, Frans C.S. Ramaekers and Monique Mulder, unpublished data). Heverin et al. (2005) showed a net flux of 27-hydroxycholesterol from the circulation into the brain using plasma samples collected from the internal jugular vein and an artery of healthy male volunteers. 27-hydroxycholesterol in human brain was distributed consistent with an extracerebral origin, with a concentration gradient from the white to the grey matter – a situation opposite to that of 24S-hydroxycholesterol. 27-Hydroxycholesterol was suggested to be an important link between intra- and extracerebral cholesterol homeostasis (Heverin et al. 2005). Similarly, other circulating cholesterol metabolites were found to enter the brain. For example Panzenboeck et al. (2007) demonstrated an efficient transfer of 7 $\alpha$ -hydroxy-4-cholesten-3-one, a metabolite of cholesterol, across cultured porcine brain endothelial cells (a model for the blood–brain barrier; Panzenboeck et al. 2007). Accordingly, accumulating evidence indicates an effect of a high-fat/high-cholesterol diet on brain (Mulder et al. 2001). Mateos et al. (2008) reported that a high-fat diet and also 27-hydroxycholesterol down regulates cytoskeleton associated protein (Arc), a protein that is also down regulated in AD brains.

Evidence was obtained that plant sterols, that can be obtained only from the diet and have a structure very similar to that of cholesterol, can end up in the brain of mice (Jansen et al. 2006). The addition of supplements to the diet could therefore provide be an alternative strategy to modulate brain cholesterol metabolism and, thereby, the development and/or the progression of AD. Possible ways to achieve this may be for example via the addition of specific compounds that have been found to affect the LXR pathway (Wagstaff et al. 2003; Yang et al. 2004).

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# Chapter 6

## Intracellular Cholesterol Transport

Daniel Wüstner

**Abstract** Cholesterol is the single most abundant lipid species in mammalian cells. More than  $2 \times 10^9$  years of evolution designed this molecule to perfectly fit into phospholipid bilayers regulating the fluidity, permeability and bending stiffness of biological membranes. Cholesterol also serves as a precursor of steroid hormones, bile acids and oxysterols, and its cellular synthesis is regulated by a complex machinery. While the molecular mechanisms underlying cholesterol synthesis are known in great detail, knowledge is rather sparse about the inter-compartment transport of cholesterol, including trafficking modes and kinetics, as well as control of endomembrane cholesterol content. This chapter provides an overview of our recent understanding of intracellular transport of cholesterol. It is aimed to create a link between the well characterized biophysical properties of cholesterol in model membranes and its behavior in living cells.

### 6.1 Biophysical Properties of Cholesterol in Model Membranes

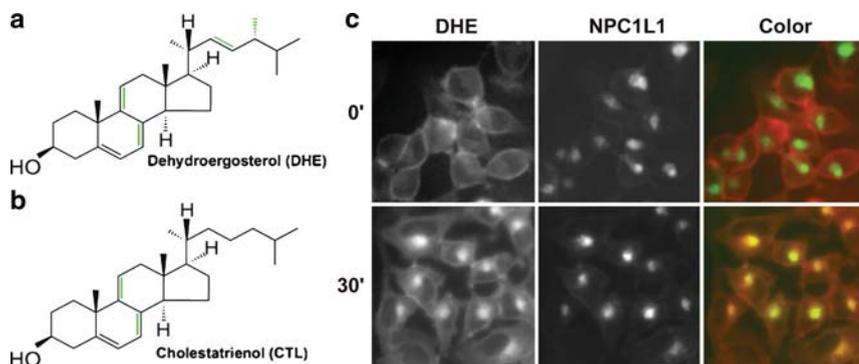
Cholesterol is a very hydrophobic lipid molecule with the C3-connected OH group as the only polar constituent. Consequently, it has a very low solubility in water, forming micelle-like aggregates at concentrations as low as 20–30 nM (Haberland et al. 1973; Loomis et al. 1979). Its very hydrophobic character combined with its planar steroid ring structure and stereochemistry means that cholesterol perfectly fits into phospho- and sphingolipid bilayers. Single-component phospholipid bilayers show a temperature-dependent phase transition characterized by a highly co-operative chain melting process and a transition from the gel (so) to the liquid-crystalline ( $L\alpha$ ) phase. To efficiently shield cholesterol from contact with water, it is located underneath the polar lipid head groups and additionally straightens their fatty acyl

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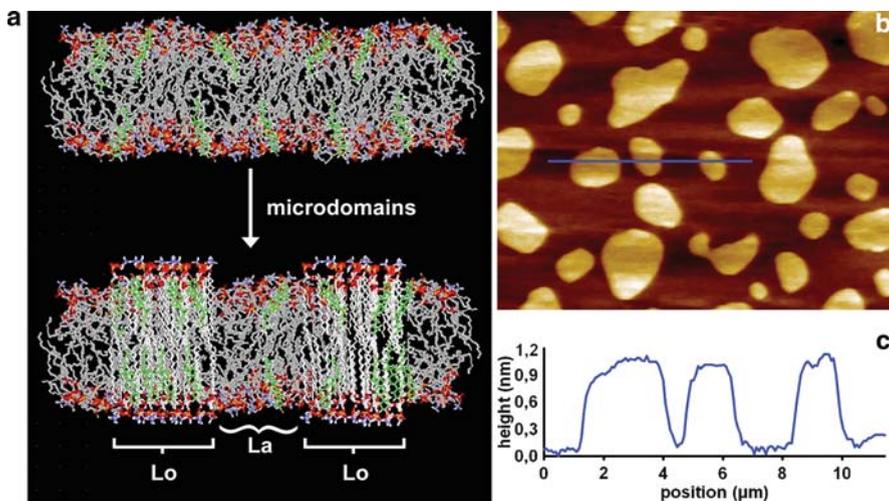
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chains in the fluid lipid bilayer. This so-called condensing effect of cholesterol decreases the lateral distance of the host phospholipids, resulting in tighter packing in phospholipid/cholesterol bilayers compared to pure phospholipids above the phase transition temperature (Yeagle 1985; Silvius et al. 1996). A consequence of this process is that the lipid bilayer gets thicker, while at the same time its area decreases in such a way that the total bilayer volume remains constant (Lindahl and Edholm 2000; Nagle and Tristram-Nagle 2000; Hofsass et al. 2003). The condensing effect of cholesterol can be measured and is conveniently represented by the ordering of the phospholipid acyl chains determined by NMR spectroscopy. This order parameter can be related to other bilayer properties like bending stiffness and resistance against area dilation – both mechanical properties which increase in the presence of cholesterol (Needham et al. 1988; Henriksen et al. 2004, 2006). Fluorescent cholesterol analogs used for analysis of cholesterol trafficking by microscopy must resemble cholesterol as closely as possible. Importantly, intrinsically fluorescent sterols like dehydroergosterol (DHE) and cholestatrienol (CTL), but not NBD-cholesterol, have a potential to order fatty acyl chains in bilayers comparable to cholesterol at relatively low sterol concentrations (Scheidt et al. 2003), while at the same time raising the bilayer stiffness like cholesterol (Garvik et al. 2009). Due to these similarities to cholesterol, both the sterols DHE and CTL are very suitable for the analysis of intracellular sterol trafficking by sensitive low-light fluorescence microscopy (see Fig. 6.1; Hao et al. 2002; Wüstner et al. 2002, 2005; Hartwig Petersen et al. 2008). At very high cholesterol concentrations (above 30 mol%) a third phase can form in sterol/phospholipid bilayers, called the liquid-ordered (Lo) phase characterized by high acyl chain order combined with rapid lateral diffusion (Vist and Davis 1990; Ipsen et al. 1987; see Fig. 6.2). This Lo phase thus bears properties of both the gel phase (high fatty acyl chain order) and the fluid phase (rapid lateral diffusion), and it attracted great attention in the past decade due to its similarity to lipid fractions isolated from cellular membranes by detergent extraction (Ahmed et al. 1997). However, it has to be emphasized that, by definition, the Lo phase is a property of simple two- or three-component lipid bilayers, and extrapolation to cellular membrane characteristics can lead to wrong, physiologically irrelevant conclusions (see Sect. 6.2). In ternary model systems, the acyl chain-ordering effect of cholesterol in the Lo phase can be directly monitored as a height difference between fluid  $L\alpha$  and ordered Lo domains by atomic force microscopy (see Fig. 6.2). The observed domains appear round, since they are stabilized by line tension energy between the Lo and  $L\alpha$  phases. In other words, lipids in either phase are free to diffuse laterally and organize in such a way as to minimize their contact to the phase “they don’t like”, so to speak. This gives round phases, since this shape minimizes contact area, similar to the case of fat droplets in a soup, for example. Interestingly, we showed recently that DHE does not only partition preferentially into the Lo phase in ternary mixtures consisting of dioleoylphosphatidylcholine/dipalmitoylphosphatidylcholine and DHE at a 1:1:1 ratio, but can also induce Lo/ $L\alpha$  phase separation with round domains, clearly confirming that DHE behaves very much like cholesterol under the same conditions (Garvik et al. 2009).



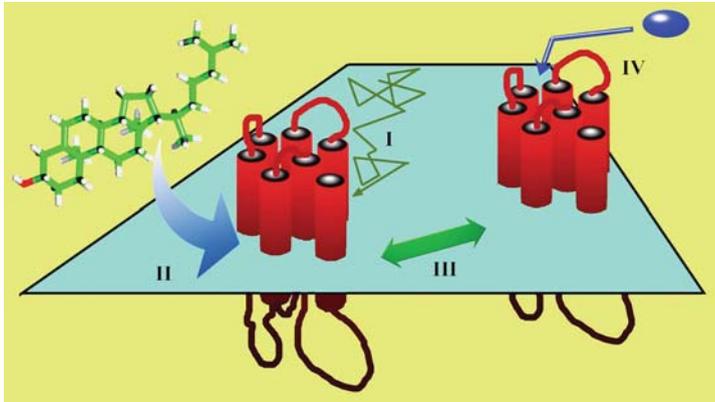
**Fig. 6.1** Direct observation of cholesterol transport by microscopy using intrinsically fluorescent sterols. **a, b** Chemical structure of dehydroergosterol (*DHE*, **a**) and cholestatrienol (*CTL*, **b**) with differences to cholesterol highlighted in green. While *CTL* has the same side-chain as cholesterol, the side-chain of *DHE* is identical to that of ergosterol, i.e., branched methyl and one double bond. The fluorescence properties of both sterols are almost identical due to the fact that the “chromophore” formed by the three conjugated double bonds in the steroid ring system is the same. **c** Distribution of *DHE* in McArdle7777 rat hepatoma cells expressing enhanced green fluorescent protein-tagged Niemann–Pick C1-like 1 (EGFP-NPC1L1) protein. Cells were pulse-labeled for 1 min with *DHE* loaded onto cyclodextrin, washed and either imaged immediately (0') or chased for 30 min at 37°C before imaging (30') on a wide-field fluorescence microscope optimized for transmission in the ultraviolet (UV) to detect *DHE* and additionally equipped with a fluorescein filter set to detect EGFP fluorescence. Right after labeling, *DHE* is found exclusively in the plasma membrane, while EGFP-NPC1L1 resides in the perinuclear endocytic recycling compartment (ERC) and to a small extent at the cell surface. After 30 min chase, *DHE* co-localizes very much with EGFP-NPC1L1 in the ERC region. The color overlay shows *DHE* in red and EGFP-NPC1L1 in green. Co-localization appears yellow to orange. For further details, see Hartwig Petersen et al. (2008) and text

It has been suggested that the cell regulates plasma membrane protein function by modulating the transverse pressure profile, e.g., via altering the lipid composition and distribution or the cholesterol content (Cantor 1999). Indeed, modulating the cholesterol content of cellular membranes has been shown to directly affect protein function, like inhibition of calcium ATPases and protein translocation machinery in the ER (Nilsson et al. 2001; Li et al. 2004). The proper function of rhodopsin and several G protein-coupled receptors depends on cholesterol. For example, modification of membrane cholesterol and sphingolipids alters the conformation and function but not intracellular trafficking of the G protein-coupled cholecystokinin receptor, while cholesterol is required for maintenance of the specific agonist binding activity of the serotonin(1A) receptor (Chattopadhyay et al. 2005; Harikumar et al. 2005; see Fig. 6.3). Much effort has been spent in the past and there is still considerable focus on the many facets of cholesterol's properties in membranes, but not all of them can be discussed here. Beside the ability to order lipid acyl chains and to induce the *Lo* phase, two other properties of cholesterol and



**Fig. 6.2** Cholesterol induces fluid–fluid immiscibility in phospholipid membranes. **a** Cartoon of a lipid bilayer made from molecular simulation of phosphatidylcholine (*PC*) with added cholesterol molecules in green. *Upper panel* shows the phospholipid bilayer in the fluid  $L\alpha$  phase with characteristic disordered fatty acid chains. Carbon atoms are shown in light gray, phosphorus in yellow, oxygen in red and nitrogen in blue, hydrogen atoms are omitted for clarity. *Lower panel* shows fluid–fluid immiscibility as in **a**, with two small domains in the ordered  $L_o$  phase surrounded by the fluid  $L\alpha$  phase. Due to straightening of the fatty acyl chains and tighter packing with cholesterol, the  $L_o$  phase appears to be thicker than the surrounding  $L\alpha$  phase. **b** Atomic force microscopy of a lipid bilayer consisting of three components, dipalmitoylphosphatidylcholine (DPPC)/dioleoylphosphatidylcholine (DOPC)/cholesterol at a molar ratio of 40:40:20 at 20°C on mica support. Round thick  $L_o$  domains (light color) surrounded by a continuous  $L\alpha$  phase (brown color) can be clearly distinguished. The  $L_o$  phase contains most of the saturated DPPC and probably most cholesterol. **c** Line scan with the AFM tip along the blue line in **b**, indicating the height difference between the  $L\alpha$  phase (set to zero height) and the  $L_o$  phase (approx. 1 nm higher). Note that, while **b** and **c** are based on experimental data, **a** is only a cartoon to illustrate cholesterol-induced phase separation in lipid membranes. This illustration was generated from an atomistic Monte Carlo simulation performed with the AMBER force field on a Linux machine by the author in collaboration with Dr. Heinz Sklenar (Max Delbrück Centrum, Berlin, Germany). **b** and **c** are courtesy of Dr. Adam Cohen Simonsen from the Department of Physics and Chemistry, Center for membrane physics (MEMPHYS) at the University of Southern Denmark

related sterols are of importance when looking at intracellular cholesterol transport. First, the transbilayer orientation: cholesterol (and DHE) show a rapid flip-flop from one leaflet to the other in model membranes, with a half-time of a few seconds (Leventis and Silvius 2001; John et al. 2002). The same has been reported for cholesterol in red blood cell membranes (Steck et al. 2002) and can be attributed to the low free energy barrier associated with moving a single polar OH group through the hydrocarbon chains of the bilayer (Hamilton 2003; Róg et al. 2008). Second, cholesterol shows a reasonable mobility out of the membrane, perpendicular to the bilayer plane (Gliss et al. 1999; Wüstner 2007; Lange and Steck 2008). This might explain how the relatively slow release of cholesterol from membranes could be



**Fig. 6.3** Possible impact of cholesterol on receptor dynamics in the plasma membrane. Cholesterol (shown on the *left* in green) could affect lateral diffusion of a symbolic receptor molecule (green zig-zag arrow) consisting of several transmembrane helices and connecting loops (shown in red; *I*). Cholesterol could bind directly to the receptor and thereby alter its conformation (curved blue arrow; *II*) and/or induce receptor clustering (green double arrow; *III*) at the cell surface. In addition, cholesterol could change binding properties of the receptor to a ligand (blue) either due to change of receptor structure or mobility (*IV*). Note that this is an idealized receptor and ligand embedded in a schematically drawn plasma membrane. See text for more details

overcome by cytoplasmic carriers: these proteins might pick up individual sterol molecules during their transient protrusion from the bilayer (see Sect. 6.5).

Finally, I want to mention that there is an upper limit of cholesterol solubility in lipid membranes. Above 50–60 mol% cholesterol in the bilayer the host phospholipids are no longer able to shield cholesterol from contacting water, causing the precipitation of cholesterol in form of monohydrate crystals from the membrane (Huang and Feigenson 1999; Huang et al. 1999). Cholesterol crystals likely formed by this mechanism have been detected in atherosclerotic lesions and in macrophage cell culture under free cholesterol loading conditions (Small et al. 1974; Tangirala et al. 1994; Kellner-Weibel et al. 1999).

## 6.2 Molecular Organization and Function of Cholesterol in the Plasma Membrane

The plasma membrane harbours most cellular cholesterol, since tight lipid packing as mediated by cholesterol's condensing effect is important to create a permeability barrier against ions, small metabolites and even oxygen in the plasma membrane (Subczynski et al. 1989; Krylov et al. 2001; Maxfield and Wüstner 2002; Khan et al. 2003). It has been shown that the two membrane leaflets independently create a diffusion barrier and that a major role is played by cholesterol to maintain this barrier function (Subczynski et al. 1989; Hill and Zeidel 2000; Krylov et al. 2001). Phospho- and sphingolipids are unevenly distributed between the two plasma

membrane leaflets (op den Kamp 1979; Maxfield and Wüstner 2002). While most sphingolipids and phosphatidylcholine (PC) reside in the exoplasmic leaflet, aminophospholipids and phosphatidylinositols (PI) are enriched on the inner, cytoplasmic membrane leaflet. This phenomenon was first described for red blood cells and is now a commonly accepted fact for mammalian cells in general. The lipid transbilayer asymmetry is actively maintained, in the case of aminophospholipids, for example, by a translocase probably belonging to the type P-ATPases (Pomorski et al. 2004). While much has been learned about transbilayer phospho- and sphingolipid transport in the past decade, the distribution of cholesterol across the plasma membrane leaflets remains obscure. Some studies state that cholesterol is enriched in the outer lamelle, others provide evidence for preferred sterol localization to the inner membrane leaflet (Maxfield and Wüstner 2002). Finally, it has been proposed that cholesterol flip-flop across the plasma membrane of red blood cells occurs within seconds, suggesting that its transbilayer dynamics are very high (Steck et al. 2002). Such rapid cholesterol flip-flop, together with reasonable mobility perpendicular to the membrane plane, could play an important role in coupling the two membrane leaflets (Gliss et al. 1999; Wüstner 2007a). Based on cholesterol's well known partition preference into membranes enriched in PC and sphingomyelin (SM) compared to aminophospholipids or PI, it is more likely that cholesterol accumulates in the outer but not in the inner leaflet of the plasma membrane (Ohvo-Rekila et al. 2002; Niu and Litman 2002). A very recent study by Maxfield and co-workers, however, found the fluorescent sterol DHE residing preferentially (i.e., by 60–70%) in the cytoplasmic leaflet of the plasma membrane in CHO cells (Mondal et al. 2008). The uncertainty regarding cholesterol distribution between the two membrane leaflets is due to methodological limitations (Maxfield and Wüstner 2002).

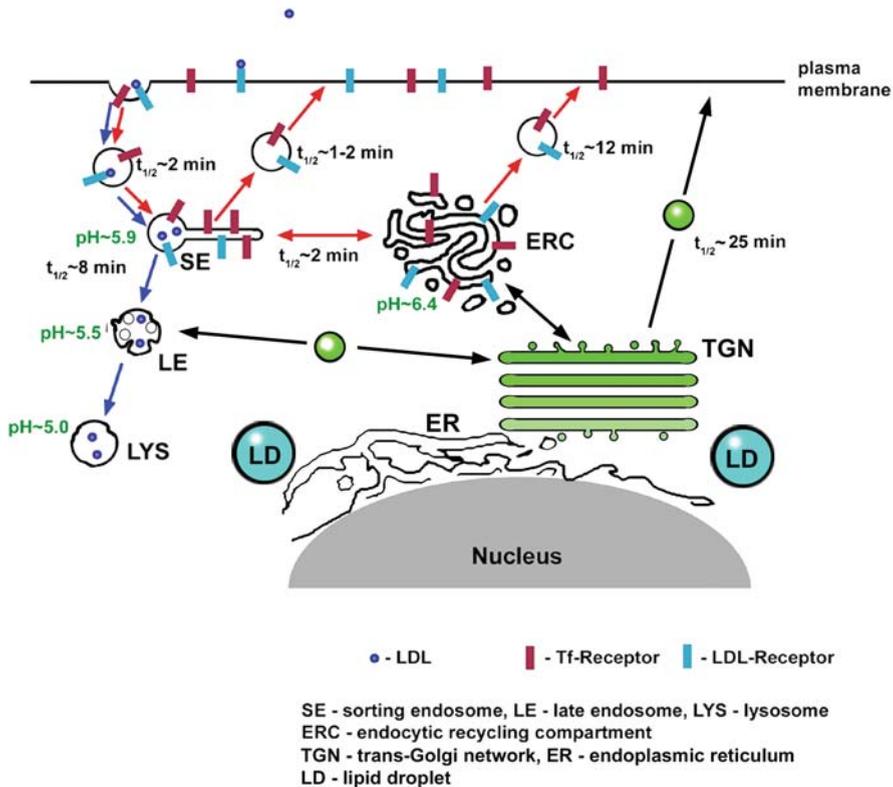
It was soon realized that membrane protein functions like signal transduction upon ligand binding to receptors require functional and probably also spatial compartmentation of proteins in the plasma membrane (reviewed by Karnovsky et al. 1982; Edidin 2003). In some cell types, like endothelial cells, specialized plasma membrane invaginations named caveolae were identified as important cholesterol-dependent “sub-compartments” of the cell surface being involved in signal transduction. Originally, it was proposed that the plasma membrane is a two-dimensional fluid with embedded proteins in a “sea” of fluid lipids (see Edidin 2003). However, a large body of evidence has accumulated during the past 15 years showing that this fluid-mosaic model of the plasma membrane is oversimplified (Subczynski and Kusumi 2003). One very popular model assumes that proteins show lateral aggregation in small microdomains in the plasma membrane together with certain lipids. According to this “raft” model, cholesterol clusters specifically with sphingolipids in the exoplasmic leaflet of the plasma membrane in so-called rafts which might play an important role in signal transduction and protein sorting (Ahmed et al. 1997; Simons and Ikonen 1997; Maxfield 2002). Raft microdomains were originally characterized by their resistance to solubilization at low temperature, using non-ionic detergents like Triton X-100, as well as by single-particle tracking techniques (Simons and van Meer 1988; Pralle et al. 2000). Detergent treatment

itself, however, can induce membrane domain formation and clustering of certain proteins in the plasma membrane (Heerklotz 2002; Mayor and Maxfield 1995). Moreover, more than 70% of the plasma membrane surface area is detergent-resistant and putative raft proteins show no difference in their (bulk) surface diffusion properties compared to non-raft proteins (Maxfield 2002; Kenworthy et al. 2004). Another model, called the “membrane skeleton fence model”, is based on extensive single particle tracking and fluorescence recovery after photobleaching studies and assumes that the membrane-attached cytoskeleton plays a crucial role in compartmentalization of the plasma membrane (Koppel et al. 1981; Sheetz 2001; Subczynski and Kusumi 2003). Fences for lateral diffusion have been characterized for the transferrin receptor, band 3 and other membrane proteins in living mammalian cells (Sako and Kusumi 1995; Tomishige et al. 1998). Even for fluorescent phospholipids, “hop-diffusion” between plasma membrane compartments has been demonstrated (Fujiwara et al. 2002). Thus, while membrane compartmentation is mainly “lipid-driven” in the “raft” model, the “membrane skeleton fence model” assumes “preformed” compartments due to the dynamic interaction of the cytoskeleton with the plasma membrane (Sheetz 2001; Maxfield 2002; Subczynski and Kusumi 2003; Mukherjee and Maxfield 2004). Without reviewing all the arguments for or against different membrane models (additionally, see Maxfield and Wüstner 2002; Munro 2003; Kenworthy 2008), I want to bring the reader’s attention to the most recent results using fluorescence nanoscopy of living cells, like stimulated emission depletion (STED) and photoactivatable localization microscopy (PALM; Hell 2007). These techniques provide images of the cell surface with high resolution below the diffraction limit of 200 nm, suggesting that these techniques are most suitable to distinguish between competing membrane models (Sieber et al. 2006, 2007; Hess 2007). For example, in a recent study using STED, it was shown that self-association of EGFP-tagged syntaxin 1 expressed in PC12 cells depends on weak homophilic protein–protein interactions without the need for rafts (Sieber et al. 2006, 2007). Importantly, syntaxin clusters require normal cholesterol levels in the plasma membrane, but this is not related to rafts defined by detergent resistance, since these clusters are Triton X-100 soluble and do not co-patch with raft markers (Lang et al. 2001). It is likely that many of the observed effects of cholesterol on lateral diffusion or interaction of proteins in the plasma membrane depend on cholesterol-mediated membrane interaction with the underlying actin cytoskeleton (Sheetz 2001; Kwik et al. 2003; Sun et al. 2007; Ganguly et al. 2008). The molecular mechanisms remain to be deciphered but likely involve cholesterol-regulated levels and distribution of phosphatidylinositols, specifically phosphatidyl-4,5-bisphosphate (PIP2) in the inner leaflet of the plasma membrane (Kwik et al. 2003). In fact, atomic force and fluorescence microscopy recently demonstrated that cholesterol depletion by incubating cells with cyclodextrin redistributed the PIP2 and F-actin, accompanied by a significant increase in the adhesion energy between the membrane and the cytoskeleton and a decrease in the membrane diffusion constants of both raft and non-raft proteins, as well as fluorescent lipid probes (Kwik et al. 2003; Kenworthy et al. 2004; Goodwin et al. 2005; Shvartsman et al. 2006; Sun et al. 2007).

While it is evident that the plasma membrane has a heterogeneous lateral organization at the nanometer to micron scale, no agreement exists on the lateral distribution of cholesterol in the plasma membrane. Early attempts to characterize sterol membrane organisation were based on filipin staining in freeze–fracture electron micrographs. Evidence for lateral heterogeneity of cholesterol based on this method with a preferred enrichment in areas of cell attachment, caveolae and filopodia has been provided (Montesano 1979; Bridgman and Nakajima 1983). Filipin, a sterol binding polyene antifungal antibiotic with a pentaene chromophore, binds non-esterified cholesterol and related sterols, and this binding to membrane cholesterol can be detected by fluorescence microscopy or by electron microscopy, where characteristic bumps show up on membrane sheets (Maxfield and Wüstner 2002). Due to the fact that filipin extracts sterols from membranes and thereby changes membrane lipid organization, using this probe gives no absolute measure of membrane cholesterol content or distribution in cells (Behnke et al. 1984a, b). Moreover, when properly used, cell fixation is required, which also is a source of artefacts. Without reviewing all results based on various biophysical methods in fixed cells (for a detailed discussion, see Wüstner 2007a, b), I will shortly review recent data obtained by wide-field UV imaging and three-photon microscopy of DHE in living cells. Both methods showed that the plasma membrane staining of DHE is heterogeneous and non-random, while the interpretation of this observation was very different (Wüstner 2005, 2007b; Zhang 2005). Schroeder and colleagues analyzed three-photon images and suggested that DHE clusters in certain lateral domains in the plasma membrane (Zhang 2005). In contrast, we used highly sensitive multicolor deconvolution wide-field microscopy to show that the same heterogeneous membrane staining is found for DHE as well as for fluid lipid probes (Wüstner 2007b). We demonstrated in an independent study that DHE completely segregates from these probes in model giant unilamellar vesicles (Garvik et al. 2009). Thus, the absence of segregation of DHE and fluid phase lipid markers in living cell membranes suggests that the heterogeneous membrane staining and co-patching of both probes is caused by the rough surface topography of living cells but not by lateral sterol-rich and -poor domains in the bilayer plane (Wüstner 2007b). We could confirm this conclusion in subsequent studies by dynamic wide-field imaging as well as in cholesterol-loaded macrophage foam cells and membrane blebs (Wüstner 2008; Wüstner and Færgeman 2008). Importantly, we could not detect higher amounts of DHE in membrane caveolae visualized by GFP-tagged caveolin in adipocytes and fibroblasts (Wüstner and Færgeman 2008). This result, obtained on living cells, suggests that caveolae have the same cholesterol content as the remaining plasma membrane and are not cholesterol-enriched membrane microdomains. In a recent analysis investigating dynamic plasma membrane staining patterns of DHE by wide-field UV and three-photon microscopy, we come to the same conclusion, namely rough surface topography as the cause for heterogeneous sterol staining (Wüstner et al. 2009). Thus, we can rule out that the two different methods of visualizing DHE in living cells provide different results.

### 6.3 Overview of Membrane Traffic Along the Endocytic and Secretory Pathways and its Dependence on Cholesterol

Our understanding of membrane traffic along the endocytic and secretory pathways is largely based on studies of protein transport. This chapter does not serve as a general reference to the endocytic or secretory pathways, since we touch only those aspects relevant to cholesterol trafficking. I begin this discussion therefore with a short introduction into clathrin-dependent endocytosis (see Fig. 6.4). For a general review of membrane traffic, the interested reader is referred to recent reviews (Mukherjee et al. 1997; Bonifacino and Lippincott-Schwartz 2003; Maxfield and McGraw 2004; Pfeffer 2007). Ligands, like LDL or iron-transporting transferrin, bind to their respective receptors at the cell surface; those receptor–ligand complexes become collected in clathrin-coated pits (CCP) which trigger – together with linking of the receptor to adaptor proteins – internalization of the complex. The internalized vesicle loses rapidly its clathrin coat by ATP-dependent uncoating, followed by fusion of the endocytic vesicle with a population of early endosomes, called sorting endosomes (SE). Due to the slightly acidic pH, ligands like LDL dissociate from their receptor within the SE and follow the degradative pathway, i.e., shuttling to late endosomes and lysosomes for degradation. Similarly, iron is released from transferrin in the SE, while apotransferrin bound to its receptor recycles to the cell surface, either directly from SE in a rapid PI3-kinase dependent circuit (half-time about 2 min) or after transit through the long-living perinuclear ERC with a half-time of 9–12 min (Mukherjee et al. 1997; van Dam et al. 2002; Maxfield and McGraw 2004). This classic, well accepted scheme was actually developed over the years, based on the pioneering studies of Brown and Goldstein, who first described LDL trafficking and metabolism in fibroblasts (Goldstein and Brown 1974). There are several sorting mechanisms at play in the SE, including segregation of ubiquitinated receptors destined for down-regulation into clathrin-coated regions of the SE and recruitment of Hsr and ESCRT proteins to Rab7-positive late endosomes, followed by lysosomal targeting and digestion of cargo (Maxfield and McGraw 2004; van der Goot and Gruenberg 2006). Ligands dissociated from their receptor due to the pH drop in the SE, like LDL, can be sorted towards the degradative pathway by a simple geometric sorting mechanism: tubules containing a high surface-to-volume ratio bud repeatedly from the SE towards the ERC thereby segregating membrane-bound (LDL) receptors from luminal cargo (Maxfield and McGraw 2004). The SE lose fusion competence for incoming vesicles within 6–8 min and mature into late endosomes, thereby acquiring the machinery for digesting internalized cargo. One characteristic of this maturation is a switch in associated rab GTPases: SE contain rab5, being important for fusion, vesicle motility and recruitment of PI3-kinases, as well as rab4, being involved in rapid recycling to the cell surface. In contrast, newly forming late endosomes acquire rab7, which plays a role in motor-mediated transport of these organelles along microtubule tracks (Miaczynska and Zerial 2002; Murray and Wolkoff 2003;



**Fig. 6.4** Overview of membrane transport between endocytic and secretory compartments. Ligands like LDL (dark blue sphere) bind on the cell surface to their receptor, which after collection in clathrin-coated pits is internalized as vesicle and targeted to early sorting endosomes (SE). There, LDL dissociates from the receptor and shuttles to late endosomes (LE), while the LDL receptor recycles to the cell surface, either directly from SE, or after passage through the endocytic recycling compartment (ERC). The fate of the LDL receptor is shared by apotransferrin bound to the transferrin receptor, a prominent marker of the SE and ERC. Maturation of SE into LE and further conversion of LE into lysosomes is accompanied by formation of internal vesicles in LE, acquisition of lysobisphosphatidic acid, a drop in pH and import of hydrolases, proteases and other lysosomal enzymes from the Golgi and specifically from the trans-Golgi network (TGN). The endoplasmic reticulum (ER) is the major site of protein and lipid synthesis including synthesis of cholesterol. Fatty acids and cholesterol are esterified in the ER and stored in cytoplasmic lipid droplets (LD) being derived from the ER membrane. Vesicles containing new proteins and lipids form continuously in the ER and shuttle through Golgi stacks (shown in light green). During this passage, proteins are modified (e.g., glycosylated) and exported to the plasma membrane in course of secretory membrane transport. Some Golgi-resident proteins take complex trafficking routes also through the endocytic system and reach the TGN either from LE (like Furin) or from the ERC (like mannose-6-phosphate receptor and TGN38). All of these pathways are probably involved in cellular cholesterol transport. Specifically the cholesterol derived from hydrolysis of cholesteryl esters in the LE or lysosomes is delivered either directly to the cell surface or probably via the TGN and ERC to the ER, where the sterol-sensing machinery resides. The released cholesterol can also be re-esterified by acetyl-CoA-acyltransferase being probably located in a sub-compartment of the ER in close proximity to TGN and ERC. Approximate half-times ( $t_{1/2}$ ) of inter-compartment vesicle transport and pH values of endosomal compartments were taken from the literature. See text for further details

Rink 2005). Mature late endosomes are characterized by a lot of internal vesicles, the unusual lipid lysobisphosphatidic acid (LBPA), enrichment of mannose-6-phosphate receptor (M6PR) and the acquisition of rab9 (van der Goot and Gruenberg 2006; Pfeffer 2007). This small rab GTPase is central for shuttling M6PR back to the trans-Golgi network (TGN), thereby linking the endocytic to the secretory pathway. Rab9 is also essential for exporting cholesterol hydrolyzed from LDL-cholesteryl esters by acidic cholesteryl esterase from late endosomes and lysosomes (Narita et al. 2005; Ganley and Pfeffer 2006). There exist several clathrin-independent endocytic pathways in mammalian cells, which are regulated by cholesterol and sphingolipids (Mayor and Pagano 2007; Sandvig et al. 2008). This is a rapidly expanding field, but it is already clear that these pathways require different machineries, import different cargo and might start from different regions at the cell surface including caveolae or membrane ruffles. For example, one clathrin-independent way involves RhoA and Cdc42 and serves a role in internalizing GPI-anchored proteins, while another requires caveolae and mediates uptake of glycosphingolipid analogs like BODIPY-lactosylceramide (BODIPY-LacCer; Mayor and Pagano 2007).

Importantly, a well defined amount of cholesterol in organelle membranes is crucial for endocytic trafficking of proteins and lipids. Acute cholesterol depletion using cyclodextrin inhibits clathrin-dependent endocytosis, while mild cholesterol extraction for shorter periods interferes with other, clathrin-independent pathways (Mayor and Pagano 2007). Stimulation of macropinocytosis and membrane ruffling via activation of Rac was found upon cholesterol-loading of macrophages, while both processes become blocked by cholesterol-depletion of cells (Grimmer et al. 2002; Pierini et al. 2003; Qin et al. 2006; Nagao et al. 2007). Lowering the cellular cholesterol content or inhibiting sphingolipid synthesis slows recycling of GPI-anchored proteins from the ERC, while return of transferrin to the cell surface occurs with unchanged kinetics (Mayor et al. 1998; Chatterjee et al. 2001). Sorting of fluorescent lipid probes with varying acyl chain length between degradative and recycling pathways depends on cholesterol (Hao et al. 2004), while caveolae-like endocytosis of sphingolipid probes is stimulated by cholesterol-loading of cells (Sharma et al. 2004). Cholesterol-loading of endosomes disturbs rab4-dependent recycling to the cell surface (Choudhury et al. 2004), rab9-dependent export of cargo from late endosomes to the TGN (Ganley and Pfeffer 2006) and rab7-dependent late endosome motility (Lebrand et al. 2002). Similarly, cholesterol-loading blocks secretory transport from the TGN to the cell surface and triggers cPLA2- and dynamin-dependent Golgi vesiculation (Ying et al. 2003; Grimmer et al. 2005). Statins, which block HMG-CoA reductase and thereby cholesterol synthesis, in combination with growing cells in cholesterol-depleted medium, retard the endoplasmic reticulum (ER)-to-Golgi transport of vesicular stomatitis virus glycoprotein and scavenger receptor A (Ridsdale et al. 2006; Runz et al. 2006). Cholesterol depletion causes lowering of the lateral mobility of these two secretory membrane proteins in the ER and thereby decreases packaging efficiency into transport vesicles (Ridsdale et al. 2006; Runz et al. 2006). The lowered ER-to-Golgi trafficking of the secretory protein ts-O45-G in response to cholesterol depletion is accompanied or maybe even caused by delayed accumulation of this protein at

ER-exit sites (ERES; Runz et al. 2006). Raising the amount of cholesterol in the ER reversibly inhibits an early step in protein translocation across the ER membrane (Nilsson et al. 2001). Similarly, cholesterol-loading of macrophages inhibits sarcoplasmic-endoplasmic reticulum calcium ATPase-2b and increases lipid order in the ER membrane (Li et al. 2004). Together, these results clearly demonstrate that secretory membrane traffic depends on well defined amounts of cholesterol in the ER and Golgi compartments.

What might be the molecular mechanisms underlying cholesterol requirements of endocytic and secretory membrane transport? This question remains open for most of the experimental observations. Based on the biophysical properties of cholesterol several hypotheses can be put forward:

1. Cholesterol in endosomes might be required for maintaining the ion permeability barrier and thereby the transmembrane electrical potential in these organelles.
2. At the same time, endosomal and Golgi membranes need to be flexible in order to bend and form tubules for inter-compartment trafficking, setting an upper limit for membrane cholesterol content, since too much cholesterol would profoundly lower bending flexibility (Henriksen et al. 2004).
3. Protein diffusion must enable formation of protein scaffolds for budding and scission decisions.

These competing demands require well defined amounts of cholesterol, and this can explain why cholesterol-depletion as well as -loading perturbs various membrane trafficking pathways. In a few cases clear experimental evidence for a molecular mechanism underlying cholesterol's function has been provided. For example, cholesterol loading of late endosomes, as occurs in Niemann–Pick type C (NPC) disease (see below, Sect. 6.6), has been reported to stabilize the prenylated rab9 at the membrane, thereby inhibiting the GDI-mediated exchange of rab9 to its GDP-bound form in the cytosol. This process, though only shown on artificial model liposomes, might underlie the observed retardation of Rab9-mediated late-endosome-to-Golgi trafficking of M6PR in response to cholesterol loading (Ganley and Pfeffer 2006). Inhibition of clathrin-dependent endocytosis by acute cholesterol depletion might be caused by a reorganization of F-actin underlying the plasma membrane due to a changed distribution or content of PIP2, both effects have been observed upon cholesterol depletion (Kwik et al. 2003). In fact, reduced plasma membrane PIP2 has a similar inhibiting effect on clathrin-dependent endocytosis as acute cholesterol depletion (Jost et al. 1998; Boucrot et al. 2006; Zoncu et al. 2007).

## **6.4 Function of Various Organelles in Cellular Cholesterol Metabolism and Transport**

Cholesterol synthesis starts by formation of hydroxymethylglutaryl (HMG)-CoA from condensation of acetyl-CoA with acetoacetyl-CoA in the cytoplasm (in contrast to the reverse reaction taking place in mitochondria to recover acetyl-CoA units from ketone bodies). Next, the HMG-CoA is reduced to mevalonate by ER-resident

HMG-CoA reductase. The activity of this enzyme is highly regulated by: (a) feed-back inhibition by cholesterol and lanosterol, via ubiquitination and degradation of the enzyme, and (b) by glucagon and insulin, acting via a cycle of phosphorylations/dephosphorylations of hormone-sensitive kinases and phosphatases. At the transcriptional level, the amount of HMG-CoA reductase is strictly controlled by sterol regulatory response element (SREBP) containing a transcription factor which – upon proteolytic cleavage in the Golgi – induces transcription of the genes for HMG-CoA reductase and other sterol-regulated genes in the nucleus (for a detailed review, see Goldstein et al. 2006). A complex of SREBP, SREBP cleavage activating protein (SCAP) and INSIG resides in the ER at normal cholesterol levels, while SCAP containing a sterol sensing domain (SSD) senses a decrease in ER cholesterol under starvation conditions. This causes INSIG to dissociate from SREBP/SCAP, and the latter protein complex becomes incorporated into COPII coated vesicles shuttling SREBP/SCAP to the Golgi, where two proteases cleave the N-terminal transcription factor from SREBP. The released transcription factor moves to the nucleus, where it initiates synthesis of HMG-CoA reductase (as well as the LDL receptor and other proteins) thereby triggering synthesis of new cholesterol as well as uptake of circulating plasma cholesterol (via LDL receptor). Importantly, INSIG controls expression of HMG-CoA reductase via SCAP/SREBP as well as ubiquitination and degradation of the enzyme. In both cases, INSIG binds to a SSD of either SCAP or HMG-CoA reductase, suggesting a competitive mechanism regulated by cholesterol and lanosterol as well as by oxysterols (Goldstein et al. 2006). In order to sense even slight changes in ER cholesterol content, the ER contains only 0.5–1.0% of total cellular cholesterol, even though the surface area of the ER exceeds that of the plasma membrane in many cells. Thus, de novo synthesized cholesterol is rapidly exported from the site of synthesis; and one pathway characterized so far comprises shuttling of new cholesterol to the plasma membrane within 10 min, or so. The ER-to-plasma membrane route of newly synthesized cholesterol is studied using radioactive  $^3\text{H}$ - or  $^{14}\text{C}$ -acetate and by measuring the appearance of  $^3\text{H}$ - or  $^{14}\text{C}$ -cholesterol at the cell surface (DeGrella and Simoni 1982; Lange and Matthies 1984; Kaplan et al. 1985; Cruz and Chang 2000; Heino et al. 2000). This pathway largely bypasses the secretory organelles, including the Golgi, but requires ATP and ceases below  $15^\circ\text{C}$ , suggesting involvement of vesicular transport (DeGrella and Simoni 1982; Kaplan et al. 1985; Heino et al. 2000). In contrast, maintenance of the high concentration gradient of cholesterol between plasma membrane and ER did not require metabolic energy (Kaplan et al. 1985). Mitochondria have a low cholesterol content as well, despite the fact that they are the target organelles for cholesterol being metabolized to steroid hormones in steroidogenic cells (Maxfield and Wüstner 2002). The plasma membrane contains most, i.e., about 60% of total cellular cholesterol corresponding to about 40% of the lipids in this membrane (Maxfield and Wüstner 2002; Warnock et al. 1993). While early endosomes including the ERC harbor about 30–35% of total, the remaining cellular cholesterol of about 5–10% is distributed between late endosomes, lysosomes, Golgi apparatus and other organelles, including mitochondria, ER and peroxisomes. This does not preclude that certain compartments, like internal vesicles of multivesicular bodies/late endosomes contain relatively large amounts of cholesterol when normalized to the total membrane

area of these organelles (Möbius et al. 2003). Similarly, due to their close apposition and continuous inter-compartment membrane traffic, the estimate for the ERC might include to some extent the TGN, even if we and others failed to detect high amounts of DHE or cholesterol in the latter compartment (Hao et al. 2002; Wüstner et al. 2002, 2005; Möbius et al. 2003). Moreover, it has to be emphasized that the values of compartmental cholesterol given above are associated with a reasonable degree of uncertainty. In fact, the standard deviation of these values as provided in the literature can be as large as 30% of the reported mean value (Warnock et al. 1993; Hao et al. 2002; Möbius et al. 2003). Beside technical limitations reviewed by Maxfield and Wüstner (2002), this uncertainty is due to variations between investigated cell types as well as due to a reported dependence of the cholesterol-to-phospholipid ratio in the plasma membrane on cell density (Lange et al. 1989; Corvera et al. 2000; Takahashi et al. 2007). It is remarkable, however, that most late endosomes and lysosomes have a low cholesterol content despite the fact that cholesterol-rich LDL particles are digested in these organelles. It is a central open question, as to how ingested LDL cholesterol is released from LDL after hydrolysis of cholesteryl esters and to which sites it is subsequently targeted (see Sects. 6.4–6.6). The fact that internal vesicles of some late endosomes are cholesterol-rich while lysosomes are cholesterol-poor is one hint that release of LDL-derived cholesterol occurs in late endosomes. Parallel measurements have been performed for the appearance of radioactive cholesterol at the cell surface, using cyclodextrin as acceptor, and for the re-esterification as a measure of transport to the ER after incubating cells with  $^3\text{H}$ -cholesteryl ester containing LDL (Neufeld et al. 1996). In such experiments, cholesterol was first enriched in the lysosomes using progesterone, and it was found that, upon wash-out of progesterone, 70% of cholesterol released from LDL traffic first to the plasma membrane and subsequently to the ER, where most esterification takes place (Neufeld et al. 1996). The remaining 30% of LDL-derived cholesterol shuttle directly from the late endosome/lysosome to the ER bypassing the plasma membrane (Neufeld et al. 1996; Underwood et al. 1998). Molecular details of the cholesterol trafficking pathway from plasma membrane to the ER are lacking. The arrival of cholesterol in the ER is often measured by cholesterol esterification, based on the assumption that acyl-CoA-acyltransferase (ACAT) – the enzyme catalyzing linkage of activated fatty acids to the OH group of cholesterol to form cholesteryl esters – is located in the ER (Lange et al. 1999). Recent data, however, questioned the reliability of this assay for monitoring cholesterol transport to the ER. First, it was shown that ACAT resides in just a small sub-compartment of the ER located adjacent to the ERC and TGN (Khelef et al. 1998, 2000). Second, based on the well known effects of the oxysterol 25hydroxycholesterol on cholesterol esterification and sensing in the ER, it has been demonstrated that cholesterol esterification by ACAT is dissociable from cholesterol transport to the SREBP-based sensing machinery in the ER (Du et al. 2004). Third, activity of a second cholesterol esterase with an activity optimum at acidic pH, in contrast to ACAT, which works best at neutral pH, has been detected in endosomes or lysosomes of macrophages and other cells (Hornick et al. 1997; Wang et al. 2005). Thus, current knowledge of cholesterol trafficking to the ER (based solely on cholesterol esterification) may need to be critically re-assessed (Du et al. 2004). It is likely that measurement of cholesterol esterification monitors to

a large extent cholesterol trafficking to a sub-compartment of the ER, where ACAT is located.

There is evidence for involvement of the Golgi in targeting LDL derived cholesterol to this ER sub-compartment. First, treatment of cells with brefeldin A, a drug which induces rapid mixing of Golgi and ER, strongly increased re-esterification of cholesterol released from LDL (Neufeld et al. 1996). Second, electron microscopy of filipin indicated enrichment of Golgi membranes with cholesterol after LDL ingestion (Coxey et al. 1993). Third, TGN specific SNARE proteins, like VAMP4, syntaxin 6 and 16, are involved in re-esterification of LDL-derived cholesterol (Urano et al. 2008). A problem with brefeldin is that it interferes with many trafficking steps in cells, including recycling from the ERC to the cell surface (Lippincott-Schwartz et al. 1991; van Dam et al. 2002). The above-mentioned SNARE machinery regulates also early/recycling endosome to TGN transport of Shiga toxin B (Mallard et al. 2002). This transport step, i.e., early endosome/ERC to TGN, was inhibited by overexpression of wild-type or dominant-negative rab11, and in these cells free cholesterol accumulation in the ERC and cholesterol esterification were reduced (Wilcke et al. 2000; Hölttä-Vuori et al. 2002). Given that the ERC is a cholesterol-rich organelle (Hornick et al. 1997; Hao et al. 2002; Hartwig Petersen et al. 2008), it is likely that transport of LDL-derived cholesterol to ACAT involves sorting and recycling endosomes in addition to the TGN. This conclusion is fully in line with the data described above. Again, the close apposition of ERC, TGN and the ACAT-containing sub-compartment of the ER makes this also very likely (Khelef et al. 2000). Importantly, the reduced cholesterol esterification in rab11-overexpressing cells could be rescued by incubating the cells with cholesterol loaded onto cyclodextrin, further supporting the view that ACAT is fed by different pathways, one probably directly from the plasma membrane (Hölttä-Vuori et al. 2002). Supporting this notion, enhanced non-vesicular transport of DHE to ER associated lipid droplets was found in cholesterol-loaded macrophages, and ACAT stimulation is regulated by a cholesterol oxidase accessible pool (Tabas et al. 1988; Wüstner et al. 2005). Another way to assess ER targeting of LDL derived cholesterol beside measurement of re-esterification by ACAT (with all its limitations) is by quantifying the processing of SREBP (Du et al. 2004; Kristiana et al. 2008). Strikingly, both methods provided very different kinetic results, suggesting that there are either different cholesterol pools and/or transport pathways feeding ACAT and SREBP within the ER (Kristiana et al. 2008). In line with this conclusion is recent morphological data demonstrating high sub-compartmentalization of the ER beyond the simple discrimination of smooth and rough ER (Snapp et al. 2003).

## **6.5 Vesicular and Non-Vesicular Transport of Cholesterol: Targets, Kinetics and Regulation**

Although it is well accepted that the heterogeneous distribution of cholesterol among various organelles must be tightly regulated, the molecular mechanisms underlying intracellular cholesterol transport are largely unknown. There is accumulating

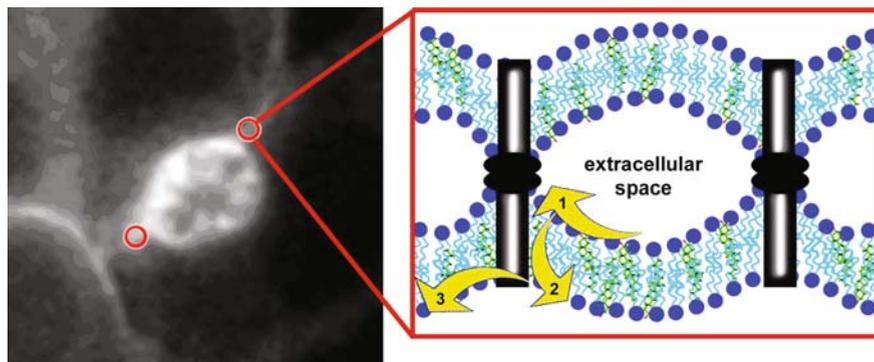
evidence that sterols, like cholesterol in mammalian cells and ergosterol in yeast cells, move by vesicular modes following general membrane traffic but also by non-vesicular mechanisms (Hao et al. 2002; Wüstner et al. 2002, 2005; Li et al. 2004; Baumann et al. 2005). ATP-depletion or low temperature, both treatments which inhibit vesicular transport, only partially inhibit the targeting of DHE to the ERC (Hao et al. 2002), while the trafficking of newly synthesized cholesterol to the plasma membrane of mammalian cells largely bypasses the secretory pathway (Kaplan et al. 1985). Similarly, ergosterol transport between plasma membrane and ER occurs by a non-vesicular pathway (Li et al. 2004; Baumann et al. 2005).

Spontaneous cholesterol transfer between lipid membranes occurs with a characteristic half-time of 2–3 h (Bar et al. 1986, 1987). Partition coefficients measured between liposomes consisting of various host lipids clearly established that cholesterol prefers membranes being enriched in SM and/or phospholipids bearing saturated fatty acyl chains (Bar et al. 1987; Leventis and Silvius 2001; Niu and Litman 2002). Some 25 years ago, Wattenberg and Silbert (1983) observed that cholesterol partitions preferentially into plasma membrane fractions, compared to membrane fractions made of mitochondria or ER. The authors did another smart experiment and measured cholesterol partition between lipids extracted from each of these membrane fractions and got the same result. Thus, the relative enrichment of cholesterol in plasma membrane compared to ER or mitochondria is a result of the different lipid composition of the isolated membrane fractions (Wattenberg and Silbert 1983). The authors could also show that an important lipid property causing this preferred cholesterol enrichment is the high content of SM and saturated lipids in the plasma membrane, compared to the intracellular organelle membranes. Finally, it was established in this pioneering study that the differences in cholesterol partition preference between isolated membrane fractions are not as high as the observed *in vivo* differences in organelle and plasma membrane cholesterol content (Wattenberg and Silbert 1983). The latter conclusion is very important. It emphasizes that intracellular cholesterol distribution is maintained by active transport processes in living cells, such as vesicular transport of cholesterol itself. Supporting this assumption, Ikonen and co-workers have shown that the ERC-associated rab-GT-Pase, rab11, regulates recycling and esterification of plasma membrane cholesterol, while rab8 is involved in cholesterol egress from late endosomes after ingestion of LDL (Hölttä-Vuori et al. 2002; Linder et al. 2006). Using fluorescence imaging of DHE, we demonstrated that cholesterol (analogs) can be internalized by several endocytic pathways and recycle from the ERC in a manner dependent on EHD/rme1, known to regulate recycling of transferrin and other receptors (Hao et al. 2002; Wüstner and Færgeman 2008). Further, active processes can also involve maintenance of specific organelle lipid and protein composition by vesicular, ATP-dependent membrane traffic. Such active transport might generate favorable conditions for relative cholesterol accumulation, like in the ERC, or an environment characteristic for sterol-poor organelle membranes, like in the ER. Although nobody knows exactly what those membrane environmental conditions are, a hypothesis has been put forward based on the chemical potential of free cholesterol in lipid monolayer and bilayer studies (Huang and Feigenson 1999; Radhakrishnan

and McConnell 2000; Maxfield and Menon 2006; Lange and Steck 2008). This model proposes the existence of two pools of cholesterol in various intracellular membranes – a phospholipid-complexed and a free pool, the latter being in non-vesicular exchange with other membranes. The proportion of free to complexed cholesterol is characteristic for every organelle and specific lipid composition and reflects the specific cholesterol affinity of the host phospho- and sphingolipids. Again, this specific lipid environment and thereby the ratio of free and complexed cholesterol could be a result of active membrane transport processes. Now, the key point of the model is that despite large differences in total membrane cholesterol content, the chemical potential of free, non-complexed cholesterol in for example the ER could be identical to that found in the plasma membrane (Radhakrishnan and McConnell 2000; Maxfield and Menon 2006; Lange and Steck 2008). Slight changes in the chemical potential of the free sterol pool, for example by adding extra cholesterol to the plasma membrane or by changing the phospholipid composition, could trigger non-vesicular cholesterol efflux to extracellular acceptors or uptake and targeting to intracellular acceptor membranes like the ER. The nature of the cholesterol in complexes is a matter of debate. While some physico-chemists argue that cholesterol indeed forms stoichiometric complexes with phospholipids (McConnell and Radhakrishnan 2003), others state that cholesterol keeps shielded from the water interface under phospholipid head groups, requiring multibody interactions and even formation of superlattice-like arrangements (Huang et al. 1999). Both ideas have in common that the capacity of phospholipids to either bind cholesterol or to shield it from water in superlattice-like structures (Huang and Feigenson 1999; Somerharju et al. 1999), is limited and specific for certain phospholipid compositions. Accordingly, raising cholesterol in a membrane beyond this capacity will shift cholesterol towards the free exchangeable pool. Independent of the exact underlying physico-chemical mechanisms for the limited bilayer capacity to accommodate cholesterol, this model is attractive because it explains, or is at least in line with many experimental observations. For example, the sigmoid dependence between plasma membrane and ER cholesterol can be explained by rapid influx of cell surface cholesterol above a critical threshold (Lange et al. 1999; Lange and Steck 2008). Similarly, activity of HMG-CoA reductase drops rapidly (half-time 10–20 min) and almost completely when plasma membrane cholesterol is raised slightly (by about 10%) above normal levels (Lange and Steck 2008). We observed a strongly increased non-vesicular uptake of DHE from the plasma membrane upon cholesterol loading of macrophages, demonstrating that the plasma membrane has a limited capacity to accommodate cholesterol (Wüstner et al. 2005). The latter observation could even be the underlying cause of the well known threshold phenomenon of ACAT stimulation in these cells above certain levels of cellular cholesterol (see Sect. 6.6). Using kinetic fluorescence imaging of DHE, we established that sterol moves in a non-vesicular, ATP-independent manner from the basolateral to the apical canalicular membrane of polarized epithelial HepG2 cells and that the forward rate constant is higher than that of the transport back from the canalicular to the basolateral domain (Wüstner et al. 2002). This is likely due to the favorable environment in the apical membrane being enriched in sphingolipids,

for example. Continuous vesicle traffic between the subapical recycling compartment and the canalicular membrane might be crucial for maintenance of the specific lipid composition of the apical membrane with higher sterol affinity, compared to the basolateral domain. The chemical potential theory is basically an extension of early considerations on the different sterol affinity of cellular membrane lipids by Wattenberg and Silbert (1983) described above. Its new point is the proposal that membrane cholesterol is always close to a physiological set-point given by the distinct lipid and protein composition of the organelles and the plasma membrane (Maxfield and Menon 2006; Lange and Steck 2008). Slight changes in the free cholesterol pool near the set-point can trigger non-vesicular cholesterol exchange between organelles and thereby large cellular responses. Of course, much remains to be done to really prove this idea; in fact, the major task is to identify these different cholesterol pools and to characterize proteins mediating the responses to cellular cholesterol perturbation. The observed relations, however, are indeed intriguing and might pave the way for future research in this direction.

What are the mechanisms underlying non-vesicular cholesterol transport in cells? In the case of polarized epithelial cells, non-vesicular sterol transport between the plasma membrane domains, as observed for hepatoma HepG2 cells, might occur by lateral diffusion (Wüstner et al. 2002). While tight junctions would prevent diffusion of lipids in the exoplasmic leaflet of the plasma membrane (Dragsten et al. 1981), sterols can rapidly flip-flop across the bilayer and thereby circumvent this diffusion barrier (see Fig. 6.5). Rapid non-vesicular



**Fig. 6.5** Transport of cholesterol to the apical canalicular membrane of hepatic cells. Polarized human hepatoma HepG2 cells were pulse-labeled with DHE loaded onto cyclodextrin for 1 min, washed and imaged on a wide-field fluorescence microscope optimized for transmission in the ultraviolet (UV) to detect DHE. The canalicular membrane forming the biliary canaliculus is strongly labelled with DHE including several microvilli (*left panel*). Tight junctions seal the canalicular from the basolateral membrane by several rows of proteins including occludins (symbolized in black, *right panel*). DHE can be rapidly shuttled from the basolateral to the canalicular membrane by ATP-independent, non-vesicular transport. This trafficking mode probably involves lateral diffusion in the exoplasmic membrane leaflet (1), transbilayer migration of sterol to the inner membrane half (2), when DHE encounters the diffusion barrier generated by tight junctions proteins in the outer layer and (3) continued lateral diffusion in the cytoplasmic leaflet of the plasma membrane (see text for details)

shuttling of cholesterol from the basolateral to the apical, bile canaliculi-forming membrane of hepatocytes could be an efficient way to deliver cholesterol from the plasma to the bile compartment during the course of reverse cholesterol transport (Robins and Fasulo 1999; Wüstner et al. 2002, 2004). In the case of intracellular non-vesicular trafficking, cholesterol has to somehow cross the aqueous phase between two compartments. The rate-limiting step in passive cholesterol transport between two bilayers is cholesterol's desorption from the donor membrane (Bar et al. 1986; Steck et al. 1988). Due to its low aqueous solubility, partition of cholesterol into the water phase is thermodynamically unfavorable. However, once released or just partially protruded from a membrane, cholesterol can be caught by an acceptor, like a transfer protein or another membrane, if located in sufficient proximity. The nebulous free cholesterol pool in the chemical potential model explained above could in fact resemble cholesterol with a high out-of-plane mobility, partly protruding from the bilayer (cf. Sect. 6.1). This could raise the probability of a protruded cholesterol molecule being transferred to a lipid-binding protein. A few candidates for cytoplasmic cholesterol carriers have been described and their potential to transfer cholesterol between membranes has been shown *in vitro*. The definitive proof of their intracellular function as a cholesterol transporter, however, is still missing. The most prominent example is the steroidogenic acute regulatory protein (StAR), which mediates cholesterol import into mitochondria for steroid hormone synthesis in steroidogenic tissues. StAR is the prototype of the StAR-related lipid transfer family of proteins; other members are the phosphatidylcholine transfer protein (PC-TP/StarD2), the ceramide transfer protein (CERT/StarD11) and the late endosomal transmembrane protein MLN64 (StarD3; Alpy and Tomasetto 2005). While PC-TP might be involved in many process involving lipid transfer steps, CERT mediates specifically ER-to-Golgi trafficking of ceramide (Alpy and Tomasetto 2005). MLN64 is probably part of the machinery moving cholesterol released from ingested LDL cholesteryl esters out of the late endosome and lysosome (Alpy and Tomasetto 2005; Hölttä-Vuori et al. 2005). Knock-down of MLN64 results in the dispersion of late endosomes and lysosomes, due to a missing recruitment of actin to these organelles (Hölttä-Vuori et al. 2005). Members of the StART family share structural similarities, including a hydrophobic pocket limited by alpha helices and a flexible lid which can open and close for the binding and releasing of a cholesterol molecule (Alpy and Tomasetto 2005; Murcia et al. 2006). Other soluble proteins with the ability to bind and even transfer cholesterol in model membranes are oxysterol-binding proteins and Niemann–Pick C protein 2 (NPC2; see Sect. 6.6). Once expelled from the donor membrane and being bound to a soluble protein with a hydrophobic pocket, the next problem is to create specificity by transferring the bound cholesterol or other lipid to the correct target membrane. Some lipid-binding proteins bear targeting sequences for two compartments, like CERT, which associates with the ER as well as with the Golgi (Alpy and Tomasetto 2005; Levine and Loewen 2006). It has been speculated that sites of close membrane apposition observable by electron microscopy play a central role in inter-compartment, non-vesicular lipid transfer via proteins like CERT and others (Levine and Loewen 2006). A central role in this hypothesis involves the ER-making membrane contact

zones with the plasma membrane (Becker et al. 2005), lipid droplets (Prattes et al. 2000), mitochondria (Rusiñol et al. 1994), Golgi (Ladinsky et al. 1999) and eventually ERC and other endosomes (Khelef et al. 2000). The close proximity of donor and acceptor membrane in these contact zones plus the existence of targeting sequences in the (putative) transfer protein for donor and target compartment could provide a highly efficient and specific way of non-vesicular lipid/cholesterol transport between organelles and plasma membrane. These ideas are largely based on observations in yeast cells and certainly need experimental back up for mammalian cells.

## **6.6 Alterations in Intracellular Cholesterol Trafficking in Atherosclerosis and Lipid Storage Diseases**

Much has been learned about normal intracellular cholesterol trafficking from disorders causing alterations in lipid transport or metabolism. The classic example for a genetic disease of cholesterol transport is familial hypercholesterolemia, studied by Joseph Goldstein and Michael Brown, leading to the discovery of the central role played by the LDL receptor system in the regulation of cellular cholesterol uptake (Goldstein and Brown 1974). Another example is Tangier disease, where strongly reduced plasma HDL and increased cholesteryl ester accumulation in macrophages could be ascribed to a defect in ATP-binding cassette (ABC) transporter A1 (ABCA1; Tall et al. 2002). This defect resulted in dramatically lowered capacity of macrophages to efflux cholesterol and thereby to reduced HDL and progression of atherosclerosis. Plant sterols accumulate in the body due to a defect in the plant sterol efflux ABC transporters ABCG5/8 (Graf et al. 2002; Yu et al. 2002). This leads to reduced extrusion of these sterols from the enterocytes in the intestine or hepatocytes in the liver of affected patients in sitosterolemia (Maxfield and Tabas 2005). Like in the case of Tangier disease and hypercholesterolemia, a cholesterol transport system and its regulation have been identified by studying the underlying causes of the disease sitosterolemia; the two half-transporters ABCG5/8 together mediate the biliary secretion of cholesterol (Yu et al. 2002).

Another group of diseases, the lysosomal storage disorders like Tay–Sachs, Fabry, Niemann–Pick and Sandhoff disease, are characterized by the accumulation of sphingolipids and cholesterol in late endosomes and lysosomes (Maxfield and Tabas 2005). In Tay–Sachs, Fabry and Sandhoff disease, the primary cause of lysosomal lipid storage is a defect in sphingolipid degradation (Futerman and van Meer 2004). The same is true for Niemann–Pick disease types A and B, also called sphingomyelin lipidosis, while the exact etiology of Niemann–Pick disease type C is not known (Futerman and van Meer 2004). It is believed that defective export of LDL-derived cholesterol from the lysosomal compartment causes sterol and sphingolipid accumulation in late endosomes and lysosomes also containing NPC1 (Puri et al. 1999; Mukherjee and Maxfield 2004). Since the transport of bulk-phase markers like sucrose or membrane proteins like M6PR or transferrin is also affected in NPC

disease, it is possible that mutation of the NPC1 protein causes a general traffic jam in the endocytic system (Kobayashi et al. 1999; Neufeld et al. 1999; Liscum and Sturley 2004; Mukherjee and Maxfield 2004; Pipalia et al. 2006). Importantly, knock-out of late endosomal/lysosomal Lamp1/Lamp2 or expression of dominant-negative dynamin as well as treatment with anti-lysobisphosphatidic (anti-LBPA) antibodies causes a similar phenotype with lysosomal or late endosomal cholesterol accumulation (Kobayashi et al. 1999; Eskelinen et al. 2004; Robinet et al. 2006). In contrast, overexpression of the small, late endosome-associated GTPases rab8 and rab9 can restore the trafficking defect and lower cholesterol accumulation (Narita et al. 2005; Linder et al. 2006). Very recent results support the idea that mutations of NPC1 cause a traffic jam in the endosomal/lysosomal system. Lloyd-Evans et al. (2008) reported that an initiating factor in NPC1 disease pathogenesis is the accumulation of sphingosine, a precursor of sphingolipids, which by an unknown mechanism causes the depletion of calcium in acidic endosomal compartments. This, in turn, disturbs normal trafficking along the degradative pathway, since the formation of lysosomes via a late endosome-lysosome hybrid organelle is calcium-regulated (Piper and Luzio 2004; Luzio et al. 2007). The authors found that drugs which elevate the cytoplasmic calcium concentration could not correct the low calcium content of late endosomes/lysosomes in NPC1 mutant cells, but they could compensate for a lack of calcium release from the acidic compartments in these cells, thereby rescuing normal transport to lysosomes (Lloyd-Evans et al. 2008). Strikingly, such drugs, like curcumin and thapsigargin, increased life span and slowed disease progression in the NPC1 knock-out mouse, paving the way for a new therapeutic approach. Although Lloyd-Evans et al. (2008) could demonstrate reduced cholesterol levels in NPC1 mutant fibroblasts upon thapsigargin treatment, the molecular mechanisms linking lysosomal calcium to cholesterol transport remain to be established. Another very interesting observation in this direction was made recently by Kaufmann and Krise (2008): they observed that NPC1 functions in regulating late endosomal/lysosomal content of amines. Based on their data, the authors suggest that NPC1-mediated clearance of amine containing molecules from lysosomes involves a vesicular transport pathway and eventually NPC1-mediated fusion of late endosomes with lysosomes (Kaufmann and Krise 2008). Most research groups report that initial export of cholesterol derived from hydrolysis of LDL-associated esters to the plasma membrane or ER is inhibited by the NPC1 mutation (Liscum and Munn 1999; Wojtanik and Liscum 2003; Liscum and Sturley 2004; Millard et al. 2005; Infante et al. 2008). Fibroblasts bearing this mutation but also cells from NPC1null individuals do not down-regulate LDL-receptor expression via the SCAP/SREBP2 system upon cholesterol loading and have a strongly reduced ability to re-esterify LDL-derived cholesterol by ACAT in the ER (Wojtanik and Liscum 2003; Liscum and Sturley 2004; Millard et al. 2005; Kristiana et al. 2008; Urano et al. 2008). The same, however, has been found in HeLa cells overexpressing dominant-negative dynamin, suggesting that several proteins which regulate membrane traffic from late endosomes interfere with cholesterol egress after LDL ingestion (Robinet et al. 2006). It has been suggested that NPC1's function as a cholesterol exporter out of late endosomes/lysosomes depends on the mobility of the

NPC1 protein in organelles (Ko et al. 2001; Zhang et al. 2001). While functional NPC1 protein traffics bidirectionally in late endosomal vesicles along microtubules, mutated NPC1 (e.g., with a point mutation P692S in NPC1's sterol-sensing domain) shows no directed movement and strongly increases the accumulation of LDL and free cholesterol in the lysosomal compartments (Ko et al. 2001; Zhang et al. 2001; Millard et al. 2005). Purified NPC1 binds cholesterol with low affinity on a luminal loop, but the role played by this process in living cells remains to be determined (Infante et al. 2008). It has been suggested that NPC1 functions as a fatty acid transporter due to its similarity to bacterial RMD permeases; however, fatty acid flux through late endosomes was not affected by NPC1 mutation (Davies et al. 2000; Passeggio and Liscum 2005). Some evidence suggests that the NPC1 protein is involved in delivering cholesterol released from LDL in late endosomes to the TGN (Coxey et al. 1993; Urano et al. 2008). This would be supported by defect trafficking of M6PR from late endosomes to TGN in NPC disease (Ganley and Pfeffer 2006), inhibited transport of fluorescent sphingolipids to the TGN in NPC mutant fibroblasts (Puri et al. 1999, 2001) as well as by the observed transient association of the NPC1 protein with Golgi elements (Higgins et al. 1999; Garver et al. 2002). However, the close association of early and recycling endosomes with the TGN, as stated above, as well as some conflicting results regarding NPC1's intracellular location, leave this issue open for further studies. While defects in NPC1 protein are responsible for 95% of all observed cases of NPC disease, the remaining 5% are caused by defects in NPC2 protein with identical phenotype (Naureckiene et al. 2000). The gene defective in NPC1 disease encodes a 1278-amino-acid protein containing 13 putative transmembrane domains, whereas NPC2 is a soluble, heterogeneously N-glycosylated 131-amino-acid protein (Liscum and Sturley 2004). Human NPC2 is modified by a terminal mannose-6-phosphate group targeting the protein to lysosomes (Willenborg et al. 2005). NPC2 is also present in high amounts in mammalian epididymal fluid and bovine milk, it binds cholesterol and DHE with high affinity in an equimolar complex and it was shown to transfer both sterols between model membranes (Naureckiene et al. 2000; Friedland et al. 2003; Cheruku et al. 2006; Babalola et al. 2007; Infante et al. 2008; Xu et al. 2008). Importantly, sterol binding and inter-membrane transfer are significantly stimulated in the presence of LBPA and PI and work optimally at acidic pH, as found in late endosomes (Cheruku et al. 2006; Babalola et al. 2007; Xu et al. 2008). Antibodies against LBPA block NPC2-mediated transfer of the fluorescent sterol CTL between membranes (Xu et al. 2008). Based on these and other observations, a working model has been developed which proposes that NPC2 shuttles cholesterol released via hydrolysis of LDL-derived cholesteryl esters and maybe other sterols from internal LBPA-rich vesicles to the limiting membrane of late endosomes. From here, NPC1 transports the cholesterol to other organelles like ER or TGN and maybe plasma membrane and early/recycling endosomes (Sleat et al. 2004; Liou et al. 2006; Infante et al. 2008). The recent observation that sphingosine accumulation precedes cholesterol build-up in late endosomes/lysosomes and that calcium and amine homeostasis are intimately linked to NPC1 function; and lysosomal biogenesis might open the research field to new directions (Kaufmann and Krise 2008; Lloyd-Evans et al. 2008).

Atherosclerosis and coronary heart disease (CHD) are the major cause of death in Western society. Although cholesterol accumulation in the body during the life-span of a normal individual is an important factor in development of atherosclerosis, the molecular mechanisms underlying this disease are manifold and very complex. In fact, there exist genetic and patho-biochemical connections between atherosclerosis, CHD, insulin resistance, obesity and metabolic syndrome, demonstrating the overwhelming complexity of these diseases. One model of etiology of atherosclerosis based on lipoprotein modification, retention and aggregation in the subendothelium (intima) of the vessel wall has been put forward (Tabas 2000). Based on this model, development of the disease can be divided into an early and a late phase, as excellently described in a recent review (Maxfield and Tabas 2005). Briefly, the first visible sign in atherogenesis is the retention of otherwise circulating lipoproteins in the intima of the vessel wall, followed by recruitment of monocytes into the area. These cells differentiate into macrophages and try to engulf the aggregated and retained lipoproteins in a process with similarities to phagocytosis and clathrin-dependent endocytosis. Importantly, during this initial prolonged contact of the modified lipoproteins with the macrophages, cholesterol uptake into cells dramatically exceeds the internalization of the apoprotein of oxidized and/or aggregated LDL (Buton et al. 1999). Uptake of soluble, oxidized LDL by macrophages is mediated by various scavenger receptors, while the receptor(s) mediating internalization of aggregated and retained LDL are not known (Tabas 2000). It is important to emphasize that, in contrast to the LDL receptor, the known receptors for atherogenic, modified LDL, like scavenger receptor A/CD36, are not under control of the SREBP/SCAP system, and their expression is therefore not down-regulated in response to cellular cholesterol loading. Accordingly, macrophages continue to internalize cholesterol beyond physiological levels. Once cellular cholesterol content is expanded above a threshold level of about 25% of normal, ACAT is activated by an unknown mechanism and catalyzes a massive synthesis of cholesteryl esters out of the excess free cholesterol (Xu and Tabas 1991). These cholesteryl esters are stored by the formation of lipid droplets; and the large number of these organelles gives the cholesterol-loaded macrophages a foamy appearance, hence the name foam cells. Early atherosclerotic lesions containing foam cells are not large enough to compromise blood flow and remain therefore mostly undetected. During the progression of atherosclerosis, however, the ability of macrophages to esterify the excess cholesterol declines, and the cells begin to accumulate free non-esterified cholesterol. This is a hallmark of late lesional macrophages, which try to accommodate to this situation by enhancing the synthesis of phospholipids, in particular PC via activation of CTP:phosphocholine cytidyl transferase (CT) (Shiratori et al. 1994; Tabas 2000). This cellular response to free cholesterol loading is likely an adaptation to overcome cholesterol's limited solubility in lipid bilayers of about 50–55 mol%, as explained in Sect. 6.1. In the tissue culture of macrophages, this stage is easily recognized by many internal membrane whorls containing the excess phospholipids and cholesterol (Shiratori et al. 1994; Tabas 2000). Cell-culture macrophages loaded with non-esterified cholesterol additionally show increased cell ruffling and spreading, Rac activation and actin re-organization, as well as strongly inhibited migration capability (Qin et al. 2006;

Nagao et al. 2007). If this also happens in vivo, actin-mediated membrane protrusions could further enhance cholesterol uptake from aggregated lipoproteins, while the lowered ability to migrate prevents egress of these cholesterol-loaded macrophages from the lesional area (Maxfield and Tabas 2005).

A turning point in late-stage atherogenesis is macrophage death as a consequence of continuing free cholesterol loading of the cells. The unfolded protein response (UPR) in the ER is activated upon acute free cholesterol loading, which stimulates expression of the cell death effector CHOP, leading to apoptosis (Feng et al. 2003a). Nanomolar concentrations of hydrophobic amines – which specifically block the egress of LDL-derived cholesterol from late endosomes and its targeting to the ER – inhibited UPR and protected macrophages from apoptosis (Feng et al. 2003a). Similarly, macrophages with a heterozygous mutation in the NPC1 protein showed no free-cholesterol-induced UPR or apoptosis (Feng et al. 2003b). These results demonstrate that cholesterol derived from ingested atherogenic lipoproteins in macrophages travels through late endosomes like normal LDL cholesterol, indicating that the overall cholesterol trafficking route is functional in lesional macrophage foam cells. Macrophage death is accompanied by an increase in necrotic areas in lesions, plaque rupture and acute thrombosis (Maxfield and Tabas 2005).

## 6.7 Future Prospects

This chapter summarizes our current understanding of intracellular cholesterol trafficking in health and disease. It emphasizes the two different transport modes of cholesterol in cells, vesicular and non-vesicular, and highlights known regulators of both trafficking mechanisms. Future work might concentrate on the following aspects:

1. Understanding the relationship between transport and metabolism of cholesterol in cells: how do cholesterol precursors move in cells? Newly synthesized zymosterol, a precursor of cholesterol, traffics from the ER to the plasma membrane with a half-time of 9 min and turns over faster than cholesterol in the latter compartment (Lange et al. 1991). Thus, trafficking of cholesterol's precursors might be important in establishing the steady-state distribution of cholesterol among various organelles. Similarly, one might ask, what role is played by continuous sterol modification, like oxidation, in establishing the heterogeneous intracellular cholesterol distribution?
2. What exactly are the biophysical properties of cholesterol determining its cellular targeting and its impact on membrane trafficking in general? Is the ability to induce a Lo phase in ternary model membranes indeed related to cholesterol trafficking in cells?
3. What is the transbilayer distribution of cholesterol in the plasma membrane and in organelle membranes? New methods based on imaging of fluorescent sterols and side-specific quenchers might be used to address this question (Mondal et al. 2008).

4. How is non-vesicular cholesterol transport regulated in cells, and what are the exact proportions of vesicular and non-vesicular trafficking modes under normal and patho-biological conditions? It remains to be determined whether there are many cytoplasmic cholesterol transport proteins with low specificity for donor and target membrane, or whether inter-membrane shuttling of sterols is indeed mediated by a few specific transfer proteins. Elucidating the molecular mechanisms underlying specific interaction/binding of cholesterol to carrier and transmembrane proteins is crucial to answer this question.
5. How is cholesterol transported from the late endosomal/lysosomal pool after digestion of LDL and from the plasma membrane to the sterol-sensing machinery and to ACAT in the ER? Deciphering the molecular mechanisms underlying concerted action of NPC1 and NPC2 might clarify this issue.

Answers to the questions raised above certainly require further improvements in technology to monitor inter-organelle sterol trafficking by either microscopy in living cells or by new *in vitro* reconstitution approaches.

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# Chapter 7

## Role of the Endothelium in Lipoprotein Metabolism

Arnold von Eckardstein and Lucia Rohrer

**Abstract** For a long time the endothelium was considered as a passive exchange barrier of lipoproteins between plasma and extravascular tissues. During the past two decades many data from clinical studies, cell culture, and animal experiments have shown that endothelial cells are a target of physiological and pathological actions of lipoproteins: Whereas lysosphingolipids and apolipoprotein (apo)A-I in native high-density lipoproteins (HDL) exert protective effects on the integrity and function of endothelial cells, modified low-density lipoproteins (LDL) and remnants of lipoproteins tend to disturb endothelial function. One central function of the endothelium is the control of protein trafficking between intravascular and extravascular compartments. Both LDL and HDL can pass the intact endothelium through transcytosis by processes which involve caveolin-1 and the LDL-receptor (for LDL) or ATP-binding cassette (ABC) transporters and scavenger receptor (SR)-BI for apoA-I and HDL, respectively. Finally the endothelium has evolved as a regulator of lipoprotein metabolism: By expressing or presenting lipases [lipoprotein lipase (LPL), hepatic lipase (HL), endothelial lipase (EL)] as well as LPL-receptors (glycerophosphatidyl inositol anchored HDL binding protein 1; GPIHBP1) the endothelium contributes to the remodelling of all lipoprotein classes. Selective conditional knock-outs of widely expressed genes like peroxisome proliferator agent receptor gamma (PPAR $\gamma$ ) in mice are starting to reveal additional specific effects of the endothelium on lipid and lipoprotein metabolism.

### 7.1 Introduction

A monolayer of polarized endothelial cells lines the intima of blood vessels. Locally the intact endothelium forms a barrier for the exchange of molecules and cells between intravascular and extravascular compartments. Thereby and in

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addition the endothelium regulates many systemic processes, including tissue fluid homeostasis, vascular tone, angiogenesis, hemostasis, and host defense. As the clinical consequence endothelial dysfunction contributes to many pathological conditions, such as atherosclerosis, diabetic microangiopathy, thrombosis, and inflammation, as well as cancer growth and metastasis.

Lipoproteins have three principal relationships with the endothelium:

1. Endothelial cells express or expose several proteins which regulate lipoprotein metabolism, notably lipases.
2. The endothelium forms a selectively permissive barrier for the passage of lipoproteins between intra- and extravascular compartments.
3. The endothelium is a target of physiological and pathological actions of lipoproteins.

## 7.2 Expression of Proteins Involved in Lipoprotein Metabolism

Endothelial cells express many proteins that play an important role in intravascular lipoprotein metabolism including lipoprotein receptors [e.g. low-density lipoprotein (LDL) receptor, scavenger receptor B1 (SR-B1)], apolipoproteins (e.g. apoA-I in porcine brain capillary endothelial cells), lipid transfer proteins (e.g. phospholipid transfer protein) and lipases. Except for the lipases (Hasham and Pillarisetti 2006; Wong and Schotz 2002) and the recently discovered glycosylphosphatidylinositol-anchored HDL binding protein 1 (GPIHBP1; Beigneux et al. 2008), endothelial cells are believed to contribute only minor amounts of each respective protein to the whole-body pools. However, in view of the huge surface of the endothelium and the resulting large exposure area to lipoproteins, the endothelium may in fact play a much bigger role in lipoprotein metabolism than usually acknowledged. This hypothesis can only be addressed by the analysis of conditional knock-out mice with selective and endothelium specific inactivation of the various lipoprotein genes. In fact, the targeted knock-out of the peroxisome proliferator activator receptor gamma (PPAR $\gamma$ ) in the endothelium revealed an as yet unappreciated role of the endothelium in the regulation of metabolism, as these mice presented with elevated plasma concentrations of triglycerides and free fatty acids but increased insulin sensitivity (Kanda et al. 2009).

In the current absence of such conditional knock-out models for lipoprotein genes, one can only discuss the contribution of the endothelium to lipoprotein metabolism by referring to proteins that are predominantly produced or exposed by endothelial cells, namely lipoprotein lipase (LPL), hepatic lipase (HL), and endothelial lipase (EL). These three lipases, which are attached to the glycocalyx of the endothelium, mediate the hydrolysis of triglycerides or phospholipids of lipoproteins and thereby contribute to the remodelling of lipoproteins (Wong and Schotz 2002). The primary products of their lipolytic activities are free fatty acids, diacyl-, and monoacylglycerols or lysophospholipids. Notably lysophosphatidylcholine and unesterified polyunsaturated fatty acids are biologically active because they themselves or their metabolic products bind to cognate cell surface or nuclear receptors, for example

lysophosphatidylcholine receptors and peroxisome proliferator activator receptors, respectively. Because of their close proximity to lipoprotein hydrolysis, endothelial cells are the immediate targets of any pro- or anti-inflammatory/atherogenic effects exerted by these lipids (Hasham and Pillarisetti 2006; see also Sect. 7.3).

### ***7.2.1 Lipoprotein Lipase and GPIHBP1***

LPL is predominantly synthesized by the parenchymal cells of adipose tissue and striated skeletal or cardiac muscle and then translocated by basolateral-to-apical transcytosis through a process which requires both heparan sulfate proteoglycans and the very low density lipoprotein (VLDL) receptor (Obunike 2001). Attached onto the luminal surface of capillaries, LPL-dimers hydrolyse triglycerides into chylomicrons and VLDL and thereby provide adipocytes and myocytes with fatty acids for energy storage and production, respectively (Wong and Schotz 2002). LPL activity depends on the presence of apoC-II and is modulated by apoA-V, apoC-III, and apoE (Forte et al. 2008; Pollin et al. 2008; Wong and Schotz 2002). Previously the enzyme was postulated to be attached to negatively charged heparan sulfate proteoglycans of the endothelial glycocalyx (Wong and Schotz 2002). Most recent data however indicate that GPIHBP1 (expressed by endothelial cells) binds both the enzyme and its lipoprotein substrates (Beigneux et al. 2008). It has been proposed that GPIHBP1 binds the fast heparin-releasable LPL pool on the luminal surface of endothelial cells, whereas heparansulfate proteoglycans bind a slow heparin-releasable pool of LPL in the sub-endothelial space (Weinstein et al. 2008). The limiting role of GPIHBP1 for lipolysis is highlighted by the hypertriglyceridemia of humans and mice with GPIHBP1 deficiency, which is as severe as that of patients or mice deficient in LPL or apoC-II (Beigneux et al. 2007, 2008; Kanda et al. 2009). The lipolysis of chylomicrons and VLDL results in the formation of surface remnants, which contain water-soluble apolipoproteins and phospholipids and contribute to the maturation of HDL, as well as core remnants, which contain apoB and apoE and are enriched in cholesteryl esters. In addition to its enzymatic activity LPL also plays a role in the catabolism of the core remnants, since LPL monomers that are detached from the endothelium associate with these remnants and act together with apoE or apoB as ligands for their binding to heparansulfate proteoglycans, the LDL-receptor or the LDL-receptor related protein (LRP) in the liver (Bishop et al. 2008; Wong and Schotz 2002).

### ***7.2.2 Hepatic Lipase***

HL is produced by the liver and attached to heparansulfate proteoglycans on the surface of sinusoidal endothelial cells and hepatic parenchymal cells in the space of Disse (Perret et al. 2002; Wong and Schotz 2002). HL contributes to the remodelling of almost all lipoprotein classes by hydrolysing triglycerides and phospholipids of

VLDL, IDL, LDL, and HDL (Perret et al. 2002). The hydrolysis of phospholipids in HDL liberates lipid-poor or lipid-free apoA-I, which induces cellular lipid efflux by interaction with the ATP-binding cassette transporter (ABC) A1 and can thereby initiate the de novo formation of HDL (Barrans et al. 1994; Perret et al. 2002; Rye and Barter 2004). In addition and like LPL, it acts as a co-receptor for hepatic uptake of IDL and LDL by members of the LDL receptor gene family and of HDL by an as yet unknown holoparticle receptor (Guendouzi et al. 1998; Perret et al. 2002). The crucial role of HL for lipoprotein metabolism is highlighted by the severe mixed hyperlipidemia affecting all lipoprotein classes, including HDL in both patients and mice with HL deficiency (Zamboni et al. 2003).

### 7.2.3 *Endothelial Lipase*

As indicated by its name, EL is synthesized mainly by the endothelium but also by liver, lung, macrophages, testis, ovary, and placenta. Like LPL and HL, EL contains heparin-binding domains which are needed for the attachment of EL to the glyco-calix of endothelial cells. In contrast to LPL and HL, EL exerts almost exclusively phospholipase activity (Badellino and Rader 2004; Jaye and Krawiec 2004; Lamarche and Paradis 2007; Wong and Schotz 2002). Both humans and mice with EL deficiency have elevated levels of HDL cholesterol (Ishida et al. 2004; Ko et al. 2005). In contrast, transgenic overexpression of EL was found to decrease HDL cholesterol levels (Ishida et al. 2003). The impact of EL on HDL metabolism is also highlighted by the inverse correlation of EL levels in postheparin plasma with HDL cholesterol concentrations (Badellino et al. 2006). A closer analysis by using NMR spectroscopy revealed an inverse correlation between postheparin plasma concentration of EL and large HDL particles and a positive correlation with small HDL particles (Badellino et al. 2006). Interestingly EL concentrations do not correlate with apoA-I levels (Paradis et al. 2006). Taken together the data indicate that EL-mediated phospholipids hydrolysis remodels large HDL into small ones. Interestingly these smaller HDL particles formed by EL have a diminished ability to interact with scavenger receptor B type I (SR-BI; Gauster et al. 2004). Whether HDL remodelling by EL increases or decreases cholesterol efflux capacity is controversial (Gauster et al. 2004; Qiu and Hill 2009). Both in vitro and in vivo data from mice indicate that EL also hydrolyses phospholipids in apoB containing lipoproteins and thereby enhances catabolism of LDL (Lamarche and Paradis 2007). However, in humans positive correlations between postheparin EL concentration and levels of triglyceride and apoB were found, possibly as the result of increased EL expression in obesity (Badellino et al. 2006; Paradis et al. 2006). Therefore it is questionable whether EL plays any role in the remodelling of apoB-containing lipoproteins in humans (Lamarche and Paradis 2007). Data on the role of EL on atherosclerosis are controversial. EL deficiency was found to modulate atherosclerosis in atherosclerosis-prone mice in one study (Ishida et al. 2004) but not in another (Ko et al. 2005). By contrast, concentrations of EL in postheparin plasma correlated with coronary calcification in humans (Badellino et al. 2006).

## 7.3 Lipoprotein Transport Through the Endothelium

### 7.3.1 *General Aspects of Transendothelial Lipoprotein Transport*

During their metabolism, lipoproteins pass endothelial barriers on several occasions, namely after secretion from the liver and intestine into the blood and lymph, respectively, from the blood into extravascular compartments, where they exert their (patho)physiological functions (e.g. in the arterial wall), from the extravascular compartments back into the circulation (e.g. through vasa vasorum and lymphatic vessels), and finally from the circulation into the catabolic organs (notably liver). These transendothelial transport processes have been studied to a little degree, probably because they have been largely envisioned to occur through fenestrae of sinusoidal vessels or through discontinuities of a damaged endothelium.

Excess transendothelial lipoprotein transport plays a pivotal role in the pathogenesis of atherosclerosis. In fact, according to the response-to-retention hypothesis, the entry of remnants of lipid-rich lipoproteins (i.e. chylomicron remnants, IDL, LDL) from the blood stream into the intima of large arteries initiates and then perpetuates atherosclerosis (Tabas et al. 2007). HDL must also pass the endothelial barrier of arteries to exert their anti-atherosclerotic properties, for example to induce cholesterol efflux from lipid laden macrophages. Even more so, to continue the reverse transport of cholesterol from these foam cells in the arterial intima to the liver, HDL must again leave the arterial wall, probably via vasa vasorum which have grown into the thickened intima. It hence crosses an endothelial barrier a second time, this time from basolateral-to-apical. The accumulation of both pro-atherogenic and anti-atherogenic lipoproteins observed in atherosclerotic arteries is hence the result of increased influx and decreased efflux as well as enhanced binding to the extracellular matrix of the subendothelial intima (Simionescu 2007; Tabas et al. 2007). Although it is generally stated that subendothelial retention rather than unbalanced influx and efflux of lipoproteins is the major determinant of lipoprotein accumulation in the intima (Schwenke and Carew 1989; Tabas et al. 2007), it is important to note that the fluxes of lipoproteins into and out of the arterial wall have not been much investigated.

Initial studies in animals and men demonstrated that influx of lipoproteins into the vascular wall increases with the plasma concentration and decreases with the lipoprotein size (Nordestgaard et al. 1995). It has therefore been believed for a long time that lipoproteins enter the vascular wall by leakage through physiological fenestrae or damaged parts of the endothelium. However, since apoptosis of endothelial cells and disintegration of the endothelial layer occur in late and advanced atherosclerotic lesions and since lipoprotein entry into the subendothelial space is an early if not the first event in the pathogenesis of atherosclerosis (Simionescu 2007; Tabas et al. 2007), there is little reason to believe that endothelial injury is a prerequisite for lipoprotein entry into the vascular wall. In agreement with this, recent clinical studies did not find any effect of atherosclerosis or microalbuminuria on the fractional escape rate of radiolabeled LDL or albumin, which is

considered as an index of transendothelial exchange (Jensen et al. 2004; Kornerup et al. 2004). However, transendothelial exchange rates of LDL and albumin correlated with one another and were increased in patients with diabetes mellitus or non-diabetic patients with hyperinsulinemia suggesting that disturbed insulin action precedes and contributes to the increased arterial entry of LDL leading to atherosclerosis (Jensen et al. 2004; Kornerup et al. 2003). Moreover, interventions such as blood pressure lowering have been found to reduce the entry of LDL into the subendothelial space of carotid arteries (Born et al. 2003). All these findings argue against the concept of unspecific and unregulated lipoprotein transudation into the subendothelium and rather suggest that transendothelial transport of lipoproteins is a regulated process. This statement does not exclude that endothelial dysfunction (e.g. as the result of shear stress) modulates the permeability of the endothelium for lipoproteins. For example confocal laser scanning microscopy studies in mice revealed that the endothelial glycocalix is thinner at arterial sections which are exposed to increased shear stress. The thinner glycocalix at the carotid bifurcation as compared to the common carotid region was associated with a two- to three-fold increase in intimal LDL accumulation (van den Berg et al. 2009).

In general the transport of molecules through a barrier is determined by their water solubility, size, and charge. However, systematic permeability studies in endothelial monolayers revealed that this correlation is true only for water-soluble molecules with a diameter below 6 nm. The transport of larger molecules is much slower and does not show any correlation with size (Mehta and Malik 2006). Thus the transendothelial transport of lipoproteins ranging in diameter between 8 nm (HDL) and hundreds of nanometers (chylomicrons) requires special prerequisites. In fact morphological, biochemical, and physiological studies provide strong evidence that proteins pass the intact endothelium by both paracellular and transcellular routes which involve the regulated opening and closure of interendothelial junctions and vesicular pathways, respectively.

### ***7.3.2 Paracellular (Lipo)protein Transport***

The barrier function of the endothelium is primarily formed by the intercellular connection of adjacent cells through adherens junctions, gap junctions, and tight junctions. It is secondarily enforced by the glycocalix formed by negatively charged surface proteins (proteoglycans, glycosaminoglycans) and the extracellular matrix on the abluminal (i.e. basolateral) side which is predominantly produced by endothelial cells themselves and constituted of collagen type IV, fibronectin, laminin, entactin, and proteoglycans. Adherens junctions are composed of vascular endothelial cadherin molecules (VE-cadherin) which are connected with the actin cytoskeleton through associated  $\alpha$ -,  $\beta$ - and p120-catenins (Komarova et al. 2007; Mehta and Malik 2006). Also the tight junction proteins occludin and claudin are connected with the actin cytoskeleton, however via zona occludens proteins (Mehta and Malik 2006). The number and composition of the different junctions vary among endothelia of different vascular beds. In general arteries and capillaries of

the blood–brain barrier contain many tight junctions, whereas endothelial cells of postcapillary venules do not express any (Mehta and Malik 2006).

The passage of molecules with a diameter larger than 6 nm requires the opening of interendothelial junctions. The opening and closing of adherens junctions are regulated by two antagonistic signalling cascades elicited by the binding of thrombin and sphingosine-1-phosphate (S1P) to their cognate G protein-coupled receptors, namely the protease activated receptor 1 (PAR1) and the sphingosine-1-phosphate receptor 1 (S1P1) (Komarova et al. 2007; Mehta and Malik 2006).

Thrombin cleaves and thereby activates the protease activated receptor PAR1 which thereby couples to the  $\alpha$ -subunits of the heterotrimeric G proteins  $G\alpha_q$  and  $G\alpha_{12/13}$ .  $G\alpha_q$  activates phospholipase C  $\beta$ - which in turn generates inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). These second messengers activate protein kinase C (PKC) $\alpha$  and serine/threonine phosphatase by stimulating both  $Ca^{2+}$  release from the endoplasmic reticulum and extracellular  $Ca^{2+}$  uptake. The PKC $\alpha$ -mediated phosphorylation of p120-catenin and the dephosphorylation of VE-cadherin and  $\beta$ -catenin by the two phosphatases finally disrupt the VE-cadherin–catenin complex. The increase in intracellular  $Ca^{2+}$  leads to the activation of the myosin light-chain kinase (MLCK) either directly or indirectly via the tyrosine kinase src (Hu and Minshall 2009). In parallel, PAR1-mediated recruitment of  $G\alpha_{12/13}$  activates the GTPase RhoA which activates downstream kinases ROCK and LIM and thereby inhibits both actin polymerization (via cofilin activation) and crosslinking of actin with the plasma membrane [via ezrin/radixin/moesin (ERM) proteins]. Both myosin light-chain phosphorylation by MLCK and inhibition of actin polymerization finally destabilize the microtubule actin cytoskeleton and thereby increase paracellular permeability. Thus PAR1-mediated signalling opens adherens junctions by disassembling the junction proteins and destabilizing the cytoskeleton (Komarova et al. 2007; Mehta and Malik 2006).

S1P is generated by the enzymatic breakdown of sphingomyelin and the subsequent phosphorylation of sphingosin by sphingosin kinase in erythrocytes and platelets and is transported in plasma by HDL (50%), albumin (40%), and LDL (10%). It binds to at least five G protein-coupled receptors, four of which are expressed in endothelial cells (Argraves and Argraves 2007; Yatomi 2008). Activation of S1P1 recruits  $G\alpha_i$  which in turn activates Rac1 and Cdc42. This induces the reorganization of actin into cortical bundles which favor the reassembly of adherens junctions (Argraves et al. 2008; Komarova et al. 2007).

As yet this regulated paracellular transport has not been investigated for lipoproteins. However, since HDL transports about 50% of S1P in plasma, it is very likely that this lipoprotein contributes to the regulation of endothelial integrity and hence transendothelial macromolecule transport. In fact, HDL was previously described to increase the endothelial barrier integrity as measured by electric cell substrate impedance sensing (Argraves et al. 2008). Pertussis toxin, an inhibitor of Gi-coupled S1P receptors, as well as antagonists of the S1P receptor, S1P1, inhibited barrier enhancement by HDL, so it is very likely that S1P mediates this protective effect, at least in part (Argraves et al. 2008). HDL-associated S1P is known to stimulate the Erk1/2 and Akt signaling pathways in endothelial cells (for details, see Sect. 7.3). However, both HDL-induced barrier enhancement and HDL-induced motility

showed a greater dependence on Akt activation as compared with Erk1/2 activation. In addition to these direct effects of HDL on endothelial permeability, HDL was previously described to favor endothelial integrity by inducing several endothelial repair mechanisms, such as proliferation and migration of endothelial cells as well as the recruitment of endothelial progenitor cells (see Sect. 7.3.3). This involves the interaction of HDL with its receptor SR-BI which is localized in caveolae of endothelial cells and signals via a carboxy-terminal PDZ domain to the multi-PDZ domain-containing adaptor protein PDZK1 which in turn phosphorylates src and activates Akt and Erk1/2 (Mineo and Shaul 2007; Zhu et al. 2008).

### **7.3.3 Transendothelial (Lipo)protein Transport**

The transendothelial vesicular transport has been termed transcytosis and consists of endocytosis, vesicular transport, and exocytosis of the cargo at the opposite site (Mehta and Malik 2006; Simionescu 2007). This process can be receptor-dependent and then frequently involves caveolae or clathrin coated pits, or receptor-independent (i.e. fluid phase-mediated; Mehta and Malik 2006; Simionescu 2007). Until recently the physiological relevance of transcytosis through the endothelium was controversial, as no limiting factor was known (Rippe et al. 2002). This general scepticism is diminishing after detailed description of transendothelial albumin transport and the delineation of caveolin-1 as a rate-limiting factor (Frank et al. 2009; Mehta and Malik 2006). Since albumin binds lipids, notably fatty acids and lysolipids, transendothelial albumin transport not only serves as a model but is probably very relevant for transendothelial lipid transport. In addition biochemical and morphological experiments provide evidence for transcytosis of LDL, HDL or lipid-free apoA-I through microvascular endothelial cells of lymphatic vessels and the blood-brain barrier, as well as through the macrovascular endothelial cells of arteries.

Morphological studies have indicated that the transendothelial transport of LDL involves caveolae rather than clathrin-coated pits (Frank et al. 2009; Rippe et al. 2002). In agreement with a limiting role of caveolin-1 in LDL transcytosis, aortic rings of caveolin-1 knock-out mice showed a 50% reduction in the uptake of radiolabeled LDL at 37°C compared to aortic rings of wild-type mice (Frank et al. 2008). In vivo caveolin-1 knock-out mice showed a reduced fast-phase clearance (45 min) of radiolabeled LDL (which reflects transendothelial loss of the label into the extravascular space) but no alteration in LDL clearance after 2 h (which predominantly reflects hepatic uptake). At 24 h after injection caveolin-1 knock-out mice showed a 50% reduced uptake of radiolabeled LDL into arteries but a 30% increased uptake into liver (Frank et al. 2008). Taken together the results suggest that caveolin-1 plays an important role for the transendothelial uptake of LDL into the arterial wall. Interestingly, upon a Western-type diet apoE/caveolin-1 double knock-out mice had less atherosclerosis than apoE-only knock-out animals although they had more severely elevated LDL and VLDL plasma levels. However, it is not yet known whether this atheroprotective effect of caveolin-1 ablation is due

to reduced arterial LDL influx or due to reduced VLDL production and increased HDL levels, which are also observed in these animals (Frank et al. 2004).

Transport studies in porcine brain capillary endothelial cells identified the LDL receptor as a rate-limiting factor for the transcytosis of LDL through an *in vitro* blood–brain barrier model (Goti et al. 2002; Dehouck et al. 1997). However, it is important to note that the brain does not contain apoB, so it is questionable whether the LDL receptor mediates transcytosis of LDL holoparticles through the blood–brain barrier *in vivo*. It is more likely that, after internalization, LDL is dissociated so that only some LDL-associated components like cholesterol,  $\alpha$ -tocopherol, and water-soluble apolipoproteins but not apoB are transported into the brain (Goti et al. 2002). Also the LDL receptor-related proteins LRP1 and LRP2 were found to mediate transport of macromolecules, for example of the receptor-associated protein RAP, through the blood–brain barrier (Pan et al. 2004). Since the blood–brain barrier is very tight for the transendothelial transport of macromolecules and since the expression of many multi-drug resistance proteins by brain capillary endothelial cells also impedes the delivery of small molecules into the brain, the LDL receptor and both LRP1 and LRP2 have become interesting targets for therapeutic drug and protein delivery into the brain (Chao et al. 2003; Spencer and Verma 2007).

Less experimental data have been obtained on the transendothelial transport of HDL. Immunohistochemical studies of bovine aortic endothelial cells recovered DiI-labeled HDL in caveolae and co-localized gold-labeled HDL with caveolin-1 (Chao et al. 2003). Our own laboratory has investigated the transport of HDL and its main protein constituent apoA-I through monolayers of bovine aortic endothelial cells (Cavelier et al. 2006; Rohrer et al. 2006, 2009). Endothelial cells bound and associated  $^{125}\text{I}$ -apoA-I and  $^{125}\text{I}$ -HDL with high affinity and in a saturable and specific manner. Biotinylation experiments, fluorescence microscopy, and immunoelectronmicroscopy found that endothelial cells internalize labeled apoA-I and HDL. Only minor amounts of the internalized  $^{125}\text{I}$ -apoA-I and  $^{125}\text{I}$ -HDL were degraded. Cultivated in a Transwell system, the cells transported  $^{125}\text{I}$ -apoA-I or  $^{125}\text{I}$ -HDL from the apical to the basolateral compartment in a competitive and temperature-sensitive manner (Rohrer et al. 2006, 2009). Furthermore, after specific transport the originally lipid-free and pre $\beta$ -mobile apoA-I molecules were recovered as lipidated particles which have electrophoretic  $\alpha$ -mobility (Rohrer et al. 2006). Using pharmacological inhibitors and RNA interference, we also showed that ABCA1 but not SR-BI modulate cell surface binding, internalization and transport of  $^{125}\text{I}$ -apoA-I (Cavelier et al. 2006). In contrast, inhibition of SR-BI and ABCG1 but not inhibition of ABCA1 decreased the binding and transport of  $^{125}\text{I}$ -HDL (Rohrer et al. 2009). Taken together the data suggest that arterial endothelial cells transcytose apoA-I and HDL by distinct pathways which involve transcytosis.

Also transport studies in a blood–brain barrier cell culture model provided evidence for the apical-to-basolateral transcytosis of HDL-holoparticles as well as associated  $\alpha$ -tocopherol and apoA-I through porcine brain capillary endothelial cells by a process which involves SR-BI and caveolin-1 (Balazs et al. 2004). ApoA-I also facilitates the transport of protamine-oligonucleotide nanoparticles through brain

capillary endothelial cells by an SR-BI-regulated pathway (Kratzer et al. 2007). Thus, similar to ligands of the LDL receptor and LRP, apoA-I may help to target therapeutic molecules into the brain (Kratzer et al. 2007; Spencer and Verma 2007).

## **7.4 Target for Physiological and Pathological Effects of Lipoproteins**

As exchange barrier between plasma and tissue, endothelial cells are exposed to lipoproteins to a higher degree than any other cell type in the organism and, consequently, are the target of many physiological and pathological effects which influence endothelial function and survival. The lipoprotein components, receptors and signaling pathways which mediate the various protective or harmful effects of lipoproteins on endothelial cells are very heterogenous. From a general and hence oversimplified view one can summarize most of the present findings that (modified) apoB-containing lipoproteins [i.e. remnants of chylomicrons, LDL, Lp(a)] favor endothelial dysfunction whereas HDL mediate endothelial protection and repair. However, in several clinical conditions (e.g. inflammation) HDL can become dysfunctional and lose its protective effects on the endothelium or even gain paradoxical and harmful effects (Ansell et al. 2007).

### **7.4.1 Regulation of the Vascular Tone**

The vascular tone of arteries is regulated by endothelium-dependent and endothelium-independent mechanisms. Endothelium-dependent vasoreactivity depends on several hormones and mediators which are secreted by the endothelium, for example nitric oxide (NO), prostacyclin (PGI<sub>2</sub>), C-type natriuretic peptide (CNP), and endothelin-1 (Félétou and Vanhoutte 2007). Disturbed endothelium-dependent vasodilation is considered as a hallmark of endothelial dysfunction and an early sign of (presymptomatic) atherosclerosis (Deanfield et al. 2007; Félétou and Vanhoutte 2007). Endothelium-dependent vasorelaxation has been assessed in clinical studies by various invasive and non-invasive methods (Deanfield et al. 2007). Classically, endothelial vasoreactivity is assessed by angiographic measurement of lumen changes of coronary or brachial arteries in response to acetylcholine and other drugs which activate endothelial NO synthase (eNOS) and thereby elicit NO release. Less invasive and therefore also applicable to healthy volunteers is the measurement of flow-mediated brachial artery vasoreactivity which is monitored by high-resolution Doppler ultrasonography. In this assay hyperemia, which is induced by rapid deflation of a blood pressure cuff occluding the upper arm, leads to shear stress and thereby NO release in the forearm arteries, which results in vasodilation (Deanfield et al. 2007). Independently of the method used, many clinical studies observed that elevated plasma concentrations of

LDL cholesterol, triglycerides or chylomicron remnants, as well as low concentrations of HDL cholesterol, are associated with decreased endothelium-dependent vasodilation of coronary or brachial arteries (Brunner et al. 2005; Davignon and Ganz 2004; Norata and Catapano 2005; Wheeler-Jones 2007; Zhang et al. 2008; Zheng and Liu 2008). Even acute changes in the lipoprotein composition had strong effects on endothelium-dependent vasoreactivity: consumption of a single high-fat meal but not consumption of a low-fat meal was found to transiently impair flow-mediated vasodilation for 4 h. The change in this endothelium-dependent vasodilation correlated with the 2-h change in postprandial triglyceride or remnant lipoprotein levels (Plotnick et al. 1997). Conversely rapid lowering of LDL cholesterol by LDL apheresis (Tamai et al. 1997) as well as the infusion of artificially reconstituted HDL consisting of apoA-I and phosphatidylcholine were found to improve endothelium-dependent vasodilation in patients with hypercholesterolemia or low HDL-cholesterol (Bisoendial et al. 2003; Spieker et al. 2002).

Both the inhibitory effects of chylomicron remnants and the stimulatory effects of HDL on endothelium-dependent vasorelaxation are at least in part mediated by changes in NO production (Norata and Catapano 2005; Zheng and Liu 2007): chylomicron remnants were found to suppress eNOS phosphorylation by inducing the focal adhesion kinase FAK and downstream activation of the phosphatidylinositol-3-kinase (PI3K)/Akt (protein kinase B) pathway (Kawakami et al. 2002). The structural component of chylomicron remnants mediating its adverse effect on eNOS activation is not known. In addition modified lipoproteins and chylomicron remnants stimulate NADPH oxidase to synthesize superoxides which may inactivate NO by peroxynitrite formation (Dunn et al. 2008; Shin et al. 2004).

Conversely to chylomicron remnants, HDL stimulates eNOS phosphorylation by activating the PI3K/Akt signaling cascade (Norata and Catapano 2005). eNOS activation by HDL requires SR-BI (Yuhanna et al. 2001). Mineo et al. proposed that eNOS activation via binding of HDL to SR-BI involves MAP kinase signaling and subsequent Akt phosphorylation (Mineo and Shaul 2007; Mineo et al. 2003). Nofer et al. (2004) however showed that the stimulatory effect of HDL is not exerted by lipid-free apoA-I or reconstituted HDL consisting of apoA-I and phosphatidylcholine but by lysosphingolipids, including S1P, sphingosylphosphorylcholin (SPC), and lysosulfatide (LSF). It has therefore been proposed that the binding of HDL to SR-BI increases the abundance of HDL on the endothelial surface and hence the presentation of the agonist S1P to its cognate receptors (Nofer and Assmann 2005). In fact the physiological relevance of S1P and the need of its receptors for the vasodilatory effects of HDL have been proven in experiments on isolated arteries and mice which do not express the receptor S1P3 (Nofer et al. 2004). HDL-induced phosphorylation of Akt and eNOS as well as vasorelaxation of pre-contracted aortic rings from S1P3-deficient mice were reduced by 60% (Nofer et al. 2004). The residual ability of HDL to induce vasorelaxation may be mediated by other S1P receptors or even by completely different pathways and/or mediators. In agreement with the former explanation S1P was also found to mediate vasoconstriction by as yet unidentified S1P receptors expressed in vascular smooth muscle cells (Argraves and Argraves 2007).

In agreement with the latter explanation, HDL was found to stimulate the endothelial secretion of PGI<sub>2</sub> and CNP which both induce smooth muscle cell relaxation and hence vasodilation and to inhibit abluminal secretion of endothelin-1 which stimulates vasoconstriction (Norata and Catapano 2005). The stimulatory effect of HDL on PGI<sub>2</sub> release is dose-dependent and involves induction of cyclooxygenase 2 (COX-2) via the MAP kinase pathway (Norata et al. 2004). It is also exerted by delipidated HDL apolipoproteins although at a weaker degree than by intact HDL (Norata et al. 2004). Interestingly chylomicron remnants also induce COX-2 expression by activation of nuclear factor kappa B (NFκB) and MAP kinase signaling pathways, namely ERK1/2 and P38MAPK (Evans et al. 2004; Wheeler-Jones 2007). The common effects of HDL and chylomicron remnants on MAP kinase signaling and COX-2 induction suggest the action of a joint agonist. In fact, ω-6-fatty acids carried by both chylomicron remnants and HDL have been suggested to account for at least some of the effects from either lipoprotein class on COX-2 induction (Norata and Catapano 2005; Wheeler-Jones 2007). The most likely sources of arachidonic or linoleic acids are phospholipids, from which they are liberated by endothelial phospholipases such as EL. Whether COX-2 activation in endothelial cells is protective or harmful is a matter of controversy, since COX-2 products exert both pro- and anti-inflammatory properties. The preponderance of physiological or pathological effects may hence depend on the local environment and the expression of downstream enzymes such as prostacyclin synthase.

#### ***7.4.2 Leukocyte Adhesion and Extravasation***

Endothelial activation by inflammatory stimuli induces the expression of E-selectins, vascular cellular adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1), which mediate reversible (rolling) and then irreversible binding (adhesion) of leukocytes to the endothelium (van Gils et al. 2009). Monocyte chemoattractant protein 1 (MCP1; which is also produced by endothelial cells) ultimately stimulates the extravasation (diapedesis) of leukocytes into the extravascular tissue. The induction of cell adhesion molecules as well as MCP-1 mainly depends on the expression of the transcription factor NFκB, which is activated by cytokines and reactive oxygen species (ROS; Pantano et al. 2006; van Gils et al. 2009). Native LDL (and even more so oxidatively modified LDL) as well as chylomicron remnants were found to stimulate the production of ROS (e.g. by NADPH-oxidase) to activate NFκB, and to thereby induce the expression of cell adhesion molecules in endothelial cells (Gleissner et al. 2007; Wheeler-Jones 2007; Zheng and Liu 2007). The binding of modified LDL or chylomicron remnants to the lectin-like oxidized LDL receptor 1 (LOX-1) appears to play a pivotal role in this scenario, since this receptor is known to signal to NADPH oxidase and NFκB (Dunn et al. 2008; Gleissner et al. 2007; Wheeler-Jones 2007; Zheng and Liu 2007) and since inhibition of LOX-1 by monoclonal antibodies or antisense oligonucleotides abrogates the stimulatory effect of oxidized LDL and chylomicron remnants on NADPH-oxidase activation and cell adhesion molecule expression (Dunn et al. 2008). Interestingly, the binding of oxidized LDL itself increases the expression of LOX-1

in a positive feedback loop (Dunn et al. 2008). At least a part of the proinflammatory effects of oxidized LDL and chylomicron remnants are exerted by lysophosphatidylcholine (i.e. a lipolytic product of phosphatidylcholine generated by endothelial phospholipases; Gleissner et al. 2007; Wheeler-Jones 2007; Zheng and Liu 2007). Several research groups demonstrated that HDL inhibits the expression of E-selectin, VCAM-1, ICAM-1, and MCP-1 by endothelial cells as well as by the endothelial diapedesis of monocytes (Mineo et al. 2006; Norata and Catapano 2005; Zhang et al. 2008). In addition HDLs were found to inhibit ROS formation by NADPH oxidase as well as  $\text{NF}\kappa\text{B}$  activation (Robbesyn et al. 2003). The inhibitory effects of HDL on the transmigration of leukocytes through the endothelium appear to be exerted by different components and pathways. Barter and colleagues initially showed that reconstituted HDL but not lipid-free apolipoproteins inhibit VCAM-1 and E-selectin expression (Baker et al. 2000). The inhibitory effect of reconstituted HDL is strongly influenced by its phosphatidylcholine moiety. Phosphatidylcholines containing poly-unsaturated fatty acids have the strongest inhibitory effect (Baker et al. 2000). In addition Barter and colleagues showed that the inhibitory effect of native and reconstituted HDL on cytokine-induced VCAM-1 expression involves the inhibition of sphingosin-1-kinase and  $\text{NF}\kappa\text{B}$ , so that they concluded that HDL prevents endothelial monocyte adhesion by inhibiting endogenous S1P synthesis (Xia et al. 1999). In contrast to this harmful effect of intracellular S1P, Kimura et al. (2003) and Nofer et al. (2003) demonstrated that extracellular S1P carried by HDL inhibits NADPH oxidase, subsequent ROS formation, and the expression of E-selectin and cell adhesion molecule. Again this protective effect appears to be exerted by the dual interactions of HDL and S1P with two receptors, namely the lipoprotein receptor SR-BI and the sphingolipid receptor S1P1 (Kimura et al. 2006).

### ***7.4.3 Platelet Aggregation, Coagulation, and Fibrinolysis***

The endothelium plays an important role in hemostasis by providing a surface for platelet aggregation and coagulation and by secreting several pro- and anti-aggregatory small molecules, such as NO,  $\text{PGI}_2$  and platelet-activating factor (PAF), as well as pro-coagulant and anti-fibrinolytic proteins such as tissue factor (TF), von Willebrand factor (vWF), and plasminogen activator inhibitor type 1 (PAI-1).

As described above, both HDL and chylomicron remnants stimulate  $\text{PGI}_2$  production by endothelial cells, whereas NO production is inhibited by LDL and chylomicron remnants but stimulated by HDL (Evans et al. 2004; Mineo et al. 2006; Norata and Catapano 2005; Norata et al. 2004; Wheeler-Jones 2007). The pro- and anti-aggregatory effects of apoB-containing lipoproteins and HDL, respectively, is also mediated by their stimulating and inhibiting effects, respectively, on the expression of selectins (Baker et al. 2000; Gleissner et al. 2007; Kimura et al. 2006; Mineo et al. 2006). In addition HDL has been shown to inhibit thromboxan A2 formation (Oravec et al. 1998). The concerted effects on the endothelium and platelet activities make HDL act anti-platelet aggregatory (Mineo et al. 2006).

Native and even more so oxidized LDL and VLDL were reported to stimulate the secretion of TF and PAI-1 by endothelial cells and to thereby favor the prothrombotic state (Bai et al. 2006; Rosenson and Lowe 1998; Zhao et al. 2008) known especially for patients with metabolic syndrome and hypertriglyceridemia. HDL rather inhibits the VLDL-stimulated TF and oxLDL-stimulated PAI-1 secretion (Bai et al. 2006; Ren and Shen 2000; Viswambharan et al. 2004). These data as well as other data on the effects of lipoproteins on coagulation and fibrinolysis factors suggest that HDL inhibits coagulation and supports fibrinolysis, whereas triglyceride rich lipoproteins and their remnants exert opposite prothrombotic effects.

#### ***7.4.4 Endothelial Survival and Repair***

Due to their exposure to much chemical and physical harm, endothelial cells have a high risk of death. To maintain the integrity of the vasculature the endothelium needs factors that support cellular survival and the replacement of damaged endothelial cells. The latter may occur via proliferation and migration of neighboring cells or via repopulation with blood-borne endothelial progenitor cells (EPCs) which are ultimately derived from bone marrow (Zampetaki et al. 2008). In more exaggerated situations such as organ growth and tissue repair the additional need for blood supply leads to angiogenesis and neovascularization. Lipoproteins, notably HDL, affect many aspects of endothelial cell apoptosis, proliferation, migration, and differentiation (Argaves and Argaves 2007; Mineo and Shaul 2007; Mineo et al. 2006; Nofer and Assmann 2005).

Many local mediators, including cytokines, activated killer cells, and oxidized LDL, can cause the apoptosis of endothelial cells. At least some of the pro-apoptotic effects of modified LDL are exerted via binding to LOX-1, which increases the Bax/Bcl2 ratio via NF $\kappa$ B activation (Li and Mehta 2000). Also triglyceride-rich lipoproteins promote endothelial cell death by NF $\kappa$ B activation (Norata et al. 2003; Shin et al. 2004). In contrast, HDL was found to inhibit endothelial cell death being induced by oxidized LDL, TNF $\alpha$ , the terminal complement complex or growth factor deprivation (Argaves and Argaves 2007; Mineo and Shaul 2007; Mineo et al. 2006; Nofer and Assmann 2005; Norata and Catapano 2005). Interestingly and depending on the pro-apoptotic stimuli, the anti-apoptotic activity of HDL has been assigned to different components of HDL, namely to apoA-I for cell death induced by oxidized LDL, VLDL or cytokines (Suc et al. 1997; Speidel et al. 1990; Sugano et al. 2000), to lysosphingolipids for protection from cell death by growth factor deprivation (Kimura et al. 2003; Nofer et al. 2001; Kimura et al. 2001), and to apoJ for the inhibition of the terminal complement complex (Rosenfeld et al. 1983). The lysosphingolipids S1P, SPC, and LSF interfere with apoptosis by activating the PI3K/Akt pathway, which in turn prevents the activation of caspases 3 and 9 (Nofer et al. 2001).

S1P has also been identified as the agonist of HDL which stimulates endothelial cell proliferation by activating the small G protein Ras and the P42/44 MAP kinase pathway

(Miura et al. 2003a; Nofer et al. 2000; von Otte et al. 2006). Interestingly the same pertussis-toxin-sensitive signaling cascade has been made responsible for the S1P-stimulated migration of endothelial cells and the formation of endothelial tubes (i.e. capillary-like structures; Kimura et al. 2003; Miura et al. 2003b). Antisense oligonucleotides against the S1P receptors S1P1 and S1P3 blocked both the HDL-induced endothelial migration and tube formation, suggesting that S1P is the only agonist for these endothelial repair activities (Miura et al. 2003b).

However in another laboratory pertussis toxin did not inhibit endothelial cell migration stimulated by HDL, so that Mineo, Shaul and colleagues postulated the presence of another pathway and agonist which induces the migration of endothelial cells by HDL (Mineo and Shaul 2007; Mineo et al. 2006). In fact, reconstituted HDL consisting of apoA-I, palmitoylcholine, and cholesterol were also able to induce endothelial cell migration (Seetharam et al. 2006). In this case interaction of HDL with SR-BI is thought to activate the small G protein Rac via src-kinase, PI3K, Akt, and MAP kinase, which in turn stimulates the formation of lamellipodia, a prerequisite for cell migration (Seetharam et al. 2006). Interestingly and in contrast to VEGF-induced endothelial cell migration, this process does not involve eNOS and NO formation (Seetharam et al. 2006). Studies in genetic mouse models also indicate that HDL and SR-BI are involved in re-endothelialization. ApoA-I-deficient and hence HDL-deficient mice show decreased re-endothelialization as compared to wild-type mice. Normalization of apoA-I and HDL levels by liver-directed gene apoA-I transfer restored the re-endothelialization capacity. SR-BI knock-out mice also showed decreased re-endothelialization despite elevated HDL cholesterol levels supporting the critical role of this HDL receptor in endothelial repair (Seetharam et al. 2006).

As pointed out in the beginning of this section, re-endothelialization can occur as the result of proliferation and migration of neighboring cells as well as of repopulation with blood-borne EPCs. Several data from animal studies point to the modulatory activity of HDL in the latter process. In atherosclerosis-prone animal models, the infusion of reconstituted HDL as well as apoA-I gene transfer increased the number of EPCs in the blood (Tso et al. 2006). In an allograft heart transplant model, apoA-I gene transfer also promoted incorporation of EPCs in allografts and attenuated transplant arteriosclerosis (Feng et al. 2008a; Sumi et al. 2007). Interestingly, the beneficial effect of apoA-I gene transfer was offset in mice with SR-BI knock-out (Feng et al. 2008b, 2009). The accompanying *in vitro* studies also showed that the beneficial effect of HDL on EPC migration involved signaling via SR-BI and extracellular signal-regulated kinases (ERK) as well as increased NO production in EPCs (Noor et al. 2007).

Also, data from humans point to the improvement of EPC function by HDL: In patients with coronary artery disease a positive correlation between circulating EPC and HDL concentrations was demonstrated (Noor et al. 2007; Petoumenos et al. 2008). *Ex vivo* HDL was observed to enhance the differentiation of human monocytic cells into EPCs, inhibit apoptosis of EPCs, increase eNOS protein expression in EPCs, but decrease pro-MMP-9, stimulate proliferation of early outgrowth colonies after extended cell cultivation, and improve adhesion EPCs on human coronary

artery endothelial cells by up-regulation of  $\beta 2$  and  $\alpha 4$  integrins (Petoumenos et al. 2008). Pilot studies showed an increase in EPCs in the blood of diabetic patients treated with reconstituted HDL (van Oostrom et al. 2007).

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# Chapter 8

## Receptor-Mediated Endocytosis and Intracellular Trafficking of Lipoproteins

Joerg Heeren and Ulrike Beisiegel

**Abstract** Members of the low-density lipoprotein receptor (LDLR) gene family are structurally related receptors involved in receptor-mediated endocytosis and signal transduction that regulate a wide range of physiological processes. Receptor-mediated endocytosis of cholesterol-rich LDL and triglyceride-rich lipoproteins (TRL) into the liver via the LDLR and the LDLR-related protein 1 (LRP1) determine the plasma concentrations of proatherogenic lipoproteins. Recent studies indicate that LDLR-mediated internalisation of LDL and very (V)LDL engages a differentially regulated intracellular sorting machinery, suggesting that the LDLR is more than a simple constitutive endocytotic receptor. The binding and internalisation of TRL via hepatic LRP1 is even more complex. After internalisation, LDLR and LRP1 facilitate a different intracellular fate of their ligands. Whereas LDL follows the classical pathway for degradation, TRL disintegrate in late and peripheral endosomes, allowing a differential sorting of TRL components. This chapter summarises current understanding of the molecular mechanisms which are important for the internalisation and subsequent intracellular transport of LDL and TRL mediated by the LDLR and LRP1.

### 8.1 Lipoproteins and Their Receptors

Unravelling the mechanisms regulating the clearance of proatherogenic lipoproteins such as triglyceride-rich lipoproteins (TRL) and low-density lipoprotein (LDL) are of great importance, as the understanding of these cellular processes may lead to

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the development of new therapies for dyslipidemia. Therefore we first introduce the metabolism of LDL and TRL and the physiological importance of lipoprotein internalisation for extrahepatic organs such as the adrenals and bone.

### **8.1.1 Metabolism of LDL**

LDL is generated from liver-derived very (V)LDL by intravascular lipolytic processing mediated by lipoprotein lipase (LPL), hepatic lipase (HL) and lipid exchange proteins, such as phospholipid transfer protein (PLTP; Huskonen et al. 2004) and cholesteryl ester transfer protein (CETP; de Grooth et al. 2004), which are described in more detail in Chap. 9. Importantly, the conversion of VLDL into intermediate-density lipoproteins (IDL) and further to LDL reduces the binding affinity of apolipoprotein E (apoE) and other apolipoproteins (such as apoC) to the LDL particle. IDL to LDL conversion finally results in LDL particles that contain a single copy of the non-exchangeable apoB100 molecule only, thereby prolonging the short half-life of IDL from a few minutes to hours for LDL. The low-density lipoprotein receptor (LDLR) plays a central role in co-ordinating the internalisation of circulating LDL. Demonstrating the importance of the LDLR pathway, mutations in the LDLR cause severe hypercholesterolemia and premature atherosclerosis (Brown and Goldstein 1986). LDLR-mediated removal of LDL from the circulation occurs mainly in peripheral cells and hepatocytes. The liver removes approximately 50% of the circulating LDL (Vance 2002). Interestingly, the LDLR is not only able to interact with LDL-associated apoB100 at the basolateral surface of parenchymal liver cells. Several studies (Twisk et al. 2000; Gillian-Daniel et al. 2002; Larsson et al. 2004) have demonstrated that the LDLR is also directly involved in the secretory pathway of VLDL, at least in vitro. In general, apoB100 lipidation and VLDL secretion is regulated by the amount of hepatocellular lipids. Under conditions of low lipid availability, substantial amounts of newly synthesised apoB100 are degraded. Thus, in general VLDL secretion is reduced when the amount of hepatocellular lipids is low. However, the absence of the LDLR results in higher apoB100 secretion even under conditions of low hepatocellular lipids (Twisk et al. 2000; Gillian-Daniel et al. 2002; Larsson et al. 2004). This indicates that binding of the LDLR to immature VLDL can occur intracellularly and thereby influence the secretion of apoB100-containing lipoproteins. In contrast, independent in vivo studies in the mouse system show normal apoB100 production and secretion in the absence of the LDLR after the injection of Triton WR 1339 (Jones et al. 2007). There could be various reasons for the discrepancy of results obtained in vitro and in vivo. In particular the utilisation of Triton derivatives to measure lipoprotein synthesis in vivo might influence not only the interaction of lipoproteins with the cell surface. These detergents also have an impact on the structure and functionality of internal membranes, possibly altering intracellular membrane and ligand-sorting events. Future experiments using novel approaches without the co-injection of detergents will probably help to clarify whether binding of apoB100 to the LDLR in the endoplasmic reticulum is of physiological importance for the secretion of lipoproteins.

The extrahepatic sites of native LDL uptake *in vivo* have been characterised by the “trapped ligand” approach, which determines the accumulation of radioactivity after injection of tyramine-cellobiose-labelled LDL (Pittman and Steinberg 1984). Although the liver is the dominant site of LDL uptake, steroid-producing tissues such as the adrenals and the ovaries show a higher specific LDLR activity compared to the liver. Interestingly, LDLR deficiency in mice did not impair corticosterone synthesis in adrenals, indicating that *de novo* cholesterol production or selective cholesterol uptake from HDL via scavenger receptor class B1 (SR-B1) may be equally important and can compensate for lack of LDLR activity in adrenal steroidogenesis (Kraemer 2007). In the human system it has also been shown that patients with homozygous familial hypercholesterolemia have normal corticosteroid production (Illingworth et al. 1984). However, these studies were carried out in the absence of the LDLR, which might underestimate the importance of LDL uptake into adrenals under physiological conditions. It has been shown that LDL can be a significant source for cholesterol delivery into human adrenals in the presence of an active LDLR (Carr and Simpson 1981; Liu et al. 2000).

### ***8.1.2 Metabolism of Triglyceride-Rich Lipoproteins***

Intestinal chylomicrons (CM) and liver-derived VLDL represent the two classes of TRL that are responsible for the transport of lipids to the various body cells. CM mediate the transport of dietary lipids, including lipophilic vitamins, whereas VLDL carry lipids from the liver to peripheral tissues. In humans, TRL can be distinguished by their apoB composition. apoB48, synthesised as a result of apoB mRNA editing (Anant and Davidson 2001), is the major apolipoprotein of intestinal-derived CM, while apoB100 is required for the assembly of VLDL in the liver (Kane 2001). In contrast to humans, mice exhibit hepatic apoB mRNA-editing activity, which results in the formation of apoB100- and apoB48-containing TRL in the liver.

In the bloodstream TRL are first hydrolysed by lipoprotein lipase (LPL) and subsequently by HL, leading to the formation of TRL remnants (Mahley and Ji 1999; Merkel et al. 2002). During lipolysis the particles simultaneously become enriched with HDL-derived apoE. After hydrolysis LPL and HL remain associated predominantly with postprandial apoB48 containing CM remnants (CR; Dichek et al. 1999, 2004; Xiang et al. 1999; Heeren et al. 2002). The majority of the resulting CR and VLDL remnants (also called IDL), which are depleted in triglycerides, are then rapidly internalised into the liver through binding of apoE, LPL and HL to the LDLR-related protein 1 (LRP1; Beisiegel et al. 1989; Rohlmann et al. 1998; Mahley and Ji 1999; Verges et al. 2004) or apoB100 and apoE interacting with the LDLR (Ishibashi et al. 1994; Mahley and Ji 1999). The current knowledge of remnant internalisation and subsequent intracellular processing is presented in Sect. 8.3.

Next to the liver, extrahepatic tissues have the capacity for uptake of TRL (Hussain et al. 1989). To permit such an uptake into bone cells, the endothelium in the bone is fenestrated and thereby allows direct contact with the TRL particles and the cells. Recently it could be demonstrated that osteoblasts participate in CR

metabolism and that CR components are relevant for osteoblast function (Niemeier et al. 2008). ApoE and LRP1 are expressed by osteoblasts (Niemeier et al. 2005; Schilling et al. 2005); and interestingly apoE deficient mice display a high bone formation rate (Schilling et al. 2005). LRP1 mediates the uptake of CR-associated vitamin K1 into human osteoblasts (Niemeier et al. 2005) and thereby this process has a direct impact on the degree of osteocalcin carboxylation and secretion by osteoblasts in mice (Niemeier et al. 2008). The adipose tissue is also involved in lipoprotein receptor-mediated uptake of TRL particles. In rodent adipocytes, LRP1 activation mediated by insulin is associated with an increased uptake of TRL remnants *in vitro* (Corvera et al. 1983; Descamps et al. 1993). In adipose-specific LRP1-deficient mice, adipose tissue mass was substantially reduced and TRL internalisation was abolished in cultivated LRP1-deficient adipocytes. It was suggested that reduced adipose tissue mass can be explained by reduced particle uptake *in vivo* (Hofmann et al. 2007). Since most functional studies on adipocyte function have been performed in the mouse system, there is a strong demand to study lipoprotein metabolism in human adipocytes. In a recent report we described that immortalised mesenchymal stem cells can be stably differentiated into human adipocytes (Prawitt et al. 2008). After differentiation, these cells maintained numerous morphologic and functional features of mature adipocytes. Interestingly, adipocyte differentiation is associated with a concomitant up-regulation of LRP1 and apoE indicating that these proteins are also important for TRL particle uptake into human adipose tissue. However, at least in this human adipocyte cell model an inverse regulation of receptor expression and remnant uptake was observed. Therefore, LRP1 and apoE might have an additional role next to TRL uptake in human adipose tissue that is independent of lipoprotein internalisation. Such a putative alternative function could include the regulation of signal transduction cascades involved in adipocyte differentiation and proliferation.

The VLDL receptor (VLDLR), expressed in adipose tissue, heart and muscle, also functions as a receptor mediating CR internalisation into adipocytes *in vitro* (Niemeier et al. 1996; Takahashi et al. 2004). However, VLDLR is expressed on endothelial cells rather than in the underlying parenchymal cells (Wyne et al. 1996). This observation probably indicates that the VLDLR primarily supports LPL-mediated lipolysis, as suggested by Goudriaan et al. (2004). Furthermore, in contrast to the fenestrated endothelium of liver and bone, the capillary endothelium of adipose tissue is continuous and therefore impermeable for lipoprotein particles. Thus, the role of LRP1 and VLDLR for the internalisation of lipoprotein particles in adipose tissue *in vivo* is unclear and need to be addressed in future work.

## 8.2 Receptor-Mediated Endocytosis of LDL

The pathway of receptor-mediated LDL uptake and the negative feed-back regulation of LDLR gene expression mediated by cellular cholesterol levels is one of the best studied cellular processes and was rewarded by the Nobel Prize in 1985 (Goldstein

and Brown 1986). However, recent studies have revealed novel insights in the cell physiology of the LDLR which have great impact on the intracellular targeting of internalised LDL. The next section gives an overview of the **classical pathway** and presents new findings in the LDLR pathway.

### ***8.2.1 Structure and Function of the LDL Receptor***

The mature LDLR is a type-I integral transmembrane glycoprotein (Fig. 8.1) containing five different domains: (1) the ligand-binding domain, (2) the EGF-precursor homology domain, (3) the O-linked sugar domain, (4) the transmembrane domain and (5) the cytosolic domain. The ligand-binding domain is localised at the N-terminal region of the LDLR and consists of seven “LDLR class A repeats” (Sudhof et al. 1986). Each repeat is encoded by a single exon and contains approximately 40 amino acids. Six cysteine residues within the LDLR class A repeats form characteristic disulfide bonds, which facilitate the presentation of a characteristic tripeptide acidic signature at the surface of the domain, thought to mediate the binding of positively charged residues to apoB100 and apoE.

Three epidermal growth factor (EGF) repeats, together with a so-called  $\beta$ -propeller domain, form the EGF-precursor homology region. The EGF domains contain characteristic disulfide bonds similar to the ligand-binding repeats. Two of these repeats are located next to the ligand-binding repeats whereas one repeat lays C-terminal to the  $\beta$ -propeller. Thus, the EGF repeats are adjacent to an approximately 260 amino acid domain containing six YWTD motifs forming the  $\beta$ -propeller domain (Jeon et al. 2001). This structure is responsible for the pH-dependent release of LDL from the LDLR in endosomal compartments. The elegant model illustrated by Jeon and Blacklow (2005) clarified that, at neutral pH, LDL-derived apoB100 bind to LDLR class A repeats 3 and 7 of the ligand-binding domain. At the acidic pH found in endosomes, LDLR class A repeats 4 and 5 interact with the  $\beta$ -propeller domain, which results in the release of LDL from its receptor.

The O-linked sugar domain is highly enriched in serine and threonine residues that serve as anchor for O-linked sugars. Although detailed structural knowledge is available, the precise function of this region is not known. The deletion of this region does not impair the function of LDLR with respect to ligand binding, internalisation and degradation. It has been postulated (Gent and Braakman 2004) that this domain might: (1) modulate the presentation of the ectodomain, (2) stabilise the LDLR at endosomal pH and/or (3) prevent proteolytic cleavage of the ectodomain by metalloproteases at the cell surface. This so-called “shedding” of the ectodomain has been described for other members of the LDLR family which lack an O-linked sugar domain such as LRP1 and SorLA. However, the physiological function of this domain remains unclear. Next to the O-linked sugar domain, a hydrophobic transmembrane domain (22–24 amino acids in length) ensures the LDLR is anchored in the plasma membrane.



The cytoplasmic tail of the LDLR consists of approximately 50 amino acids and is responsible for endocytosis and intracellular transport. Site-directed mutagenesis revealed several amino acids which are important for correct LDLR function. The most prominent motif within the LDLR tail is the characteristic NPXY sequence (NPVY in the case of the LDLR), which is known to bind phospho-tyrosine-binding (PTB) domains of adaptor proteins. The NPVY sequence is, at least in hepatocytes, important for the clustering of LDLR within coated pits (Keyel et al. 2006). A more detailed view of the importance of this sequence is provided in Sect. 8.2.3. A comparison of the domain organisation between LDLR and LRP1 is illustrated in Fig. 8.1.

### 8.2.2 *Ligands of the LDL Receptor*

The LDLR binds and mediates the internalisation of lipoproteins that contain apoB100 and/or apoE. The most important lipoproteins that are internalised by the LDLR are LDL and their respective precursors VLDL and IDL (Goldstein et al. 1985). Liver-derived VLDL contain a single copy of apoB100 and multiple copies of apoE (Shelness and Sellers 2001), which together facilitate high-affinity binding to the LDLR. The interaction of apoE to the LDLR is highly dependent on the association of apoE with lipids. As determined by different spectroscopic techniques, lipidated apoE has an approximately 500-fold higher affinity for LDLR compared to the delipidated form (Wilson et al. 1991). The lipidation of apoE probably causes a conformational rearrangement and increased exposure of basic residues at the surface of the apoE molecule (Sehayek et al. 1991). Furthermore, LPL-mediated conversion of VLDL into IDL leads to a higher ratio of apoE to total particle protein (Goldberg 1996). Both effects explain the higher binding affinity of IDL to the LDLR compared to VLDL. The basic residues arginine and lysine of apoE located between amino acids 120–160 are responsible for the binding to the LDLR (Saito et al. 2004). The significance of these basic residues for LDLR binding is supported by the differential internalisation rate of apoE isoforms. ApoE exists in three isoforms which affect their binding properties to the LDLR. ApoE3 and E4, but not apoE2, exhibit a strong binding affinity to the LDLR (Schneider et al. 1981). In contrast to apoE3 and apoE4, the arginine residue at position 158 is replaced by a cysteine residue in the isoform apoE2. This amino acid change modulates the receptor binding domain and inhibits the LDLR-mediated internalisation of apoE2-TRL in the liver.

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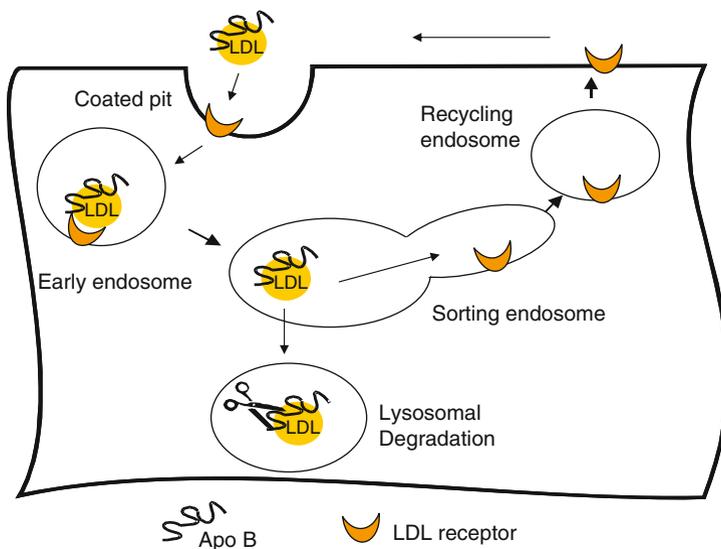
←  
**Fig. 8.1** (continued) remain non-covalently associated with the smaller C-terminal  $\beta$ -subunit. The  $\alpha$ -subunit contains four clusters of ligand-binding repeats which are encircled by eight  $\beta$ -propeller domains, each adjacent to EGF-like repeats. Both LRP1 subunits lack the O-linked sugar domain. The cytoplasmatic LRP1  $\alpha$ -subunit contains diverse potential endocytosis and signaling motifs: two NPXY motifs whereas the distal NPXY sequence overlaps with the endocytosis signal XYYL and two dileucine motifs. The structures are illustrated according to Herz and Strickland (2001), Jeon and Blacklow (2005) and Gent and Braakman (2004)

This leads to the accumulation of remnant particles in plasma, thereby contributing to dyslipidemia in homozygous apoE2 carriers, and is an essential factor for the development of type III hyperlipoproteinemia (Mahley et al. 1999).

Enzymatic conversion of IDL to LDL is associated with the loss of all exchangeable apolipoproteins including apoE. This results in an LDL particle with one single molecule of apoB100, which compared to apoE-containing IDL has a lower binding affinity for the LDLR. The binding domain of apoB100 is located at the C-terminal end explaining why TRL-derived apoB48 cannot bind to the LDLR. Boren et al. (1998) identified residues 3359–3367 in apoB100 as those interacting with the LDLR. However, since the mutation R3500Q immediately upstream of the LDLR binding region in apoB100 disrupts binding to the LDL receptor and causes hypercholesterolemia, additional sequences must contribute to apoB100 binding capability. To investigate the entire sequences required for effective binding of apoB100 to the LDLR, the same group reconstituted human wild-type and mutated apoB100 into LDL by the use of several transgenic mouse lines (Boren et al. 2000). They concluded that normal receptor binding in apoB100 depends on an interaction between arginine 3500 and tryptophan 4369, because both mutations disrupt the binding of LDL to the LDLR. Thus, the ability of apoB100 to interact with the LDLR depends on the conformation in the C-terminal domain, which is probably essential for the correct exposure of the arginine- and lysine-rich binding motif between residues 3359 and 3367 on the surface of LDL particles.

### **8.2.3 Intracellular Processing of LDL**

After endocytosis within clathrin-coated vesicles, LDL enter the lysosome where the cholesteryl esters are hydrolysed to cholesterol and free fatty acids and the apoB100 moiety is degraded to free amino acids (see Fig. 8.2; Goldstein et al. 1985). New aspects in the early internalisation events which influence sorting and targeting of receptors and their respective ligands along the lysosomal pathway have recently been described. Initially, sorting endosomes within the early endosomal compartment were considered as the primary location that disintegrates the receptor/ligand complex to enable receptor recycling back to the cell surface (LDLR) and lysosomal targeting of the ligand (LDL). This model has now been challenged, as ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes (Lakadamyali et al. 2006). Live cell microscopy to follow individual endosomes and ligand particles in real time showed that early endosomes comprise two distinct populations: (1) a dynamic population that matures rapidly toward late endosomes and (2) a static population that matures much more slowly. LDL was targeted preferentially into the dynamic pool of early endosomes, a process associated with the recruitment of specific adaptor proteins. The importance of the adaptor protein ARH1 for uptake and subsequent intracellular sorting LDL is described in more detail in Sect. 8.2.4. Interestingly, a fraction of endocytosed LDL escapes lysosomal degradation and is secreted in an unaltered



**Fig. 8.2** Uptake and intracellular processing of LDL. After LDL receptor-mediated internalisation of LDL, the change in pH within the lumen of early endosomes causes a conformational change in the receptor. This results in the release of bound LDL in early endosomes. Whereas LDL receptors are recycled back to the plasma membrane via tubular extensions of the sorting endosomes, the content of the endosomal lumen including LDL follows the lysosomal pathway (adapted from Goldstein and Brown 1985)

or only slightly altered form by a process termed retroendocytosis (Greenspan and St Clair 1984; Aulinskas et al. 1985). Edge et al. (1986) observed that, in hepatocytes, much less endocytosed apoB100 is degraded compared to skin fibroblasts. Although these observations indicate that the degradation of apoB100 may not be the sole metabolic fate of the protein moiety of endocytosed LDL in the liver, its potential implications for the homeostasis of the apoB containing lipoproteins in the circulation have not yet been addressed in further detail.

### 8.2.4 Regulation of LDL Receptor Function

LDLR gene expression is transcriptionally regulated via sterol regulatory element-binding proteins (SREBP) in dependence of cellular cholesterol levels. These ER-derived transcription factors are the major regulatory element that determines LDLR activity and are the subject of excellent reviews (Brown et al. 2000; Rawson 2003). Since defects in the LDLR gene were not detected in all patients with familial hypercholesterolemia, great efforts were made to identify further candidate genes

that can cause inherited forms of severe hypercholesterolemia (Rader et al. 2003). Indeed, gene defects in proprotein convertase subtilisin-like kexin type 9 (PCSK9) and the LDLR adaptor protein ARH1 correlate with elevated LDL plasma levels (Cohen et al. 2003; Horton et al. 2007). LDL-elevating PCSK9 mutations cause an autosomal dominant hypercholesterolemia (ADH), leading to reduced levels of LDLR protein and consequently to high plasma levels of LDL cholesterol. Since overexpression of wild-type PCSK9 specifically accelerates the degradation of LDLR protein, these findings are indicative of gain-of-function mutations within the PCSK9 gene promoting lysosomal targeting of LDLR, thereby reducing the amounts of LDLR at the cell surface being available for LDL internalisation and clearance, ultimately causing ADH. The posttranslational regulation of LDLR protein levels by PCSK9 as well as its clinical importance has been summarised in detail elsewhere (Attie and Seidah 2005; Horton et al. 2007).

Autosomal recessive hypercholesterolemia (ARH) results from a mutated gene located on the short arm of chromosome 1. The respective gene product, a protein with a phospho-tyrosine binding (PTB) domain, is called ARH1. This adaptor protein binds to the internalisation sequence NPVY within the cytoplasmic tail of the LDLR and connects the LDLR to the endocytotic machinery of the cell. Retroviral expression of normal ARH1 in transformed lymphocytes from an affected individual with ARH restored LDLR internalisation, confirming that ARH is caused by defective adaptor protein function (Eden et al. 2002). The C-terminal domain of ARH1 directly interacts with clathrin and adaptor protein 2 (AP2), which are both essential components for receptor-mediated endocytosis via clathrin coated pits (He et al. 2002; Mishra et al. 2002). Interestingly, ARH1 is only required for hepatic LDLR-dependent LDL uptake (Keyel et al. 2006; Jones et al. 2007). In fibroblasts and probably also in other tissues, the adaptor protein disabled 2 (dab2) can also mediate LDLR-dependent endocytosis. Since hepatic dab2 levels are very low, defective ARH1 protein resulted in a reduced LDL clearance only into the liver. Somewhat unexpectedly, VLDL clearance via the LDLR was not affected in ARH1-deficient mice (Jones et al. 2007). The authors concluded that the clearance of VLDL and IDL mediated by the LDLR is independent of ARH1, which could explain the lower LDL-cholesterol levels in patients with ARH1 deficiency compared to patients lacking the LDLR. These findings also indicate that the NPVY internalisation sequence within the cytoplasmic tail of the LDLR interacting with ARH1 is differentially involved in VLDL and LDL internalisation; and a report by the same investigators support this assumption (Michaely et al. 2007). Although the exact determinants for lipoprotein internalisation have yet to be identified, the presence of specific uptake mechanisms for different lipoproteins (LDL, VLDL) via the same receptor (LDLR) suggest that: (1) the differential expression and activity of adaptor proteins in the various tissues can modulate LDLR function and (2) the ligands themselves determine the internalisation process mediated by the LDLR. The relevance of these observations in regard to intravascular VLDL and LDL metabolism and intracellular processing of VLDL/LDL constituents needs to be addressed in the future and will probably provide new therapeutic options to lower the plasma levels of these proatherogenic lipoproteins.

### 8.3 Receptor-Mediated Endocytosis of Chylomicron Remnants

Hepatic lipoprotein uptake is a complex but well regulated process involving multiple different players. Although not all steps are completely understood, recent advances from *in vitro* studies and mouse models have allowed the development of a general concept for the internalisation and processing of postprandial lipoproteins in the liver. From the liver sinusoids, CR enter the space of Disse through the fenestrated endothelium. The vast majority of these particles first attaches to cell surface structures such as heparin sulfate proteoglycans (HSPG; for reviews, see Havel and Hamilton 2004; Mahley and Huang 2007; Bishop et al. 2008). Recent studies in genetically engineered mice showed that a liver-specific knock-out of N-deacetylase/N-sulfotransferase (resulting in significant changes in liver heparin sulfate composition) led to an increase in plasma triglyceride levels under both fasted and fed conditions. This study proves that HSPG have a prominent role for the binding of TRL to the surface of parenchymal liver cells (McArthur 2007). The authors suggested that HSPG can also be considered as an endocytotic receptor which is directly involved in the internalisation process. However, it has been shown that postprandial apoB<sub>48</sub> containing CR are internalised into the liver via binding of apoE and LPL to LRP1 (Beisiegel et al. 1989; Rohlmann et al. 1998; Heeren et al. 2002; Verges et al. 2004) and via apoE interacting with the LDLR (Mahley and Ji 1999; Ishibashi et al. 1994). LDLR knock-out mice accumulated LDL-sized particles (Ishibashi et al. 1993), whereas in fasted animals the liver-specific loss of LRP1 expression did not influence lipoprotein profiles (Rohlmann et al. 1998). This is due to the up-regulation of hepatic LDLR which compensates for the loss of LRP1 to ensure clearance of fasting TRL. Only double-knockout mice, deficient for LDLR and hepatic LRP1, showed an accumulation of remnant lipoproteins. Thus, both receptors are essential for the clearance of TRL *in vivo* (Rohlmann et al. 1998).

#### 8.3.1 Structure and Function of LRP1

LRP1 is one of the largest members of the LDLR family and is a multifunctional type I cell surface glycoprotein that participates in endocytosis, phagocytosis and signalling pathways (Herz and Strickland 2001; Lillis et al. 2005). It is synthesised in the ER as a 600-kDa precursor protein which is proteolytically cleaved within the Golgi complex to generate two subunits: (1) the N-terminal 515-kDa  $\alpha$ -subunit containing the ligand-binding domains and (2) the C-terminal 85-kDa  $\beta$ -subunit containing an extracellular part, the transmembrane-spanning domain and the cytoplasmic intracellular domain (Herz et al. 1990). The N-terminal domain lacks a membrane-spanning region but remain non-covalently associated with the smaller C-terminal  $\beta$ -subunit. Compared to the extracellular part of the LDLR, the extracellular domain organisation of LRP1 is much more complex. The  $\alpha$ -subunit contains four clusters of ligand-binding repeats, the sizes of which differ due to variations in the number of LDL class A repeats (cluster 1 has two, cluster 2 has eight, cluster 3

has ten, cluster 4 has 11). Most of the ligands interact only with ligand-binding clusters 2 and 4. Interestingly, only apoE and the chaperone RAP bind to cluster 3 (Herz and Strickland 2001). The ligand-binding domains are encircled by eight  $\beta$ -propeller domains, each adjacent to EGF-like repeats, suggesting that pH-dependent release might be regulated in dependence of the internalised ligands. In contrast to the LDLR, the extracellular parts of both LRP1 subunits lack the O-linked sugar domain. The extracellular element of the  $\alpha$ -subunit contains only EGF-like repeats which are located next to transmembrane domain. The cytoplasmatic LRP1  $\alpha$ -subunit contains diverse potential endocytosis and signalling motifs: two NPXY motifs whereas the distal NPXY sequence overlaps with the endocytosis signal **XXYL** and two dileucine motifs (Li et al. 2000). The tyrosine residue within the distal NPXY motif is phosphorylated upon treatment with platelet-derived growth factor (PDGF), influencing the binding of adaptor proteins as described below. In addition, serine and threonine phosphorylation, mediated by protein kinase A and C, generates a cytoplasmatic receptor tail that has increased affinity for adaptor proteins such as **disabled 1** (Dab1) and **cell death abnormal/engulfment adapter protein** (CED-6/GULP) which is associated with a reduced internalisation rate (Li et al. 2001; Ranganathan et al. 2004). Thus, phosphorylation of the  $\alpha$ -subunit represents a molecular mechanism to modulate the different functions mediated by LRP1 (see Sect. 8.3.4).

### 8.3.2 *Ligands of LRP1*

LRP1 is widely expressed in several tissues and interacts with more than 30 unrelated ligands (Lillis et al. 2005) including apoE-containing lipoproteins, proteinase/ proteinase inhibitor complexes, bacterial toxins, viruses, growth factors, matrix proteins and intracellular proteins such as the ER-resident chaperon receptor-associated protein (RAP). Efficient binding to LRP1 makes RAP a suitable tool for investigating LRP1 function. Studies with “apoB48-only” and “apoB100-only” mice on a LDLR-deficient background identified that RAP-mediated inhibition of LRP1 resulted in the accumulation of apoB48 containing remnants in the plasma (Veniant et al. 1998). These findings imply that LRP1 facilitates the clearance of postprandial CR but plays no significant role for the catabolism of apoB100-containing lipoproteins. As mentioned above, not only CR-derived apoE, but also LPL binding to LRP1 efficiently initiates the internalisation of CR (Beisiegel et al. 1991; Heeren et al. 2002). Furthermore, LRP1 mediates the uptake of LPL into the liver (Verges et al. 2004) and hepatic LRP1 deficiency leads to the accumulation of LPL in plasma (Espirito Santo et al. 2004). This non-enzymatic function of LPL as a LRP1 ligand probably compensates for other defects that impair TRL clearance, such as apoE variants (Mann et al. 1999), but may also contribute to the delayed clearance of TRL in patients with dyslipidemias caused by LPL mutations (Merkel et al. 2002). Some evidence also points to a potential role of hepatic lipase in LRP1-mediated CR internalisation (Krapp et al. 1999; Gonzalez-Navarro et al. 2004;

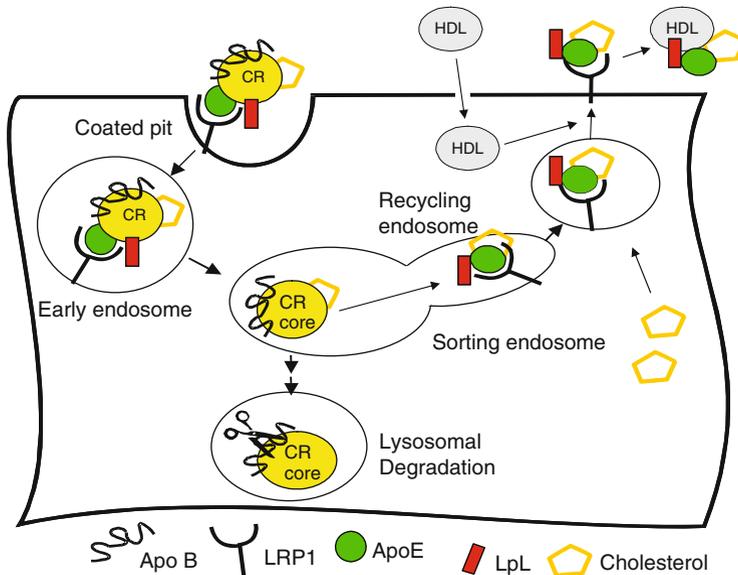
Verges et al. 2004). Thus, both LRP1 and LDLR are involved in the endocytosis of postprandial lipoproteins, suggesting that HSPG are predominantly involved in tethering lipoproteins at the cell surface. Even though LRP1 has been shown to be responsible for the hepatic clearance of CR, only a very small proportion of the endocytotic receptor is present at the surface of liver cells under normal conditions *in vitro* and *in vivo* (Harasaki et al. 2005; Tamaki et al. 2007). However, the postprandial hormone is known to stimulate a rapid LRP1 translocation to the plasma membrane of rodent adipocytes (Corvera et al. 1989; Descamps et al. 1993) and in hepatocytes (Tamaki et al. 2007). Recent data indicate that insulin-stimulated translocation of LRP1 correlates with an increased LRP1-mediated uptake (Laatsch et al. 2008). In wild-type mice, insulin triggers the hepatic uptake of LRP1 ligands, whereas in the absence of hepatic LRP1 expression or in mice with impaired hepatic insulin-signalling, insulin-triggered uptake of LRP1-specific ligands was abolished (Laatsch et al. 2008). Thus, this suggests that impaired hepatic LRP1 translocation can contribute to the postprandial hyperlipidemia observed in subjects with insulin resistance.

### **8.3.3 Intracellular Processing of Chylomicron Remnants**

After internalisation of CR, lipoproteins are immediately disintegrated in late and peripheral endosomes, which allows a differential sorting of CR components. While core lipids and apolipoprotein B (apoB48) are targeted via late endosomes to lysosomes, the majority of CR-derived apoE together with surface lipids remain in peripheral recycling endosomes. The current model proposes (see Fig. 8.3) that the pool of CR-derived apoE is then mobilised by high-density lipoproteins (HDL) or HDL-derived apoA-I to be recycled back to the plasma membrane, followed by apoE re-secretion and the concomitant formation of apoE containing HDL. The HDL-induced recycling of apoE is accompanied by cholesterol efflux and involves the internalisation and targeting of HDL-derived apoA-I to endosomes containing both apoE and cholesterol (Heeren et al. 2006). The cellular mechanisms of apoE recycling and its connection to cholesterol efflux is described in more detail in the following sections.

#### **8.3.3.1 Recycling of Apolipoprotein E**

In initial studies it was observed that internalised VLDL particles, but not different from LDL, were poorly degraded in HepG2 hepatoma cells (Lombardi et al. 1993, Rensen et al. 2000). When analysing the internalisation of rabbit  $\beta$ -VLDL in mouse macrophages, apoE was resistant to lysosomal degradation and accumulated in widely distributed vesicles (Tabas et al. 1990). Similarly, VLDL-sized apoE containing triglyceride-rich emulsion particles were more resistant to degradation in HepG2 than LDL-derived apoB (Rensen et al. 2000) suggesting that, following internalisation by liver cells, apoE can escape degradation and can be re-secreted.



**Fig. 8.3** ApoE recycling initiates HDL particle formation. After LRP1-mediated internalisation the disintegration of CR within sorting endosomes resulted in two different intracellular pathways. The lipid core is directed to lysosomal compartments where it is hydrolysed and its constituents can be used for metabolic requirements. The CR-derived “surface remnants” enriched in apoE and LpL and cellular cholesterol are transferred back to the plasma membrane via a peripheral recycling compartment. The intracellular association of apoE with HDL generates HDL<sub>E</sub>, which are re-secreted and can probably serve as apoE supply for remnant enrichment with apoE in the post-prandial period

These findings indicated that TRL are disintegrated after internalisation and that some TRL constituents are targeted along the degradative pathway, whereas others, such as apoE, are re-secreted. Similar to these TRL model particles, CR components were disintegrated and differentially sorted in a peripheral cellular compartment after internalisation into human hepatoma cells (Heeren et al. 1999). Whereas CR-derived lipids and apoB were directed to lysosomes, CR-derived apoE, apoC and LpL were recycled back to the cell surface, where re-secretion could occur (Heeren et al. 1999, 2001). Since evidence has accumulated that the complex processing of internalised TRL also exists *in vivo*, the intracellular stability of apoE after hepatic uptake was studied in C57BL/6 mice (Rensen et al. 2000). Only 15–20% of apoE was degraded in hepatocytes, whereas 75% of the cholesteryl oleate was hydrolysed. Fazio and co-workers injected radiolabelled mouse VLDL particles into C57BL/6 mice and consistently identified iodinated apoE in Golgi enriched fractions (Fazio et al. 1999). Similar results were obtained in LDLR-deficient mice, indicating that apoE recycling involves LRP1-mediated TRL internalisation. Up to 60% of internalised apoE appeared to be re-utilised in the plasma, providing further evidence of a unique intracellular pathway to process apoE-containing remnant

lipoproteins *in vivo* (Heeren et al. 2001; Swift et al. 2001). Subsequent analysis of re-secreted proteins revealed that recycled intact apoE was found in plasma associated with HDL (Heeren et al. 2001). These findings provided the first evidence that recycling of TRL-derived apoE plays an important role in the modulation of HDL particles *in vivo*. Fazio and co-workers identified internalised VLDL-derived apoE to be associated with nascent lipoprotein particles in liver Golgi fractions (Fazio et al. 1999) and they concluded that recycling of apoE could be mediated in part by Golgi-derived secretory vesicles. However, the majority of TRL-derived apoE is probably re-secreted from peripheral endosomal compartments, where apoE recycling is connected with HDL metabolism. The intracellular link between TRL-derived apoE and HDL metabolism in early endosomes was described by several groups. In these studies, HDL or purified apoA-I stimulated the release of internalised apoE in hepatocytes and fibroblasts (Hasty et al. 2003; Heeren et al. 2003). Similarly, HDL or apoA-I-induced apoE recycling also occurs in mouse macrophages and human neuronal cells (Farkas et al. 2005; Rellin et al. 2008) and therefore it has been investigated whether apoE recycling could also be linked to intracellular cholesterol transport in other cells (see Sect. 8.3.2.2).

Since epidemiological data show that apoE3 and apoE4 have differential effects on HDL metabolism, we investigated whether the intracellular processing of TRL-derived apoE4 differs from apoE3-TRL (Heeren et al. 2004). ApoE4 correlates with high LDL-cholesterol, elevated triglyceride and low HDL levels (Davignon et al. 1988; Dallongevielle et al. 1992) and is associated with atherosclerosis and Alzheimer's disease (Dallongeville et al. 1992; Roses 1996). But despite the importance of apoE4 as a risk factor, the cellular mechanisms which are responsible for the differences between apoE3 and apoE4 are not well understood. Cell surface binding, internalisation, endosomal transport and disintegration of apoE3-TRL and apoE4-TRL in early endosomes have been shown to be identically comparable in hepatoma cells (Heeren et al. 2004). Thus, a dissimilar recycling of apoE isoforms could contribute to the development of apoE4-associated diseases. Along these lines, we could demonstrate that HDL-induced recycling of TRL-derived apoE4 is impaired and associated with a decreased cholesterol efflux (Heeren et al. 2004). Although the molecular mechanisms and the potential involvement of members of the LDLR family are yet unclear, the biophysical characteristics of apoE3 and apoE4 might provide an explanation for the reduced efficiency of intracellular apoE4 processing. ApoE3 binds preferentially to HDL, while apoE4 has a higher affinity to VLDL (Dong et al. 1994), suggesting that apoE isoforms interact differently with lipids. TRL-derived apoE3 might therefore associate more rapidly with internalised apoA-I and cholesterol to form HDL-containing apoE (HDL<sub>E</sub>) particles.

### 8.3.3.2 Apolipoprotein E Recycling and Cholesterol Efflux

In human hepatoma cells and fibroblasts, HDL-induced apoE recycling is accompanied by cholesterol efflux (Heeren et al. 2003). Large amounts of internalised TRL-derived apoE co-localises with early endosome antigen 1 (EEA1), a marker for

early endosomes. ApoE and EEA1-positive endosomes also contain cholesterol and, upon HDL incubation, simultaneous apoE recycling and cholesterol efflux occurs simultaneously from peripheral early endosomes. These results point to a direct link between HDL-induced apoE recycling and cholesterol efflux from early endosomal compartments. Although internalised TRL-derived apoE was not detected in API-positive Golgi vesicles (Heeren et al. 2001) and apoE recycling was unaffected in the absence of an intact Golgi apparatus (Farkas et al. 2004), current data does not completely rule out that apoE recycling concomitantly affects cholesterol efflux via the Golgi network where cholesterol-rich membranes are assembled and moved forward to the plasma membrane. At first, we and others favoured HDL to act at the plasma membrane to promote apoE recycling and cholesterol efflux. New findings addressing this question clearly challenge this model (Heeren et al. 2003). In hepatoma cells, HDL-derived apoA-I was shown to co-localise with apoE and cholesterol in EEA1-positive endosomes, strongly suggesting that HDL-derived apoA-I is internalised and targeted to pre-existing apoE/cholesterol-containing endosomes to promote apoE recycling and cholesterol efflux. Time-lapse confocal microscopy confirmed that HDL-derived apoA-I is targeted to TRL-derived apoE/cholesterol-containing endosomes in the periphery (Heeren et al. 2003). Similarly, FPLC analysis of secreted proteins from macrophages identified recycled apoE in fractions that contained small HDL, indicating that TRL-derived apoE exits cells in a remodelled, moderately lipidated form (Hasty et al. 2005).

ApoE/cholesterol-containing endosomes are positive for EEA1, a marker for early endosomes. EEA1 is absent from the plasma membrane and restricted to a subcompartment of early endosomes that contain Rab5 and Rab5 effectors (Rubino et al. 2000). These Rab5/EEA1-positive endosomes are connected to the recycling compartment by tubular structures but clearly distinct from recycling endosomes that are targeted to the cell surface. Rather other Rab proteins, such as Rab4 and Rab11, are characteristic for recycling endosomes in the cellular periphery. This suggests that, after TRL disassembly in early sorting endosomes, apoE/cholesterol complexes are retained to allow complex formation with internalised HDL/apoA-I before entering the recycling compartment. HDL-induced apoE3 recycling also occurs in neuronal cells, but here apoE recycling is not accompanied by enhanced cholesterol efflux (Rellin et al. 2008). In summary, the association of internalised apoA-I with intracellular apoE/cholesterol complexes leads to the formation of apoE-enriched HDL particles. In macrophages, HDL-induced apoE recycling may prevent foam cell formation by enhancing the removal of cholesterol from the periphery. In liver, this mechanism secures the maintenance of high levels of HDL<sub>E</sub> in plasma in the postprandial phase and accelerates the enrichment of CR with apoE. After a high fat meal, the re-utilisation of internalised apoE would overcome any shortage of HDL<sub>E</sub> due to insufficient de novo synthesis of apoE and thereby assure the efficient hepatic clearance of CR.

### 8.3.4 Regulation of LRP1 Function

LRP1 regulates the endocytosis of a variety of different ligands, modulates PDGF, TGF $\beta$  and Wnt-dependent signalling pathways (Herz et al. 2001; Lillis et al. 2005) and, with regard to lipoprotein metabolism, LRP1 is probably responsible for the differential intracellular targeting and recycling of internalised apoE. The various functions likely involve the association of LRP1 with distinct intracellular adaptor proteins containing phospho-tyrosine-binding domains (Gotthardt et al. 2000; Uhlik et al. 2005); and therefore organ-specific functions of LRP1 might be regulated by the expression pattern of the respective adaptor proteins and/or depend on the phosphorylation status of the cytoplasmatic tail of LRP1. In general, the two NPXY motifs within the cytoplasmatic tail are able to interact with adaptor proteins in a phospho-tyrosine-independent or dependent manner. Although the importance of growth factor (e.g. PDGF-mediated LRP1 tyrosine phosphorylation) has been shown by several investigators *in vitro* and *in vivo* (Loukinova et al. 2002; Boucher et al. 2003; for reviews, see Lillis et al. 2005; Stolt and Bock 2006), the role of adaptor proteins for LRP1-mediated internalisation of triglyceride-rich lipoproteins remains elusive. Since after a meal there is a special need for effective hepatic clearance of postprandial lipoproteins, it is interesting to note that the endocytotic activity of LRP1 is at least partly regulated by the metabolic condition within the cell. In the fasted state, LRP1 is phosphorylated in dependence of the hunger signal cAMP at threonine and serine residues by activated protein kinase A (PKA). LRP1 phosphorylation results in a decrease in the endocytosis rate of LRP1 and the efficiency of ligand delivery for degradation (Li et al. 2000). Serine/threonine phosphorylation has been shown to modulate adaptor protein binding to the distal NPXY motif and reduce the association of LRP1 with adaptor molecules of the endocytotic machinery such as AP-2 (Ranganathan et al. 2004). This might explain the slower endocytosis rate of LRP1 in the presence of high cellular cAMP levels. In the postprandial phase, the drop in hepatocellular cAMP concentration mediated by increased insulin levels phase would consequently increase LRP1 endocytotic activity, which probably facilitates the LRP1-mediated clearance of postprandial lipoproteins, as shown by Laatsch et al. (2008).

The proximal NPXY within the cytoplasmatic tail of LRP1 has been identified as the binding motif for sorting nexin 17 (Snx17) and this interaction is important for the efficient recycling and basolateral targeting of LRP1 but not for the internalisation process (van Kerkhof et al. 2005). Interestingly, analysis of mutant LRP1 knock-in mice revealed that the reinsertion of an inactivating mutation in the proximal NPXY motif caused prenatal death, whereas a mutation in the distal NPXY motif did not cause any obvious pathological effects (Roebroek et al. 2006).

In summary, endocytosis and diverse signal transduction processes mediated by LRP1 are critically dependent on adaptor proteins interacting with NPXY motifs. However, the physiological importance of adaptor protein association with the

cytoplasmatic tail of hepatic LRP1 for the internalisation and subsequent recycling of apoE-containing lipoproteins are yet unclear.

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# Chapter 9

## Angiopoietin-Like Proteins and Lipid Metabolism

Sander Kersten

**Abstract** Lipoprotein lipase, hepatic lipase, and endothelial lipase play a pivotal role in the clearance and remodelling of plasma lipoproteins. Their activity is governed at the level of gene transcription as well as via changes in enzyme activity by specific activator or inhibitor proteins. The latter group includes two members of the Angiopoietin family: Angiopoietin-like proteins 3 and 4 (Angptl3, Angptl4). Angptl4 is expressed ubiquitously and is a direct target gene of the peroxisome proliferator activated receptors, whereas Angptl3 is expressed exclusively in liver and is regulated by the liver X receptor. For both Angptl4 and Angptl3, injection of recombinant protein, adenoviral over-expression, and transgene-mediated overexpression have been shown to cause marked hypertriglyceridemia. Conversely, inactivation of the *Angptl4* or *Angptl3* gene is associated with lower plasma triglyceride levels. Recent human genetic studies suggest that rare or common sequence variants in the *ANGPTL4* and *ANGPTL3* gene, respectively, impact plasma TG levels, supporting a role for *ANGPTL4* and *ANGPTL3* in lipoprotein metabolism in humans. The present chapter provides an overview of the role of Angptl4 and Angptl3 in the regulation of lipoprotein metabolism. It is postulated that alterations in Angptl4 and Angptl3 signalling might be involved in dyslipidemia.

### 9.1 Introduction

Changes in the plasma level of lipoproteins are known to affect atherosclerosis and associated coronary heart disease (CHD). Indeed, it is well established that elevated plasma low-density lipoprotein (LDL) levels increase the risk for CHD, whereas elevated high-density lipoprotein (HDL) levels are considered atheroprotective. Besides LDL, elevated plasma triglycerides (TG) are increasingly recognized as an

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important, independent risk factor for CHD. High TG levels frequently occur together with low HDL levels, often with normal levels of LDL, and are referred to as abnormalities of the TG–HDL axis. This atherogenic dyslipidemia is connected with central obesity characterized by excess fat tissue in and around the abdomen.

Depending on nutritional status, TG are present in blood plasma mainly packaged in chylomicrons or in very low-density lipoproteins (VLDL). Accordingly, plasma TG levels are determined by the balance between the rate of production of chylomicrons and VLDL in intestine and liver, respectively, and their rate of clearance in peripheral organs such as skeletal muscle, heart, and adipose tissue. Clearance of TG-rich lipoproteins is mediated by the enzyme lipoprotein lipase (LPL), which is anchored into the capillary endothelium via heparin sulfate proteoglycans and catalyses TG hydrolysis. Other lipases that target plasma lipoproteins include hepatic lipase, which promotes clearance of remnant particles in liver, and endothelial lipase, which preferentially hydrolyses (phospho)lipids within HDL.

While LPL gene expression is known to respond to changes in nutritional status, LPL is also extensively regulated at the level of enzyme activity (Merkel et al. 2002). Several modulators of LPL activity are known, including apolipoproteins Apoc3, Apoc2, Apoc1, and ApoA5. In addition to apolipoproteins, in the past few years it has become evident that two proteins belonging to the family of angiopoietin-like proteins (Angptls), Angptl3 and Angptl4, can modulate LPL activity. Here, a detailed overview is provided of the role of Angptl3 and Angptl4 in the regulation of lipid metabolism.

## 9.2 Angptl4 and Lipid Metabolism

### 9.2.1 *Discovery and Structure of Angptl4*

Angptl4 was identified by Kim et al. (2000) as hepatic fibrinogen/angiopoietin-related protein (HFARP) while searching for additional members of the angiopoietin family (Kim et al. 2000). In parallel, Yoon discovered *Angptl4* by a subtractive cloning strategy to identify target genes of the nuclear hormone receptor PPAR $\gamma$  in adipose tissue (Yoon et al. 2000). Finally, Kersten et al. (2000) identified *Angptl4* as a PPAR $\alpha$  target gene by comparing mRNA from livers of wild-type and PPAR $\alpha$ -deficient mice (Kersten et al. 2000). They named the protein fasting-induced adipose factor (FIAF), which in the literature is used interchangeably with Angptl4.

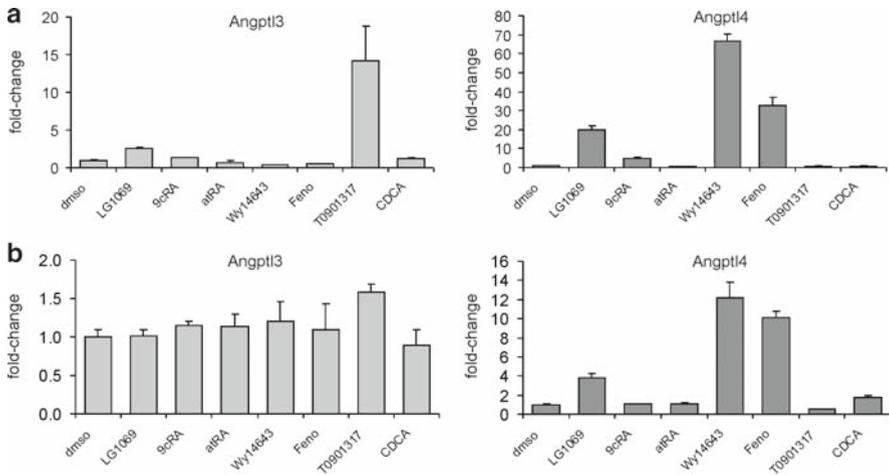
In mouse, the gene sequence encoding *Angptl4* covers 6.6 kb and consists of 7 exons (Kersten et al. 2000; Yoon et al. 2000). The human gene shows a similar organization and is located on chromosome 19, in a region close to a locus associated with atherosclerosis susceptibility (Yoon et al. 2000). The open reading frame derived from the cDNA sequence spans about 1.2 kb and gives rise to a 406- and 410-amino-acid protein in human and mouse, respectively (Kersten et al. 2000; Kim et al. 2000), that undergoes glycosylation and is secreted (Ge et al. 2004a; Kersten et al. 2000; Kim et al. 2000). Within its angiopoietin/fibrinogen-like domain,

Angptl4 contains several conserved cysteine residues that contribute to intermolecular disulfide bonding, resulting in the formation of variable-sized multimeric structures (Ge et al. 2004a; Mandard et al. 2004; Yoon et al. 2000). In addition, Angptl4 is proteolytically processed, releasing N- and C-terminal fragments that can be detected in human serum. The exact site of cleavage has yet to be exactly defined but may vary between R162 and R229. While the N-terminal fragment of Angptl4 impacts lipid metabolism, the C-terminal fragment is a structural and likely regulatory component of the extra-cellular matrix that so far has been shown to affect a number of different processes including angiogenesis, endothelial cell function, vascular leakage, and cell adhesion (Cazes et al. 2006; Galaup et al. 2006; Hermann et al. 2005; Ito et al. 2003; Le Jan et al. 2003; Padua et al. 2008). However, currently there is a lack of consensus on the direction of the impact of Angptl4 on these processes.

### 9.2.2 Regulation of *Angptl4* Expression

In mouse, *Angptl4* is expressed in a variety of tissues, but highest levels are found in white and brown adipose tissue, followed by ovary, liver, lung, heart, and intestine (Kersten et al. 2000; Yoon et al. 2000). In human, expression of *ANGPTL4* is particularly high in liver, followed by adipose tissue and pancreatic islets (Zandbergen et al. 2006). Befitting its alternative name “fasting-induced adipose factor”, expression of *Angptl4* is up-regulated by fasting in a variety of tissues (Dutton and Trayhurn 2008; Ge et al. 2005; Kersten et al. 2000; Mandard et al. 2006; Sukonina et al. 2006). The effect of fasting is likely mediated by elevated plasma free fatty acids, which stimulate *Angptl4* gene transcription via peroxisome proliferator activated receptors (PPARs). Indeed, treatment with synthetic agonists for PPAR $\alpha$  potently induces *Angptl4* expression in liver, skeletal muscle, heart, and intestine (Bunger et al. 2007; Ge et al. 2005; Kersten et al. 2000; Mandard et al. 2004). Furthermore, expression of *Angptl4* is induced by PPAR $\alpha$  agonists in white adipose tissue, liver, heart, and muscle (Kersten et al. 2000, unpublished data; Yoon et al. 2000), and by PPAR $\beta/\delta$  agonists in heart, muscle, keratinocytes, and liver (Akiyama et al. 2004; Kersten et al., unpublished data; Schmuth et al. 2004; Schug et al. 2007). Using transactivation assays, electrophoretic mobility shifts, and in vivo chromatin immunoprecipitations, *Angptl4* was shown to be a direct PPAR target gene, containing a conserved functional PPAR response element in its third intron (Mandard et al. 2004). In addition to PPARs, synthetic and endogenous ligands for RXR and possibly FXR induce *Angptl4* expression (Fig. 9.1).

Regulation of *Angptl4* mRNA or protein has been studied in several mouse models and under a variety of physiological stimuli. Expression of *Angptl4* is highly induced by hypoxia as observed in cardiomyocytes, brain, endothelial cells, and adipocytes (Belanger et al. 2002; Gustavsson et al. 2007; Le Jan et al. 2003; Wang et al. 2007; Wiesner et al. 2006), suggesting a role for *Angptl4* in angiogenesis. Alternatively, upregulation of *Angptl4* under hypoxic conditions may be linked to the inhibitory effect of *Angptl4* on cellular fat uptake (see below) and is aimed at favouring anaerobic metabolism (e.g. glucose) over aerobic metabolism (e.g. fatty acids).



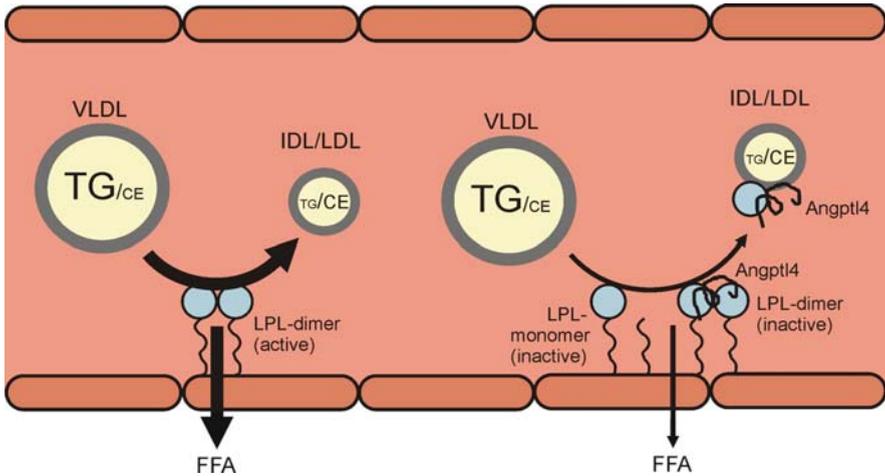
**Fig. 9.1** Angptl3 and Angptl4 expression are upregulated by different nuclear receptor agonists. Rat FAO hepatoma cells (a) or primary rat hepatocytes (b) were treated for 24 h with different nuclear receptor agonists [LG1069 1  $\mu$ M, 9cRA 0.5  $\mu$ M, atRA 0.5  $\mu$ M, Wy14643 5  $\mu$ M, fenofibrate (feno) 50  $\mu$ M, T0901317 1  $\mu$ M, chenodeoxycholic acid (CDCA) 50  $\mu$ M]. Gene expression of Angptl3 and Angptl4 was analysed by qPCR

Other conditions in which adipose *Angptl4* expression is elevated include pregnancy and lactation (Josephs et al. 2007) and obesity and diabetes (Yoon et al. 2000). In the pituitary gland but not in the hypothalamus, *Angptl4* expression is increased by food restriction, suggesting a role for *Angptl4* in the neuroendocrine response to food deprivation (Wiesner et al. 2004). In the intestinal epithelium of germ-free mice lacking gut microbes, *Angptl4* expression increases during the suckling–weaning transition. This increase is absent in germ-free mice given a normal microbiota derived from conventionally raised mice, suggesting that *Angptl4* expression is suppressed by the intestinal microbiota (Backhed et al. 2004).

### 9.2.3 Role of *Angptl4* in Lipid Metabolism

Regulation of *Angptl4* by PPARs, fasting, high-fat feeding, and obesity first suggested that *Angptl4* might be involved in lipid metabolism (Kersten et al. 2000; Yoon et al. 2000). In 2002, Yoshida and colleagues (2002) were the first to demonstrate a direct and immediate stimulatory effect of recombinant *Angptl4* on plasma levels of TG, FFA, and non-HDL cholesterol in mice (Yoshida et al. 2002). Induction of plasma TG by *Angptl4* has been confirmed in several transgenic and adenoviral mouse models of *Angptl4* over-expression, as well as in *Angptl4* knock-out mice (Backhed et al. 2004; Ge et al. 2004b; Koster et al. 2005; Mandard et al. 2006; Xu et al. 2005; Yu et al. 2005). Also, repeated injections of mice with a monoclonal

antibody against Angptl4 lowers plasma TG levels (Desai et al. 2007). Hypertriglyceridemia induced by *Angptl4* is dependent on its oligomeric state, as rendering Angptl4 defective in oligomerization resulted in a reduced hypertriglyceridemic effect (Ge et al. 2004b). Angptl4 raises plasma TG by suppressing LPL-mediated clearance of plasma TG-rich lipoproteins, (Ge et al. 2004b; Lichtenstein et al. 2007), thereby inhibiting cellular fatty acid uptake from that specific plasma fatty acid pool, and perhaps to a minor extent by raising hepatic VLDL production (Desai et al. 2007; Mandard et al. 2006). Angptl4 directly inhibits the activity of lipoprotein lipase (LPL), as shown by numerous in vitro and in vivo studies (Koster et al. 2005; Lichtenstein et al. 2007; Yoshida et al. 2002; Yu et al. 2005). In addition, Angptl4 inhibits activity of hepatic lipase, contributing to a reduced hepatic uptake of cholesteryl-esters and triglycerides from remnant particles, which in turn leads to induction of hepatic cholesterol synthesis (Lichtenstein et al. 2007). Recent in vitro studies indicate that the N-terminal fragment of Angptl4 inhibits LPL by promoting the conversion of catalytically active LPL dimers into catalytically inactive LPL monomers, thereby permanently inactivating LPL (Sukonina et al. 2006). This mechanism is supported in vivo, although it may not account for the full extent of inhibition of LPL in *Angptl4*-transgenic mice (Lichtenstein et al. 2007; Fig. 9.2). We showed that, in mouse blood, Angptl4 is physically associated with LPL monomers, which in turn are bound to LDL. Possibly, by binding LPL monomers Angptl4 pulls the equilibrium between LPL dimers and monomers toward monomers, which effectively results in an inhibition of LPL activity (Lichtenstein et al. 2007).



**Fig. 9.2** Inhibition of LPL-mediated plasma TG lipolysis by Angptl4. Catalytically active LPL dimers are bound to endothelial cells lining the capillaries via heparin-sulfate proteoglycans. Angptl4 inhibits the activity of LPL by promoting dissociation of LPL dimers into catalytically inactive LPL monomers. LPL monomers dissociate from the vessel wall and associate with circulating IDL/LDL particles. In addition, Angptl4 may inhibit LPL independently of dimer dissociation. Decreased LPL activity leads to impaired clearance of TG from the plasma and decreased uptake of fatty acids into the cell

It has been suggested that the induction of *Angptl4* mRNA levels in adipose tissue by fasting may explain the known rapid decrease of LPL activity in adipose tissue upon fasting (Sukonina et al. 2006). While this may be true, it should be emphasized that fasting also markedly induces expression of *Angptl4* in skeletal muscle, concurrent with an increase in LPL activity (Lichtenstein et al. 2007; Ruge et al. 2005).

One of the key questions regarding *Angptl4* function is to what extent *Angptl4* acts locally by selectively inhibiting LPL in the tissue in which it is produced. The specific inhibition of cardiac LPL activity in mice transgenic mice over-expressing *Angptl4* in heart is supportive of a paracrine action of *Angptl4* (Yu et al. 2005). In contrast, the marked elevation of plasma TG in mice over-expressing *Angptl4* specifically in liver, which lacks LPL, suggests that *Angptl4* acts as a circulating LPL inhibitor that mediates inter-organ communication (Xu et al. 2005).

By inhibiting activity of LPL and as a consequence cellular uptake of plasma TG-derived fatty acids, *Angptl4* would be expected to decrease fat storage in adipose tissue. Surprisingly, bodyweight and composition were reported to be unaltered in *Angptl4* knock-out mice, either on chow diet or after a high-fat/high-carbohydrate feeding for 15 weeks (Koster et al. 2005). In contrast, using the same *Angptl4* knock-out mice on a pure C57Bl/6 background, we found a significant increase in epididymal fat mass compared to wild-type mice. Furthermore, in mice over-expressing *Angptl4* in liver and peripheral tissues, epididymal and perirenal fat mass was decreased by 50% compared to their wild-type littermates (Mandard et al. 2006). While the reduction in fat mass by *Angptl4* may be explained by decreased uptake of plasma TG, other mechanisms likely contribute as well. Plasma levels of FFA and glycerol were elevated in mice over-expressing *Angptl4*, suggesting that *Angptl4* stimulates adipose tissue lipolysis (Mandard et al. 2006; Lichtenstein et al. 2007). In agreement with this notion, injection of recombinant *Angptl4* abruptly raises plasma FFA (Yoshida et al. 2002). *Angptl4* may induce lipolysis via upregulation of *Pnpla2* (adipose triglyceride lipase). Other mechanisms that may contribute to reduced fat mass in *Angptl4* transgenic mice include adipose upregulation of *Ucp1* as well as increased fatty acid oxidation, suggesting an increased abundance of brown adipocytes (Mandard et al. 2006).

In addition to lipoprotein metabolism, *Angptl4* has also been implicated in regulation of glucose homeostasis. Adenoviral-mediated over-expression of *Angptl4* markedly lowered blood glucose and improved glucose tolerance, while inducing hepatic steatosis and (transient) hypertriglyceridemia (Xu et al. 2005). In addition, in primary rat hepatocytes, adenoviral-mediated *Angptl4* over-expression decreased glucose production and enhanced insulin-mediated inhibition of gluconeogenesis. In contrast, plasma glucose levels were unchanged in *Angptl4* knock-out mice and in transgenic mice over-expressing *Angptl4* in liver (Koster et al. 2005). Furthermore, blood glucose levels were unaltered in transgenic mice with general *Angptl4* over-expression, while glucose tolerance was worsened, especially after high-fat feeding (Mandard et al. 2006). Using hyperinsulinemic–euglycemic clamp techniques it was shown that *Angptl4* over-expression causes insulin-resistance in the periphery, but improves insulin-sensitivity at the hepatic level (Lichtenstein et al. 2007). At the

present time, it is difficult to reconcile these various findings regarding the effects of Angptl4 on glucose homeostasis. Clearly, the dramatic effect of Angptl4 on plasma glucose levels as reported by Xu et al. (2005) has not been reproduced in other studies using Angptl4 transgenic or knock-out mice.

### 9.2.4 Role of Angptl4 in Human

In human, highest expression levels for *ANGPTL4* are found in liver, followed by adipose tissue (Zandbergen et al. 2006). Similar to Angptl4, *ANGPTL4* is cleaved into N- and C-terminal fragments, which together with full-length *ANGPTL4* are detectable in human blood plasma (Mandard et al. 2004).

Very little is known about possible determinants of plasma *ANGPTL4*, likely because assays to quantitatively assess *ANGPTL4* levels in human plasma are presently not commercially available. Using a homemade ELISA or by semi-quantitative Western blot, it has been shown that the plasma *ANGPTL4* concentration increases after treatment with rosiglitazone or fenofibrate, which serve as synthetic agonists for PPAR $\gamma$  and PPAR $\alpha$ , respectively (Mandard et al. 2004; Xu et al. 2005). In obese patients with type 2 diabetes, serum levels of Angptl4 were found to be lower than in healthy subjects with or without obesity (Xu et al. 2005). No correlation seems to exist between plasma *ANGPTL4* levels and body mass index (Mandard et al. 2004; Xu et al. 2005). In a very recent study, plasma *ANGPTL4* levels were negatively correlated with HDL-C and positively correlated with plasma glucose and TG. No differences in plasma *ANGPTL4* were found between healthy subjects and diabetic patients (Stejskal et al. 2008).

In support of a role of *ANGPTL4* in regulation of lipoprotein metabolism in human, carriers of a rare sequence variant of *ANGPTL4* (E40K) were shown to have decreased plasma TG and elevated plasma HDL-C levels (Romeo et al. 2007). In contrast, no association was found between four common genetic variants within the *ANGPTL4* gene and anthropometric data, family history of diabetes, plasma triglyceride and FFA levels, insulin sensitivity, or insulin secretion (Staiger et al. 2008). Also, in genome-wide association studies, no sequence variants in loci near the *ANGPTL4* gene were associated with plasma lipid parameters, including TG. These data suggest that variations within the *ANGPTL4* gene may only minimally account for inter-individual variations in plasma lipid levels.

## 9.3 Angptl3 and Lipid Metabolism

### 9.3.1 Discovery and Structure of Angptl3

Angptl3 was discovered while searching EST databases for signal sequences and amphipathic helices (Conklin et al. 1999). Mouse *Angptl3* contains 455 amino acids and is encoded by 7 exons on mouse chromosome 4, spanning 7–8 kb. The

human *ANGPTL3* gene is located on chromosome 1 and encodes a protein that is slightly larger (460 amino acids). Angptl3 is structurally highly homologous to Angptl4 and shares the modular structure consisting of a signal sequence, a small unique region, a coiled-coil domain and a large fibrinogen/angiopoietin-like domain. Also, similar to Angptl4, it contains several conserved cysteine residues involved in intermolecular disulfide bonding to form multimeric structures and is proteolytically processed at R221 and/or R224 (Ono et al. 2003).

### 9.3.2 Regulation of Angptl3 Expression

*Angptl3* is expressed almost exclusively in liver. While Angptl4 is under transcriptional control of PPARs, *Angptl3* gene expression is governed by liver X receptor (LXR), a nuclear hormone receptor involved in the regulation of hepatic TG and cholesterol metabolism. Activation of LXR using the synthetic agonist T0901317 or the endogenous agonist 22(*R*)-hydroxycholesterol markedly increases Angptl3 mRNA in mouse liver (Inaba et al. 2003; Ge et al. 2005), human HepG2 cells (Kaplan et al. 2003), rat FAO cells (Ge et al. 2005), and rat primary hepatocytes (Fig. 9.1). In mouse liver, *Angptl3* expression is also upregulated by cholesterol feeding (Kaplan et al. 2003). A functional LXR response element is present in the promoter of the human *ANGPTL3* gene, establishing *ANGPTL3* as a direct target gene of LXR (Inaba et al. 2003; Kaplan et al. 2003). Expression of *Angptl3* is suppressed by thyroid hormone, which is mediated by the thyroid hormone receptor beta. Down-regulation of *Angptl3* is achieved by TR $\beta$ -dependent interference with the HNF1 $\alpha$  signaling pathway (Fugier et al. 2006). *Angptl3* expression is also suppressed by PPAR $\beta/\delta$ , which likely competes with LXR for the common heterodimeric partner RXR $\alpha$  (Matsusue et al. 2006). Although the nuclear receptor FXR is highly expressed in liver and plays a major role in hepatic lipid metabolism, no effect of FXR activation on *Angptl3* expression is observed in either primary rat hepatocytes or rat FAO hepatoma cells (Fig. 9.1). Finally, *Angptl3* gene expression and plasma protein levels are increased in insulin-deficient streptozotocin-treated mice, in leptin-resistant C57BL/6J(db/db) mice, and in leptin-deficient C57BL/6J(ob/ob) mice, indicating that insulin and leptin inhibit *Angptl3* expression in hepatocytes (Inukai et al. 2004; Shimamura et al. 2004).

### 9.3.3 Role of Angptl3 in Lipid Metabolism

The connection between Angptl3 and lipoprotein metabolism was first made by the finding that KK/San mice have a 4-bp insertion in exon 6 of the *Angptl3* gene, thereby explaining their markedly reduced plasma FFA, cholesterol and TG levels

(Koishi et al. 2002). Conversely, adenoviral-mediated over-expression and intravenous injection of recombinant Angptl3 leads to increased plasma TGs, FFAs, and cholesterol levels (Koishi et al. 2002; Shimizugawa et al. 2002). Similar to Angptl4, the dramatic effect of Angptl3 on fasting plasma TG levels can be ascribed to suppression of VLDL-TG clearance via inhibition of the activity of LPL, rather than stimulation of hepatic VLDL production, which is only minimally affected (Ando et al. 2003; Shimizugawa et al. 2002). Targeted deletion of the *Angptl3* gene gives rise to a similar phenotype as observed in the KK/San mice: markedly decreased plasma TG levels associated with elevated heparin-releasable LPL activity (Fujimoto et al. 2006; Koster et al. 2005). A N-terminal portion of the protein corresponding to amino acids (AA) 17–165 is sufficient to elicit the hypertriglyceridemic effect, while deletion mutants of Angptl3 containing AA 207–460 or AA 79–207 are ineffective. The former region is present in the N-terminal fragment that is produced upon proteolysis of Angptl3. Mutating the putative cleavage sites reduces the potency of Angptl3 towards elevating plasma TG, suggesting that regulated proteolysis of Angptl3 may be an important mechanism to govern its activity and thus the activity of LPL (Ono et al. 2003). The plasma TG-raising effect of Angptl3 is supposedly mediated by a putative heparin-binding domain present in the N-terminal region (Ono et al. 2003).

Besides plasma TG, plasma HDL-cholesterol and HDL-phospholipids are also decreased in *Angptl3* knock-out mice. These alterations are likely explained by an inhibitory effect of Angptl3 on endothelial lipase, as demonstrated by in vitro and in vivo studies (Shimamura et al. 2007). Compared to LPL and HL, EL has a higher specificity towards hydrolysing phospholipids in HDL. Recently, Angptl3 was suggested to mediate the effect of proprotein convertases on EL activity and consequently plasma HDL levels (Jin et al. 2007). According to the mechanism proposed, proprotein convertases cleave Angptl3 to release a N-terminal fragment. This fragment inhibits EL, resulting in increased plasma HDL levels. As Angptl3 is particularly active toward LPL, this mechanism would be expected to impact plasma TG as well, although no data are currently available.

There is some ambiguity regarding the effects of Angptl3 on HL activity. In vitro studies suggest that Angptl3 is only marginally active towards HL (Shimizugawa et al. 2002). In contrast, in mice Angptl3 deletion was associated with a significant increase in post-heparin HL activity (Fujimoto et al. 2006). Similar observations were made in *Angptl3* knock-out mice on an *ApoE* knock-out background (Ando et al. 2003).

Similar to Angptl4, recombinant Angptl3 or adenovirus-mediated over-expression of Angptl3 causes elevated plasma FFA levels (Koishi et al. 2002), while Angptl3 inactivation leads to decreased plasma FFA and glycerol levels (Fujimoto et al. 2006; Shimamura et al. 2003). In line with these data, recombinant Angptl3 stimulates release of FFA and glycerol from differentiated 3T3-L1 adipocytes, probably via direct interaction with adipocytes. The data provide compelling evidence that Angptl3 stimulates adipose tissue lipolysis. At the present time, the mechanism behind this effect remains unclear.

### 9.3.4 Role of *Angptl3* in Human

Recent genome-wide association studies have shown that a common sequence variant at a locus near the *ANGPTL3* gene is associated with plasma concentration of TG, but not other lipoprotein parameters (Kathiresan et al. 2008; Willer et al. 2008). These data suggest that genetic variation within the *ANGPTL3* gene contributes to genetic variation in plasma TG levels within specific populations; and they validate the importance of *ANGPTL3* in the regulation of lipoprotein metabolism in humans.

Information on plasma *ANGPTL3* levels in humans and possible correlation with biochemical, metabolic, or clinical parameters is limited. In a recent report, plasma *ANGPTL3* levels in healthy individuals averaged 225 ng/ml (Stejskal et al. 2007). A significant correlation was found between plasma *ANGPTL3* and systolic blood pressure, plasma LDL, and plasma A-FABP. In contrast, no correlation was found between *ANGPTL3* and plasma TG. Remarkably, the plasma *ANGPTL3* concentration was about 3-fold higher in another study. In that study, a significant positive correlation was found between plasma *ANGPTL3* and carotic artery intima-media thickness in healthy human subjects (Hatsuda et al. 2007).

## 9.5 Conclusion

Overall, the effects of *Angptl3* and *Angptl4* on plasma lipoprotein metabolism are highly similar, which indicates a common mechanism of action. Evidence abounds that *Angptl4* and *Angptl3* raise plasma TG levels in mice by suppressing clearance of plasma VLDL and chylomicrons via inhibition of LPL and HL activity. In addition, *Angptl3* inhibits endothelial lipase. While these effects are very well supported, the overall role of *Angptl4* and *Angptl3* in the physiological regulation of fuel selection as well as its impact on cellular metabolism are poorly understood. Furthermore, the physiological determinants of plasma *ANGPTL4* and *ANGPTL3* levels in humans remain to be identified. Finally, the impact of genetic variants of the human *ANGPTL4* and *ANGPTL3* gene on plasma lipoprotein levels requires further investigation.

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# Chapter 10

## Thyroid Hormones and Lipid Metabolism: Thyromimetics as Anti-Atherosclerotic Agents?

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B.F. and A.W. contributed mainly to Sect. 10.1 (Thyroid hormones, thyroid hormone-receptors and lipoprotein metabolism): J.R.P., I.T. and A.R. contributed mainly to Sect. 10.2 (Thyromimetics).

**Abstract** Overt hypothyroidism most often causes moderately elevated cholesterol and triglycerides; but, in genetically predisposed individuals, it can also trigger frank hyperlipidemia, e.g. type III hyperlipoproteinemia. In contrast, in subclinical hypothyroidism,  $T_4$  lowers low-density lipoprotein cholesterol (LDL-C) by only 3.5–6.0 mg/dl LDL-C for each 1 mU/l thyroid-stimulating hormone (TSH) increase from baseline, a finding barely perceptible in individual patients. In humans,  $T_3$  decreases apoB-containing IDL, LDL and chylomicron remnant particles by up-regulation of LDL receptors and despite a concomitant increase in HMGCoA reductase in the liver. In rodents,  $T_3$  also up-regulates CYP7A1, ABCG5 and ABCG8 which interact to increase bile acid synthesis and biliary elimination of cholesterol. Thyromimetics, selective for either thyroid hormone-receptor  $\beta$  or liver, lower both LDL-C in humans and HDL-C in mice, thereby promoting reverse cholesterol transport. Interestingly, the thyromimetic T-0681 protects cholesterol-fed rabbits from atherosclerosis. If safety concerns with  $TR\beta$ -selective thyromimetics can be addressed (i.e. the observed pituitary feed-back inhibition leading to low TSH levels), this class of drugs may complement statins in tackling the burden of atherosclerosis in humans.

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## 10.1 Thyroid Hormones, Thyroid Hormone-Receptors and Lipoprotein Metabolism

### 10.1.1 Thyroid Hormone Signalling

Thyroid hormones (THs) play a key role in the regulation of development, growth, and metabolism of practically every cell and organ (for a review, see Oetting and Yen 2007). Two THs, thyroxine ( $T_4$ ) and triiodothyronine ( $T_3$ ), are synthesized by the thyroid gland, under the control of the hypothalamic-pituitary axis; and  $T_4$  is the major secreted hormone, leading to a molar ratio of serum  $T_4:T_3$  of  $\approx 40$ . However, several naturally occurring TH metabolites (e.g. Triac, Tetrac) may retain some biological activity.  $T_4$  binds to circulating TH-binding globuline (TBG), the main transporter in blood, and to prealbumin, albumin and also to lipoproteins, mainly high-density lipoproteins (HDL). In order to produce biological effects, sensitivity of the peripheral effector tissues is modulated at several levels. First, TH transporters located in the plasma membrane serve to control cellular uptake of TH; in the same vein, selective access to the nucleus mediated by the nuclear membrane has been postulated (Ichikawa et al. 2000). Binding of  $T_4$  to low-density lipoprotein (LDL) generates LDL- $T_4$  complexes which are taken up by the LDL receptor (LDL-R), thereby affording  $T_4$  an alternative mode of entry into cells (Benvenega and Robbins 1990). Second,  $T_3$  (10-fold more potent of the two TH regarding TR-binding) is formed by 5' deiodination of the outer ring of  $T_4$  by deiodinases. Type I deiodinase is present in peripheral tissues, e.g. liver, to convert circulating  $T_4$ ; whereas type II deiodinase is present in pituitary gland and brain and contributes to both peripheral and intracellular conversion of  $T_4$ . Type III deiodinase (present mainly in brain) together with type I deiodinase serves to inactivate  $T_4$  to *reverse*  $T_3$ , an enantiomer lacking relevant bioactivity. Third, and most importantly, signal transduction of  $T_3$  is brought about by binding to thyroid hormone receptors (TRs), which act as ligand-regulatable transcription factors (Lazar 1993). TRs bind to DNA as monomers, homodimers, or heterodimers with other receptors from the steroid hormone receptor family, mainly with retinoic-X receptor- $\alpha$  (RXR $\alpha$ ). Thus,  $T_3$  binding to TR activates transcription of positively regulated target genes containing a TH response element (TRE), e.g. type I deiodinase. In humans, two genes (TR $\alpha$ , TR $\beta$ ) are expressed in almost all tissues and encode several major TR isoforms which are formed as the result of alternative splicing: TR $\alpha$ 1, TR $\alpha$ 2, TR $\beta$ 1, TR $\beta$ 2. TRs are  $\approx 500$  amino acids long and contain a central DNA-binding domain, a C-terminal ligand-binding domain and a heterodimerization sequence with RXR, as well as interaction sites with co-activators and co-repressors. TR $\alpha$ 1, TR $\beta$ 1 and TR $\beta$ 2 bind  $T_3$  with similar affinity and display a comparable transcriptional activity. TR $\alpha$ 2 does not bind  $T_3$  and may act as a dominant inhibitor of TH action on some target genes. Major progress has been made in the past decade understanding the biological consequences of TR diversity. Expression of TR $\alpha$  and TR $\beta$  varies substantially between different tissues. In the heart, TR $\alpha$  makes up 75% of TR, whereas in the liver TR $\beta$  comprises 80% of TRs. However, genetic recombination studies in mice

with targeted TR mutations have questioned in much greater detail whether TR $\alpha$  or TR $\beta$  serve redundant or specific functions. TR $\alpha$ 1-/- mice show a normal anatomy and reproduction, however, they show decreased heart rate due to altered cardiac ion channel function, an effect which cannot be corrected by TR $\beta$  (Johansson et al. 1998; Gloss et al. 2001). In this context, it is very important to realize that a TR unoccupied by T<sub>3</sub> frequently acts as a dominant repressor of gene function, thus explaining why genetic ablation of a TR often displays a relatively mild phenotype compared to hypothyroidism. For example, mice with a total genetic ablation of all functional TR (TR $\alpha$ 1-/- TR $\beta$ -/- mice) are viable, but display defects in pituitary-thyroid axis, growth and bone maturation (Göthe et al. 1999). To more fully recapitulate repression by the unliganded receptor, TR $\alpha$  knock-in mice were produced, with various defects in ligand binding. Dominant negative heterozygous TR $\alpha$ 1-PV mice are dwarfs and show reduced adipose tissue (Ying et al. 2007); TR $\alpha$ 1P398H show obesity combined with impaired catecholamine-stimulated lipolysis (Liu et al. 2003); and TR $\alpha$ R384C/TR $\beta$ -/- show central hypermetabolism, leading to resistance to diet-induced obesity, probably overriding direct effects of the mutated TR $\alpha$  in adipose (Sjögren et al. 2007). Synthesizing these intriguing data, TR $\alpha$  is crucial for heart rate, cardiac contractility, cardiac relaxation and adipogenesis in the mouse model. Human mutations in TR $\alpha$  have not been reported up to now. In contrast, more than 300 families with the syndrome of resistance to thyroid hormone have been found which are due to mutations in the TR $\beta$  gene disturbing T<sub>3</sub> binding. Studies of genetic ablation of TR $\beta$ , studies of knock-in mice for TR $\beta$  and experiments with TR $\beta$ -selective analogs have conclusively indicated that metabolic effects of TH on cholesterol and lipoprotein metabolism are predominantly mediated by TR $\beta$ . Many of the target genes are directly transcriptionally regulated by TRs, whereas others are indirectly transcriptionally regulated through intermediary genes, e.g. transcription factors. TRs can also regulate transcription through protein-protein interactions with other transcription-factors, alter mRNA levels by changing mRNA stability and directly induce rapid effects by engaging other cellular proteins, e.g. PI3-kinase (Storey et al. 2006).

### ***10.1.2 Thyroid Function and Lipoprotein Metabolism***

#### **10.1.2.1 Overt Hypothyroidism and Lipoproteins**

Overt hypothyroidism (OH) is both a relatively frequent condition (0.3% of the population) and a major cause of atherosclerotic disease, as evidenced both by current clinical studies (Nyirenda et al. 2005) and by autopsy studies performed already in the 1930s (for a review, see Cappola and Ladenson 2003). OH, due to primary thyroid disease, is defined as elevated TSH levels in the presence of decreased free T<sub>4</sub> and/or free T<sub>3</sub> values. Mechanistically, atherosclerosis this is caused by a combination of at least four factors: dyslipidemia (the topic of this Section), adverse changes in “novel” cardiovascular risk factors, increased peripheral

vascular resistance with ensuing diastolic hypertension and direct adverse effects of  $T_3$  deficiency on heart and vasculature, leading e.g. to diastolic dysfunction (Klein and Ojamaa 2001). Overt hypothyroidism is probably the most important cause of secondary hyperlipidemia and has to be ruled out in any patient evaluated at the outpatient lipid clinic (reviewed by Duntas 2002; Pearce 2004). Depending on the variation in lipid transport genes in individual patients, OH can present with either a moderately disturbed lipoprotein profile or, more rarely, with a frank hyperlipidemia of (respectively) the lipoprotein pattern type IIa, type IIb, type III, type IV or type V, according to Fredrickson. In other words, total cholesterol (TC) and/or triglycerides (TG) most often are moderately elevated, but can also be normal or dramatically increased. High-density lipoprotein cholesterol (HDL-C) often is moderately elevated, but can also be normal or low. Thus, the effect of OH on lipoproteins can not only induce atherosclerosis, but also the chylomicronemia syndrome, a life-threatening disorder with excessive TG-accumulation which may cause acute pancreatitis and/or multiple organ failure. All the above abnormalities are at least partly reversible with  $T_4$  replacement, which in the setting of severe OH has a pronounced hypocholesterolemic effect of  $\approx 100$  mg/dl, similar in magnitude to standard doses of a statin. Although the reduction in LDL-C induced by  $T_4$  in OH is quite variable, currently there are no genetic markers to predict this change (Wiseman et al. 1993; Diekman et al. 2000).

It is critical to initially treat the hyperlipidemia of OH with  $T_4$  rather than statins not only because direct  $T_3$  deficiency in numerous organs needs to be replaced, but also because OH leads to both myopathy and reduced statin clearance in the liver, two factors explaining the increased risk of rhabdomyolysis during statin treatment in the presence of OH. Usually, 4–6 weeks of maintenance therapy with an optimal dose of  $T_4$  are necessary to fully correct the lipoprotein disorder due to OH. Combined treatment with  $T_3$  and  $T_4$  offers no clear advantages and is not advisable. It should be kept in mind, however, that  $T_4$  replacement in OH also increases exercise, decreases food intake and, thus, induces weight loss of several kilograms, factors which are rarely controlled in clinical studies, but clearly have the potential to improve plasma lipids.

#### 10.1.2.1.1 ApoB-Containing Lipoproteins in Overt Hypothyroidism

##### *10.1.2.1.1.1 Lipids of apoB-Containing Lipoproteins in Overt Hypothyroidism*

The typical lipid profile in pronounced OH [thyroid-stimulating hormone (TSH)  $>50$  mU/l] is moderately disturbed: TC is clearly increased (ca. +35%), mainly due to increases in LDL-C (ca. +40%), intermediate-density lipoprotein cholesterol (IDL-C; ca. +100%) and HDL-C (ca. +25%), whereas VLDL-C is unchanged. Thus, in absolute terms, LDL-C is increased most (+80 mg/dl), followed by IDL-C (+10 mg/dl) and HDL<sub>2</sub>-C (+10 mg/dl; Packard et al. 1993). However, normal or low HDL-C levels have also been reported (Agdeppa et al. 1979). Fasting TG tends to increase or shows moderate increases in OH (+20%). The postprandial TG response

to a fatty meal, i.e. postprandial lipemia, is increased even in normotriglyceridemic OH (Weintraub et al. 1999). Interestingly, VLDL-TG, the major TG-containing lipoprotein in fasting plasma, is unchanged; and increases in TG in OH pertain mainly to LDL-TG and HDL-TG, suggesting increased core lipid exchange despite low CETP activity (Packard et al. 1993; Tan et al. 1998a, b). In line with these findings, the total mass (= lipid core + lipid surface components + apoproteins) of large VLDL<sub>1</sub>, smaller VLDL<sub>2</sub> and HDL<sub>3</sub> are normal in OH, whereas IDL-, LDL- and HDL<sub>2</sub> mass are increased (Packard et al. 1993). Recently, NMR spectroscopy data complemented our understanding of lipid changes in OH (Pearce et al. 2008). The content of large VLDL particles was normal, but medium and small VLDL as well as IDL particles were increased two-fold. In line with these observations, remnant-like particles in fasting plasma, as defined by apolipoprotein composition (presumably overlapping with IDL), were increased in OH by 35% (Ito et al. 2007). Postprandial chylomicron remnant clearance, as measured by retinyl palmitate labelling, was delayed in OH (Weintraub et al. 1999). Importantly, only large LDL particles were increased by ≈100%, whereas medium and small LDL were normal (Pearce et al. 2008). As small LDL are considered to be especially atherogenic due to their propensity to be oxidized and to filter into the vessel wall, this may indicate that the increase in LDL-C in OH is confined to less dangerous LDL particle subsets (Pearce et al. 2008). It is not surprising that oxidized LDL is increased too in OH (Duntas et al. 2002). This can be explained both by an increased half-time of LDL in the circulation in OH and, alternatively, by the decrease in circulating LDL-bound T<sub>4</sub> itself, which may directly protect LDL from oxidation (Hanna et al. 1993).

#### *10.1.2.1.1.2 Apolipoproteins of apoB-Containing Lipoproteins in Overt Hypothyroidism*

Each VLDL-, IDL- and LDL-particle contains one molecule of apoB. **apoB** is consistently increased in OH, indicating an increased number of potentially pro-atherogenic particles (Tan et al. 1998a, b). **apoE** is also increased in OH, presumably as a component of smaller VLDL and IDL particles (Muls et al. 1985). apoB metabolism has been examined in some detail in OH (Packard et al. 1993). As expected from the lipid data above, VLDL-apoB is unchanged in OH, whereas IDL-apoB and LDL-apoB are increased. While VLDL-apoB kinetics were unchanged, the increased FCR of IDL-apoB was explained by an increased conversion of IDL-apoB to LDL-apoB, whereas direct liver uptake of IDL-apoB was unaltered. During OH, LDL-apoB removal was substantially delayed, explaining its increased plasma levels (Thompson et al. 1981), presumably due to down-regulation of LDL receptors.

**Lipoprotein(a)**, Lp(a), is an LDL particle in which apoB is attached to a large plasminogen-like protein called apolipoprotein(a). Lp(a) values >30 mg/dl are strongly genetically determined, are widely regarded as a “novel” risk factor for atherosclerosis and respond poorly to treatment. Some reports suggest that Lp(a) decreases after T<sub>4</sub> replacement (de Bruin et al. 1993). However, currently, most papers report no change (for a review, see Duntas 2003).

### 10.1.2.1.2 HDL in Overt Hypothyroidism

#### 10.1.2.1.2.1 HDL Lipids in Overt Hypothyroidism

In OH, HDL-C has most often been found to be high (Muls et al. 1984, 1985; Kuusi et al. 1988; Tan et al. 1998a, b; Pearce et al. 2008), however some reports also note normal values (Verdugo et al. 1987) or decreases (Agdeppa et al. 1979; Lithell et al. 1981). HDL<sub>2</sub>-C is almost doubled compared to controls, whereas HDL<sub>3</sub>-C is somewhat decreased (Tan et al. 1998a, b). Isopycnic ultracentrifugation confirmed a modified distribution of HDL subclasses in OH, with an increase in the lighter (and thus larger) HDL<sub>2b</sub> particles ( $d = 1.063\text{--}1.100$  g/ml; Muls et al. 1985). This has also been corroborated by NMR studies, which find large HDL to be increased in OH, whereas medium and small HDL are significantly decreased (Pearce et al. 2008).

#### 10.1.2.1.2.2 HDL Apolipoproteins in Overt Hypothyroidism

In OH, apoA-I is increased, whereas apoA-II, the majority of which is associated with HDL<sub>3</sub>, is unchanged (Verdugo et al. 1987). Correspondingly, HDL particles containing only apoA-I (LpA-I) are increased, whereas LpA-I:A-II are not (Tan et al. 1998a, b). Taken together, apoA-I levels during T<sub>4</sub> replacement are probably determined both by changes in production and catabolism, the latter in particular being responsive to the amount of TG-lowering afforded by T<sub>4</sub>.

### 10.1.2.2 Subclinical Hypothyroidism and Lipoproteins

Subclinical hypothyroidism (SCH) is more than 10 times as prevalent as OH (4% vs 0.3% of the population; for a review, see Biondi and Cooper 2008). SCH is even more prevalent in women, the elderly and in populations with a high iodine intake. The etiology of SCH is similar to that of overt hypothyroidism. The main causes are autoimmune thyroiditis, other forms of thyroiditis, thyroid injury after thyroid resection, external radiotherapy, radioiodine-therapy or drugs impairing thyroid function, e.g. amiodarone, which act as a competitive antagonist of T<sub>3</sub> binding to TR $\beta$ , thereby not only explaining its antiarrhythmic property but also its ability to raise cholesterol (Bakker et al. 1994, 1998).

SCH is defined as the repeated measurement of increased TSH serum levels in the presence of normal free T<sub>4</sub> and free T<sub>3</sub> levels (Biondi and Cooper 2008). However, it is difficult to establish consensus as to what constitutes a normal TSH range because TSH levels in the population show no Gaussian distribution. The “upper tail” of TSH levels has been attributed either to occult autoimmunity against the thyroid or to the presence of TSH isoforms, e.g. due to variations in glycosylation. Thus, TSH cutoff values between 2.5 mU/l and 4.0 mU/l have been defined as the upper limit of normal. TSH values >2.5–4.0 mU/l in the presence of normal TH values correspond to mild SCH. TSH values >10 mU/l in the presence of normal TH values characterize a subgroup of patients with severe SCH (Biondi and Cooper 2008).

Whether SCH causes clinical symptoms of hypothyroidism (e.g. cold intolerance) is currently under debate. Of particular importance in the current context is the discussion whether or not SCH is risk factor for atherosclerosis and, if so, whether derangements in lipids are responsible. With regard to lipids, a large body of literature has examined mainly two questions (Biondi and Cooper 2008):

1. Do patients with SCH have increases in total and LDL-C?
2. Does replacement therapy with T<sub>4</sub> lower total and LDL-C in SCH patients?

#### 10.1.2.2.1 Subclinical Hypothyroidism and Lipoproteins in Cross-Sectional Studies

Several large population-based studies have hitherto been unable to unequivocally establish increases in LDL-C in SCH especially when controlling for demographic variables and BMI. Some studies were negative, some showed even lower TC in SCH and some found the expected increase in TC, LDL-C and, more rarely, TG. HDL tends to be lower in SCH.

For example, in the study by Kanaya et al. (2002), in 2799 adults a TSH >5.5 mU/l was associated with an increase in TC of 10 mg/dl. In the study by Bauer et al. (1998), corrected LDL-C was 12% higher and HDL-C was 12% higher in women with TSH >5.5 mU/l. In the fifth Tromso study, using a nested case-control design, TC and LDL-C were higher and apoA-I was significantly lower than in the controls. Taken together, it remains biologically plausible that SCH leads to small increases in LDL-C, especially if there is more profound TSH elevation (>10 mU/l), and if background TC and BMI levels in the population are elevated. Heterogeneity regarding etiology, as well as extent and duration of disease may partly explain the variable results. The fact that the extrapolated increases in TC (3.5 mg/dl in men and 6.2 mg/dl in women for every 1 mU/l elevation in TSH) are quite small may help to explain why the noise of epidemiological, biological and analytical variation in LDL-C may have made it so difficult to clearly establish this link. However, if the above increases in LDL-C are correct, they alone may correspond to increases in 18–30% of CHD risk even within the cohort of mild SCH.

#### 10.1.2.2.2 Lipoproteins in Subclinical Hypothyroidism after T<sub>4</sub> Replacement

A related, but actually quite different question is whether or not replacement of T<sub>4</sub> in SCH induces changes in lipoprotein metabolism. Even though the literature is also somewhat divided on this issue, the most probable answer is that T<sub>4</sub> reduces LDL-C (and with it TC) without changing HDL-C or Lp(a). Two meta-analyses summarize the individual studies. Tanis et al. (1996) analyzed 13 studies from 1976 to 1995 comprising 278 patients and found that T<sub>4</sub> decreased TC by 15 mg/dl. Danese et al. (2000) analyzed 13 studies from January 1966 to May 1999 comprising 247 patients and found that T<sub>4</sub> decreased TC by 8 mg/dl and LDL-C by 10 mg/dl

with no changes in TG or HDL-C. Recently, three major RCTs resolved to some degree the above issue (Caraccio et al. 2002; Monzani et al. 2004; Razvi et al. 2007). LDL-C was reduced by  $-27.4\%$ ,  $-30.0\%$  and  $-7.3\%$ , without changes in HDL-C or Lp(a). Expectedly, apoB is also decreased by  $\approx 16\%$  (Arem and Patsch 1990). Importantly, one study also demonstrated that  $T_4$  replacement reduced intima-media thickness by 10% after 6 months and that this decrease was directly related to improvements in lipids (Monzani et al. 2004). Taken together, there is substantial evidence for clinically relevant decreases in LDL-C resulting from  $T_4$  replacement in SCH, a finding which may be especially relevant in SCH with TSH  $>10$  mU/l, hypercholesterolemia and/or atherosclerotic vascular disease.

### 10.1.2.3 Overt Hyperthyroidism and Lipoproteins

In overt hyperthyroidism (OHyper), TC is decreased due to both decreases in LDL-C (by  $\approx 50\%$ ) and HDL-C (by  $\approx 35\%$ ); apoB and apoA-I are decreased in parallel (Tan et al. 1998a, b). Regarding HDL, mainly HDL<sub>2</sub> and LpA-I are decreased, whereas HDL<sub>3</sub> and LpA-I/A-II remain normal (Tan et al. 1998a, b). In OHyper, TG have been reported to be low (Abrams and Grundy 1981), normal (Tan et al. 1998a, b), or even high (Cachefo et al. 2001). High TG have been explained by increased TG hydrolysis in adipose, high FFA levels in plasma, an ensuing increase in hepatic lipogenesis (Cachefo et al. 2001), presumably leading to increased VLDL secretion.

### 10.1.2.4 Subclinical Hyperthyroidism and Lipoproteins

Subclinical hyperthyroidism (SCHyper) is a prevalent disorder affecting 0.7–3.2% of the population. Up to 10–30% of patients receiving replacement doses of  $T_4$  for OH have exogenous SCHyper. Replacement doses of  $T_4$  leading to suppressed TSH, i.e. SCHyper, lower LDL-C more potently than doses resulting in normal TSH (Franklyn et al. 1993). Endogenous SCHyper significantly decreases TC by 12%, due to decreases of both LDL-C and HDL-C (both NS; Parle et al. 1992).

### 10.1.2.5 Thyroid Dysfunction in Rodents

Most of the animal data on thyroid dysfunction are derived from rodents. However, lipoprotein metabolism in rodents differs from humans in a number of aspects. In rodents on chow, cholesterol is mainly transported with HDL, with very little VLDL-C and LDL-C. Furthermore, rodents have extensive apoB mRNA-editing in the liver (Davidson 1988), monomeric apoA-II in plasma, hepatic lipase that circulates in plasma rather than binding to glucosaminoglycans in the liver; and, finally, they lack CETP. These changes contribute to make rodents notoriously resistant to atherosclerosis. Thus, extrapolation of lipoprotein changes in rodents with thyroid dysfunction to humans should be done with caution.

### 10.1.2.5.1 Hypothyroidism in Rodents

Hypothyroidism in **rats** increases TC, especially on a cholesterol-rich diet, due to the accumulation of apoE-rich large HDL,  $\beta$ -VLDL and LDL particles. In contrast, TG in hypothyroid rats are normal (Staels et al. 1990) or even decreased (Apostopoulos et al. 1990; Salter et al. 1991). In hypothyroid rats, **apoA-I** levels are unchanged, and apoB and apoE levels in plasma are moderately increased. Normal apoA-I levels result from a combination of normal apoA-I mRNA (Staels et al. 1990), a strong decrease in hepatic apoA-I secretion due to post-transcriptional effects of hypothyroidism (Wilcox et al. 1991) and, in turn, a reduced clearance of radiolabeled HDL proteins (Gross et al. 1987). Increased **apoB** levels in plasma are readily explained by increases in apoB mRNA in liver and intestine (Staels et al. 1990) and, also, by strongly decreased LDL-R mRNA, leading to a decreased FCR of LDL-apoB in hypothyroid rats (Gross et al. 1987). Hepatic **apoE** mRNA is unchanged; however, hepatic apoE synthesis is increased due to post-transcriptional effects (Davidson et al. 1988) which may, together with decreased HL and a strongly decreased LDL-R, explain the increased plasma levels of apoE in hypothyroid rats. Presumably, apoE in this model is associated primarily with IDL and large apoE-rich HDL.

In euthyroid **mice** fed normal chow or a cholesterol-rich diet, gene targeting of either TR $\beta$  or TR $\alpha$  does not substantially change serum cholesterol (Gullberg et al. 2000). No good data on apolipoproteins in TH-resistant mice are available.

### 10.1.2.5.2 Hyperthyroidism in Rodents

In **rats**, short-term (6–20 days) hyperthyroidism induced by T<sub>3</sub> injections does not change plasma TC levels. TG range from normal (Staels et al. 1990) to increases by 10-fold (Apostopoulos et al. 1990), presumably dependent on the degree of hyperthyroidism.

In euthyroid **mice** on chow, 14 days of T<sub>3</sub> injection resulting in severe hyperthyroidism reduces TC by 40%, whereas TG are unchanged (Tancevski et al. 2008). Reductions in TC are almost exclusively due to decreased HDL-C, with little apparent changes in VLDL and LDL. Metabolic studies show that HDL-cholesteryl ester clearance is unchanged, however, indicating that low HDL-C in hyperthyroid mice reflects decreased HDL-cholesteryl ester synthesis. In line with changes in HDL, serum apoA-I levels decrease by 50% (Tancevski et al. 2008).

Regulation of **apoA-I** by TH in rodents has been examined in some detail (Apostopoulos et al. 1990; Staels et al. 1990; Wilcox et al. 1991; Strobl et al. 1992; Lin-Lee et al. 1993, 1995; Soyak et al. 1995). Short-term treatment of euthyroid rats with T<sub>3</sub> increases transcription of apoA-I and apoA-II; however, the stimulation of apoA-I persists longer (Strobl et al. 1992). Chronic hyperthyroidism in rats increases the plasma levels of apoA-I by 2 to 3-fold, due to increased synthesis and secretion of apoA-I protein, as shown in perfused rat livers (Apostopolulos et al. 1990; Wilcox et al. 1991). Increased apoA-I is due to increases in apoA-I mRNA in the liver but not the intestine, which occur despite decreased transcription of apoA-I in chronic

hyperthyroidism, indicating a post-transcriptional mechanism stabilizing apoA-I mRNA (Apostopoulos et al. 1990; Staels et al. 1990; Strobl et al. 1992).

Further analysis of the mechanism underlying decreased transcription of apoA-I in hyperthyroidism revealed that transcription initiation was similar to control rats, but that  $T_3$ -treated rats had a much higher rate of transcriptional arrest, explaining decreased overall transcription rates (Lin-Lee et al. 1995). Contradictory data have been reported regarding TM and apoA-I. In euthyroid rats, CGS 23425 afforded substantial increases in serum apoA-I (Taylor et al. 1997). In euthyroid mice on chow, in contrast, T-0681 decreased apoA-I (Tancevski et al. 2008).

Interestingly, **apoA-II**, the evolutionally related gene coding for the other major HDL structural protein, is clearly regulated differentially from apoA-I. In euthyroid rats,  $T_3$  acutely increases apoA-II transcription (Strobl et al. 1992). In chronic hyperthyroidism, however, both apoA-II transcription and apoA-II mRNA levels decrease substantially (Staels et al. 1990; Strobl et al. 1992). Whether plasma levels of apoA-II are changed is unknown.

Acutely,  $T_3$  increases **apoA-IV** transcription in euthyroid rat liver (Lin-Lee et al. 1993). Chronic hyperthyroidism decreases apoA-IV transcription by 55%, but increases apoA-IV mRNA 2.8-fold, respectively, indicating stabilization of apoA-IV mRNA in the cytoplasm which mechanistically differs from  $T_3$  effects on apoA-I mRNA (Lin-Lee et al. 1993). Plasma levels of apoA-IV appear to be unchanged (Apostopoulos et al. 1990).

Short-term administration of  $T_3$  increases **apoC-III** transcription in euthyroid rat liver (Lin-Lee et al. 1993). Chronic hyperthyroidism decreases apoC-III transcription by 72%, but decreases in apoC-III mRNA were less pronounced presumably due to more efficient maturation of apoC-III mRNA (Lin-Lee et al. 1993).

Hyperthyroidism does not change **apoB** and **apoE** mRNA (Staels et al. 1990), but increases transcription of LDL-R, which probably explains the substantial decrease in apoB and apoE plasma levels. However,  $T_3$  enhances apoB mRNA-editing in rat liver, leading respectively to more hepatic production of apoB48 and less of apoB100 (Davidson et al. 1988).

Taken together, both transcriptional and post-transcriptional mechanisms make hepatic apolipoprotein synthesis (apoA-I, apoA-II, apoA-IV, apoC-III, apo-B) exquisitely sensitive to altered thyroid status. However, extrapolation to the human situation has to be done with caution.

### 10.1.2.6 Mechanistic Insight from Humans and Rodents

#### 10.1.2.6.1 Factors Mainly Affecting apoB-Containing Lipoproteins

##### 10.1.2.6.1.1 Cholesterol-7 $\alpha$ Hydroxylase

Rodents, in contrast to man, are quite resistant to the development of hypercholesterolemia on a cholesterol-rich diet, at least in part because they are able to increase the formation and secretion of bile acids leading to increased removal of cholesterol

from the body (Björkhem et al. 1997). However, both rats and mice accumulate lipoproteins in plasma when made hypothyroid, thus becoming susceptible to atherosclerosis. Because cholesterol-7 $\alpha$ -hydroxylase (CYP7A1) is the rate-limiting enzyme for the conversion of cholesterol to bile acids in the liver via the “classical pathway”, its regulation by TH has been thoroughly examined in rodents and man. Transcriptional, post-transcriptional and post-translational regulation of CYP7A1 activity has been documented (Pandak et al. 1997; Drover and Agellon 2004; Shin and Osborne 2006). However, transcriptional regulation appears to be the most important mechanism.

Early studies in hypophysectomized **rats** indicated that hepatic CYP7A1 mRNA and –activity respond rapidly to very low doses of T<sub>3</sub> reaching a level of 8-fold greater than control within 6 h (Ness et al. 1990). In these rats stimulation of biliary cholesterol secretion due to increased CYP7A1 preceded an increase in HMG-CoA reductase by 12 h, establishing that this effect is independent of the stimulation of cholesterol synthesis by TH (Day et al. 1989). T<sub>3</sub> induces CYP7A1 in hypophysectomized rats at the transcriptional level (Ness et al. 1990; Pandak et al. 1997).

In hypothyroid wild-type **mice**, T<sub>3</sub> substitution reduces cholesterol both on chow and on a cholesterol-rich diet, whereas no such effect of T<sub>3</sub> is seen in TR $\beta$ –/– mice, indicating that increased CYP7A1 transcription is unique to TR $\beta$  (Gullberg et al. 2000). Overexpression of TR $\alpha$ 1 in the liver cannot overcome this defect (Gullberg et al. 2002). Paradoxically, however, T<sub>3</sub>-deficient TR $\beta$ –/– mice show **augmented** CYP7A1 mRNA on a cholesterol-rich diet when compared to the wild type, making them **resistant** to dietary hypercholesterolemia despite overt hypothyroidism (Gullberg et al. 2000). This situation can presumably be explained by two mechanisms:

1. Unliganded TR $\beta$  potently suppresses CYP7A1 in hypothyroid wild-type mice, whereas in –/– mice this inhibition is relieved due to the absence of TR $\beta$ .
2. LXR $\alpha$ , an oxysterol-binding transcription factor directly activating the rodent CYP7A1 promoter is no longer competed by TR $\beta$  and gains the upper hand (Gullberg et al. 2000).

Regulation of CYP7A1 has also been analyzed in a model of human resistance to TH (Hashimoto et al. 2006a, b). On chow, CYP7A1 mRNA was undetectable in the livers of wild-type and mutant mice. T<sub>3</sub> substantially induced CYP7A1 in the wild type but, as expected with a ligand-binding defective TR $\beta$ , only marginally (<30%) in mutant mice. Paradoxically, on a high cholesterol diet, CYP7A1 mRNA rose to 3-fold the wild-type levels in mutant mice, presumably due to a lack of competition of the mutant TR $\beta$  with LXR-mediated promoter stimulation in this particular situation. Thus, cross-talk between TR $\beta$  and LXR $\alpha$  in the regulation of CYP7A1 during a hypercholesterolemic diet has also been reported in a TR $\beta$  knock-in mouse model (Hashimoto et al. 2006a, b). CYP7A1 gene transcription is inhibited by FXR by increasing the expression of small heterodimer partner (SHP), a non-DNA binding protein (Davis et al. 2002). Thus, in rodents, CYP7A1 gene transcription is, respectively, inhibited by FXR and unliganded TR $\beta$  and stimulated by liganded TR $\beta$  and liganded LXR $\alpha$ .

It has to be emphasized, however, that regulation of CYP7A1 regarding both TR and LXR is highly species-specific. Investigating the **human** CYP7A1 gene in cell culture, Drover et al. (2002) found that both TR $\alpha$  and TR $\beta$  bind to two regions in the proximal promoter. Upon addition of T<sub>3</sub> to cells, human CYP7A1 promoter activity is decreased by  $\approx 50\%$  (Drover et al. 2002). The study of CYP7A1 regulation by T<sub>3</sub> in humans in vivo has been hampered by the invasive nature of obtaining repeated liver samples. To overcome this limitation, transgenic mice were generated which expressed the human CYP7A1 gene under the control of its natural flanking sequences, but not the mouse CYP7A1 gene (Drover and Agellon 2004). Hypothyroidism did not alter human CYP7A1 mRNA levels in transgenic mice. In hyperthyroid male mice, expectedly, human CYP7A1 mRNA decreased; however, in hyperthyroid female mice human CYP7A1 mRNA remained unchanged (Drover and Agellon 2004). Thus, T<sub>3</sub> represses human CYP7A1 transcription in transgenic mice in a gender-dependent manner. Interestingly, however, despite lower CYP7A1 mRNA levels, CYP7A1 activity in liver microsomes was increased in hyperthyroid male transgenic mice, but not in female mice  $\approx 2$ -fold. This finding suggests a major role of post-transcriptional mechanisms in the regulation of human CYP7A1 activity.

Regulation of CYP7A1 by LXR $\alpha$  also differs substantially between species. Whereas the rat/mouse CYP7A1 promoter contains LXR-binding sites that increase transcription in response to the generation of oxysterols induced by a cholesterol-rich diet, functionally active LXR-sites are absent in the human CYP7A1 (Chen et al. 2002; Menke et al. 2002). This, in part, explains why rodents are resistant and humans susceptible to diet-induced hypercholesterolemia.

Different thyromimetics (TM) stimulate bile acid synthesis, as assessed by monitoring serum levels of C4, an intermediary in bile acid synthesis, both in euthyroid mice (Johansson et al. 2005) and in euthyroid humans (Berkenstam et al. 2008). These data are corroborated by findings with the liver-specific TM T-0681, which increases liver CYP7A1 mRNA levels in mice (Tancevski et al. 2008) and by reduction of SHP mRNA levels in mice by  $\approx 50\%$ , which would be expected to de/inhibit CYP7A1 expression (Davis et al. 2002). In humans with OHyper, cholic acid synthesis appears to be reduced (Pauletzki et al. 1989). Thus liver and TR $\beta$ -specific TM appear to stimulate bile acid synthesis both in rodents and in humans. However, robust data regarding the situation in humans are currently not available.

#### 10.1.2.6.1.2 LDL Receptor

In humans, elegant LDL turnover studies have demonstrated quite early that LDL-R dependent-removal of LDL from the circulation is massively disturbed in OH; upon T<sub>4</sub> replacement LDL FCR in vivo increases 3.7-fold (Thompson et al. 1981). Chait et al. found that T<sub>3</sub> increases <sup>131</sup>I-LDL binding to human skin fibroblasts indicating upregulation of LDL-Rs (Chait et al. 1979). Fibroblasts that either lack the LDL-R or are from patients with the syndrome of resistance to TH do not respond to T<sub>3</sub> by taking up more LDL, indicating that both LDL-R and TR $\beta$  are indispensable for this

effect. In hypophysectomized rats, hepatic LDL-R mRNA increased 20-fold 24 h after injection of  $T_3$  (Ness et al. 1990); however, decreased growth hormone (GH) and cortisol levels may contribute to low baseline expression in this model. Similarly, in rats made hypothyroid by feeding propylthiouracil (PTU), hepatic mRNA for the LDL-R was significantly decreased (Staels et al. 1990; Salter et al. 1991), while there was no change in intestinal mRNA. In vivo, receptor-dependent clearance of  $^{131}\text{I}$ -labelled LDL was decreased by 40% in hypothyroid rats (Gross et al. 1987). Conversely, hyperthyroid rats showed increased LDL-R mRNA levels (Staels et al. 1990). Co-transfection experiments of the human LDL-R promoter and TR $\beta$ 1 in HepG2 cells indicated a functional TRE in the proximal promoter and showed that constitutive activity is suppressed by unliganded TR $\beta$ 1, while  $T_3$  increases promoter activity up to 6-fold (Bakker et al. 1998). While the above data strongly support direct stimulation of LDL-R transcription by  $T_3$ , an indirect stimulation of transcription by  $T_3$ -induced increases in SREBP-2 was recently uncovered (Shin and Osborne 2003).

The contribution of TRs to the induction of LDL-R mRNA was clarified by Gullberg et al. (2000).  $T_3$  increased LDL-R mRNA in wild-type mice; and both TR $\beta$ -/- and TR $\alpha$ -/- mice had higher mRNA levels than hypothyroid, but failed to increase LDL-R mRNA during  $T_3$  substitution (Gullberg et al. 2000). Similar data were obtained in a TR $\beta$  knock-in model (Hashimoto et al. 2006a, b). Thus, stimulation of LDL-R transcription in vivo in mice cannot be attributed unequivocally to TR $\beta$  or TR $\alpha$ .

The liver-specific TM CGS23425 induced a 44% increase in  $^{125}\text{I}$ -LDL binding to HepG2 cells, indicating an up-regulation of LDL-R number, an effect comparable to equimolar doses of  $T_3$  (Taylor et al. 1997). Similarly, the liver-specific TM T-0681 increased LDL-R protein levels by 2.5-fold in rabbit liver (Heimberg et al. 1985; Tancevski et al. 2008).

Taken together, down-regulation of the LDL-R constitutes a major mechanism underlying the hyperlipidemia in OH.  $T_3$  upregulates LDL-R transcription, directly by binding to a TRE and indirectly both by induction of SREBP-2 and, at least in rodents, by stimulation of bile acid synthesis, thereby lowering hepatic cholesterol levels.

#### 10.1.2.6.1.3 *HMGCoA Reductase*

TH stimulate cholesterol synthesis in man, an effect alleviating both hypercholesterolemia in OH and hypocholesterolemia in OHyper (Abrams and Grundy 1981). HMGCoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, is expressed in all tissues; however, contribution by the liver is crucial for whole body cholesterol homeostasis. If cholesterol accumulates in hepatocytes, HMGCoA reductase is transcriptionally down-regulated because the release of stimulatory membrane-bound transcription factors, i.e. SREBPs is blocked.

HMGCoA reductase is sensitive to  $T_3$ . In hypophysectomized rats, HMGCoA reductase mRNA, HMGCoA protein and HMGCoA activity are up-regulated after supplementation with  $T_3$  (Ness et al. 1990). In rat liver nuclei,  $T_3$  both increases

HMGCoA reductase transcription and stabilizes HMGCoA reductase mRNA (Simonet and Ness 1988). Studies in **mice** revealed that  $T_3$  increases HMGCoA reductase mRNA only in the wild type, but not in  $TR\beta^{-/-}$  mice (Gullberg et al. 2000). In hypothyroid wild-type mice, expectedly, hepatic HMGCoA reductase mRNA was increased  $\approx 10$ -fold by  $T_3$  replacement on chow and on a high-cholesterol diet, whereas in  $TR\beta$  knock-in mice the increase by  $T_3$  was substantially blunted (Hashimoto et al. 2006a, b). Unexpectedly, in hypothyroid  $TR\beta$  knock-in mice HMGCoA reductase mRNA was increased both on chow and on the atherogenic diet, suggesting activation of expression by unliganded  $TR\beta$  (Hashimoto et al. 2006a, b). These findings support a critical role for  $TR\beta$  in the regulation of cholesterol synthesis. Interestingly, TM GC-1 failed to upregulate HMGCoA reductase in euthyroid mice highlighting that effects of  $T_3$  replacement and oversubstitution with TH may differ (Johansson et al. 2005).

Presumably, transcriptional, post-transcriptional and indirect mechanisms and indirect mechanisms (e.g. stimulation of SREBP-2) contribute to stimulation of HMGCoA reductase gene expression by  $T_3$  in rodents in a  $TR\beta$ -dependent fashion.

#### 10.1.2.6.1.4 *SREBP-1c, SREBP-2, LXR $\alpha$ and PPAR $\gamma$*

**SREBP-1c** preferentially increases the transcription of hepatic genes required for fatty acid synthesis. Recent reports indicate that  $T_3$  lowers SREBP-1c mRNA levels in mouse liver, presumably by suppressing mouse SREBP-1c promoter activity in a mouse hepatocyte cell line in a  $TR\beta$ -dependent manner (Hashimoto et al. 2006a, b). Because species differences in gene regulation by  $T_3$  are frequent, the authors repeated these experiments examining human SREBP-1c promoter activity in HepG2 cells and obtained the same result (Hashimoto et al. 2006a, b). However, stimulation of human SREBP-1c mRNA by  $T_3$  has also been reported (Kawai et al. 2004).

**SREBP-2** preferentially increases the transcription of genes required for the maintenance of hepatocyte cholesterol levels, e.g. LDL-R and HMGCoA reductase. Recent reports indicate that in hypothyroid mice, decreased SREBP-2 mRNA and decreased nuclear protein levels of SREBP are present (Shin and Osborne 2003). Interestingly, the proximal promoter of SREBP-2 contains at least one TRE and is activated directly by heterodimers of  $TR\alpha$  or  $TR\beta$  with RXR in a  $T_3$ -dependent manner (Shin and Osborne 2003). This may in part explain down-regulation of LDL-R and low cholesterol synthesis in OH.

**LXR- $\alpha$**  is abundant in liver, whereas LXR- $\beta$  is ubiquitously expressed. Although LXR and TR are structurally distinct and bind different ligands, they share some similarities, e.g. heterodimerization with RXR and a physiological role in cholesterol homeostasis. Both LXR and TR appear to regulate a series of target genes promoting reverse cholesterol transport (RCT; Naik et al. 2006), e.g. ABCA1, ABCG1, ABCG5/8, SREBP-1c, CETP and, in mice, CYP7A1. In this context, it is of interest that  $T_3$  up-regulates LXR $\alpha$ , but not LXR $\beta$  mRNA expression in mouse liver by  $TR\beta$  mediated binding to the  $-1240/+30$  proximal promoter (Hashimoto et al.

2007). As a possible counterbalance to indirect activation of SREBP-1c by LXR $\alpha$ , T<sub>3</sub> directly down-regulates SREBP-1c mRNA expression in mice, which has been interpreted as fine tuning of this pathway. Thus, while enhancement of several facets of RCT due to TH is well documented, the relative contribution of direct TH-signalling as opposed to indirect signalling via LXR- $\alpha$  still needs to be resolved.

Cross-talk between TR and **PPAR $\gamma$**  regarding differentiation of adipose tissue has been reported (Ying et al. 2007).

#### 10.1.2.6.1.5 *ABCG5, ABCG8 and NPC1L1*

**ABCG5** and **ABCG8** are expressed in liver and intestine, where upon dimerization, they enhance biliary cholesterol secretion and, possibly, direct intestinal cholesterol loss, respectively (van der Velde et al. 2007). In hypophysectomised **rats**, ABCG5 and ABCG8 mRNA was almost totally suppressed (<20% of controls) and T<sub>4</sub>, but not GH or cortisol increased their expression by >60-fold (Gälman et al. 2008). In line with these data, in hyperthyroid **mice**, hepatic ABCG5 and ABCG8 mRNA were substantially increased by 3.0- and 1.5-fold, respectively, compared to euthyroid controls (Tancevski et al. 2008). No effect on intestinal ABCG5 and ABCG8 mRNA levels was seen in hyperthyroid mice (Tancevski et al. 2008). Mice treated with T-0681 also showed moderate increases in liver ABCG5 and ABCG8 mRNA levels (Tancevski et al. 2008).

No major regulation of intestinal **NPC1L1** mRNA levels was observed in hypophysectomized rats during TH replacement (Gälman et al. 2008) and in mice on TM (Tancevski et al. 2008).

#### 10.1.2.6.2 Factors Mainly Affecting HDL

##### 10.1.2.6.2.1 *Hepatic Lipase*

HL hydrolyzes phospholipids (PL) and TG in IDL and large HDL particles, thereby facilitating IDL uptake by the liver and inducing HDL remodelling. In addition, non-catalytically active HL mediates uptake of apoB-containing lipoproteins in the liver (Amar et al. 1998). HL consistently is decreased in OH. In human OH, postheparin HL increases by 40% (Kuusi et al. 1988) to 273% (Valdemarsson et al. 1982) after T<sub>4</sub> replacement and this increase is strongly indirectly related to the parallel decrease in HDL<sub>2</sub>-C (Kuusi et al. 1988; Tan et al. 1998a, b). As HL is also regulated by sex steroids, the increase in SHBG due to T<sub>4</sub> was examined and found to be unrelated to the changes in lipids (Kuusi et al. 1988). Recently, HL was reported to be decreased by  $\approx$ 30% in SCH (Brenta et al. 2007).

Only a slight decrease in HL mRNA in the liver was observed in hypothyroid rats; however, as HL activity was not examined, it remains possible that post-transcriptional stimulation of HL by T<sub>4</sub> was missed (Staels et al. 1990). No information regarding HL is available from studies targeting TR genes in mice.

In experimental OHyper in **humans**, HL increases by 46% (Hansson et al. 1983). In contradistinction, overdosing euthyroid **rats** with  $T_4$  does not increase HL mRNA in the liver (Staels et al. 1990). Application of TM T-0681 for 4 weeks to euthyroid cholesterol-fed NZW **rabbits** failed to change postheparin HL (Tancevski et al. 2008). Thus, HL is low in OH, which in part explains distinctive features of the dyslipidemia in OH, i.e the accumulation of HL substrates, namely IDL, chylomicron remnants and HDL<sub>2</sub>. The converse pertains to OHyper. The molecular regulation of HL by TH remains to be defined.

#### 10.1.2.6.2.2 Scavenger Receptor Class B, Type I

Scavenger receptor class B, type I (SR-BI) functions as an HDL receptor in liver and steroid hormone-producing tissues. However, it also plays a major role in cholesterol efflux from cells, e.g. from macrophages. No data are available examining the role of SR-BI in **human** thyroid dysfunction. In euthyroid **mice** on chow, the liver and  $\beta$ -selective TM GC-1 and  $T_3$  did not affect liver SR-BI mRNA levels. In these mice, surprisingly, GC-1 and equimolar amounts of  $T_3$  increased SR-BI protein to levels 2.4-fold and 1.5-fold above baseline (Johansson et al. 2005). In euthyroid mice on a high-cholesterol diet, GC-1 and  $T_3$  reduced hepatic SR-BI mRNA levels. Despite lower SR-BI mRNA, GC-1 (but not  $T_3$ ) doubled hepatic SR-BI protein concentrations (Johansson et al. 2005). In line with these observations, in NZW **rabbits** fed a high-cholesterol diet, T-0681 increased liver SR-BI protein  $\approx$ 2-fold (Tancevski et al. 2008). Up-regulation of SR-BI in the liver may be an important step in the turnover of HDL-C via the stimulation of RCT in humans, mice and rabbits. Presumably, this occurs by a post-transcriptional mechanism of action. It remains to be seen whether regulation in peripheral cells differs from the above paradigm.

#### 10.1.2.6.2.3 ATP-Binding Cassette Transporter-AI

ATP-binding cassette transporter-AI (ABCA1) catalyzes cholesterol efflux from cells to lipid-poor apo A-I, the first step in RCT. Transcription of ABCA1 is regulated by promotor binding of heterodimers of LXR $\alpha$ /RXR and TR $\beta$ /RXR, which may compete for the same binding site (Huuskonen et al. 2004). No data on hypothyroidism and ABCA1 are available in humans and rodents. In severely hyperthyroid **mice**, both hepatic ABCA1 mRNA and hepatic ABCA1 protein were reduced by  $\approx$ 50%, which may conceivably contribute to decreases in HDL-C (Tancevski et al. 2008). In contrast to liver, intestinal levels of ABCA1 mRNA were increased 4-fold (Tancevski et al. 2008). In mice treated with the liver-specific TM T-0681, hepatic ABCA1 mRNA levels were unchanged (Tancevski et al. 2008). Taken together, ABCA1 is clearly sensitive to regulation by  $T_3$ , however, important questions concerning tissue- and species-selective regulation still need to be worked out.

#### 10.1.2.6.2.4 *Cholesteryl Ester Transfer Protein, Lecithin:Cholesterol Acyl Transferase and Phospholipid Transfer Protein*

Cholesteryl ester transfer protein (**CETP**) affords core neutral lipid exchange between lipoproteins. In fasting and/or postprandial hypertriglyceridemia (Föger et al. 1996), this leads to TG-enrichment of HDL and LDL, which thereby become a good substrate for intravascular remodelling by HL and phospholipid transfer protein (**PLTP**; Föger et al. 1997). CETP lowers HDL-C, which may be pro-atherogenic. However, CETP stimulates RCT via apoB-containing lipoproteins which may be anti-atherogenic. As rodents normally lack CETP, mainly **human** data contribute to our knowledge. In OH, CETP activity in plasma as measured independently of endogenous lipoproteins, is decreased by  $\approx 35\%$  (Tan et al. 1998a, b; Dullart et al. 1999). In contrast, OHyper increases CETP activity by  $\approx 32\%$  and correction of thyroid dysfunction normalizes CETP activity (Tan et al. 1998a, b). Thus, changes in CETP could help to explain high HDL-C in OH and low HDL in OHyper, respectively. However, Tan et al. (1998a, b) found no correlation between decreased CETP and increased HDL-C or HDL<sub>2</sub>. While this may pertain to the lean, normo-triglyceridemic Chinese patients studied (Tan et al. 1998a, b) low CETP would be expected to increase HDL-C in obese and/or hypertriglyceridemic patients, respectively. Interestingly, CETP mass, not measured in the above work, was found to be normal in OH (Ritter et al. 1996), suggesting decreased specific activity in OH, e.g. due to increased inhibitory factors present in plasma. Cholesteryl ester (CE) net mass transfer from HDL to apoB-containing lipoproteins, influenced both by CETP activity and endogenous lipoproteins, was significantly decreased in OH (Ritter et al. 1996). This was due to qualitative changes in hypothyroid VLDL and LDL, which have been interpreted as potentially beneficial (Ritter et al. 1996). CETP activity in **rabbits** receiving the liver-specific TM T-0681 was unchanged (Tancevski et al. 2008). Thus, it is currently unclear at which level T<sub>3</sub> stimulates CETP-activity and also if this involves LXR $\alpha$ . While stimulation of CETP should be expected to lower HDL-C, this may in fact reflect stimulation of RCT.

Lecithin:cholesterol acyl transferase (**LCAT**) activity in plasma in **human** OH tends to be decreased; however, T<sub>4</sub> replacement did not result in increased LCAT activity (Valdemarsson et al. 1983). The same appears to be the case in MMI-treated hypothyroid **rats**. Hepatocyte cultures obtained from hypothyroid rats secrete substantially less LCAT into the medium (Ridgway and Dolphin 1985). Few data regarding regulation of LCAT are available indicating that few researchers feel LCAT plays an important role in the hyperlipoproteinemia in OH.

Human data on PLTP are lacking. PLTP activity was unchanged in severely hyperthyroid mice (Tancevski et al. 2008).

#### 10.1.2.6.2.5 *Lipoprotein Lipase*

Lipoprotein lipase (**LPL**) is secreted from adipose and muscle to hydrolyze TG from large TG-rich lipoproteins, both postprandially and in the post-absorptive state, i.e. chylomicrons and VLDL. While some studies did not detect changes in

LPL during **human** OH (Krauss et al. 1974; Abrams et al. 1981; Packard et al. 1993; Tan et al. 1998a, b), others observed presumably decreased baseline values and an increase of up to 50% after  $T_4$  replacement (Lithell et al. 1981; Valdemarsson et al. 1982; Kuusi et al. 1988). LPL in post-heparin plasma increases after  $T_4$  for OH, varying in amount from marginal to +50% (Lithell et al. 1981). This is due to increases in LPL in adipose by 21% and in skeletal muscle by 42% (Lithell et al. 1981). Thus, in humans, LPL may be increased in both major tissues responsible for 75% of intravascular TG hydrolysis, leading to an increased clearance capacity after iv infusion of an artificial TG emulsion (Lithell et al. 1981). However, this effect is only observed in more profound OH with TSH >40 mU/l (Lithell et al. 1981). In line with these observations, after replacing  $T_4$  in OH, the TG increase above fasting levels, as assessed by a fat tolerance test, was decreased by 20%; however, chylomicron RP-AUC was not decreased (Weintraub et al. 1999). In summary, a mild defect in LPL activity leading to TG intolerance in susceptible patients may be present in human OH, which is alleviated by  $T_4$ . The underlying molecular mechanisms remain to be clarified.

In ad-libitum fed hypothyroid **rats**, LPL mRNA was unchanged (Saffari et al. 1992). Nevertheless, LPL activity and mass in white adipose, cardiac and skeletal muscle was increased by 4.5-fold, 5.0-fold and 10.0-fold, respectively, indicating post-translational regulation (Saffari et al. 1992). Interestingly, pair-feeding hypothyroid rats with controls abolished the above differences, highlighting the often neglected role of alterations in motor activity and feeding behavior regarding lipids. No information regarding LPL is available from studies targeting TR genes in **mice**.

In OHyper, LPL was not found to be increased (Abrams et al. 1981, Tan et al. 1998a, b). Inducing experimental OHyper in healthy young volunteers does not increase LPL in post-heparin plasma (Hansson et al. 1983). Application of TM T-0681 for 4 weeks to euthyroid cholesterol-fed NZW **rabbits** failed to change postheparin LPL (Tancevski et al. 2008).

Thus, regulation of LPL in human and rat hypothyroidism is fundamentally different indicating species-specific responses. Hyperthyroidism does not appear to alter LPL.

## 10.2 Thyromimetics and Thyromimetic Compounds

### 10.2.1 Background

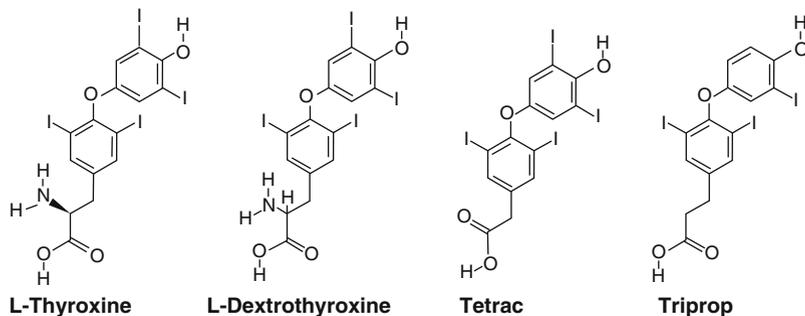
#### 10.2.1.1 Desiccated Thyroid and Thyrotoxicosis

It has been known since 1930 that hyperthyroidism is associated with reduced plasma cholesterol levels (Mason et al. 1930) and since then many efforts have been made to exploit the ability of TH to lower cholesterol (Morkin et al. 2004; Moreno et al. 2008). In initial studies during the 1950s, desiccated thyroid was administered to a small group of patients, all of whom responded with a fall in cholesterol

(Strisower et al. 1954). A low dosage of desiccated thyroid led to a significant reduction in plasma cholesterol, “escape” occurred after 20–30 weeks of treatment (Strisower et al. 1955). Patients treated with high doses of desiccated thyroid were not refractory to treatment, but a large number presented with tachycardia, angina pectoris, diarrhea, weight loss and insomnia, in brief, with overt hyperthyroidism (Galioni et al. 1957). Thus, studies with thyroid preparations were stopped at that time and the search for synthetic analogs began.

### 10.2.1.2 First Synthetic TH Analogs and the “Twisted” D-T<sub>4</sub> Study

A large number of TH analogs were synthesized and tested in experimental animal models for their lipid-lowering activity (reviewed by Jorgensen 1979). 3,5,3'-triiodothyropropionic acid (Triprop) was one of the first analogs to be demonstrated to lower plasma cholesterol without affecting basal metabolic rate (Money et al. 1960). In addition to Triprop, tetraiodothyroformic acid, Tetrac, Triac, Diac and dextrothyroxine (D-T<sub>4</sub>) were developed (Boyd and Oliver 1960; Fig. 10.1). Among all these analogs tested in animal studies, D-T<sub>4</sub> appeared to have the highest specific cholesterol-lowering action, without showing concomitant deleterious effects on the heart. In the late 1960s, a large clinical trial of D-T<sub>4</sub> therapy was conducted, as part of the **Coronary drug project** by the National Institutes of Health, which aimed to answer the question as to whether cholesterol reduction may prevent coronary heart disease (Coronary drug project research group 1972). The study was terminated after average follow-up of 36 months due to a higher proportion of deaths in the D-T<sub>4</sub>-treated group, although this difference did not reach statistical significance. However, the design and performance of this study may have not been sufficient to elucidate the lipid-lowering effect of D-T<sub>4</sub> in humans. First, subsequent investigations revealed the preparations used in the D-T<sub>4</sub> study to be contaminated with as much as 0.5% L-T<sub>4</sub>, equivalent to a dosage of 30 µg/day, which may have been the only active metabolite of the study (Young et al. 1984). Second, the deaths occurred in patients already carrying high cardiovascular risk factors at the initiation of the study, including angina pectoris, congestive heart failure and tachycardia.



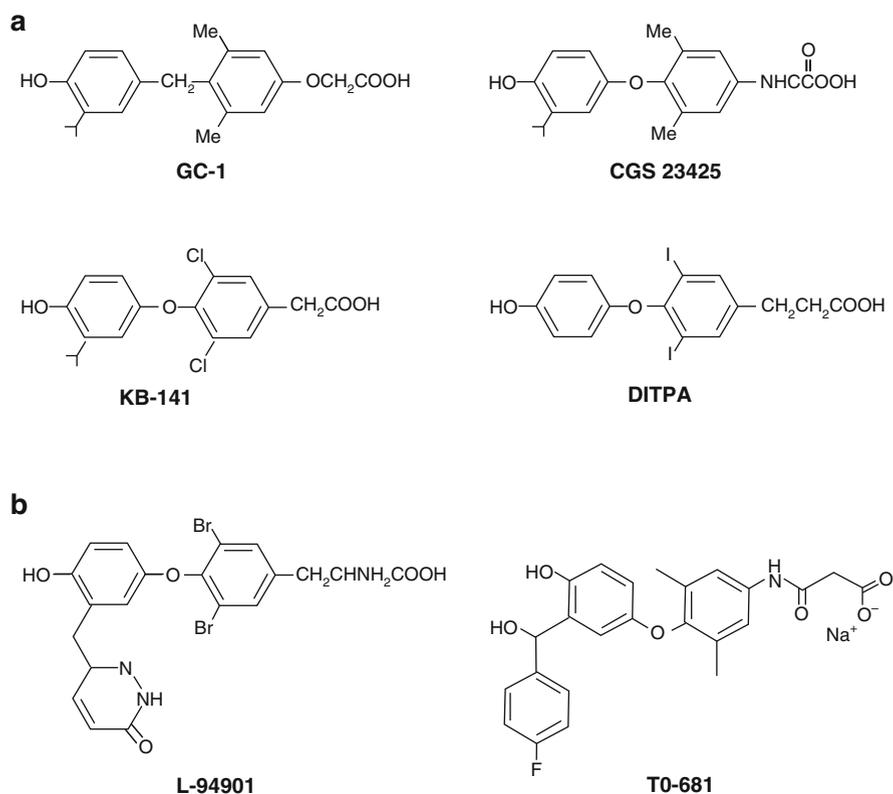
**Fig. 10.1** Early synthetic TH analogues

After exclusion of high risk patients, the overall survival in the D-T<sub>4</sub>-treated group was greater than with the controls (Baxter et al. 2001). The unfavorable recruitment of patients together with the accidental employment of preparations contaminated with the enantiomer of D-T<sub>4</sub> led to the discontinuation of clinical studies with TH analogs in the 1970s.

### 10.2.2 Selective Thyromimetic Compounds

With the introduction into clinical practice of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) inhibitors, usually known as “statins”, to lower plasma cholesterol in the mid-1980s, efforts on the development of TH analogs slowed.

It was during this time period, however, that novel “selective” compounds mimicking the cholesterol-lowering actions of TH were developed (representative selective TH analogs are shown in Fig. 10.2a, b).



**Fig. 10.2** Selective thyromimetic compounds. (a) TR $\beta$ -selective TH analogs. (b) Organ-selective TH analogs

### 10.2.2.1 Organ-Selective TH Analogs

The first described selective thyromimetic compound was 3,5-dibromo-3'-pyridazinone-L-thyronine (L-94901), which showed half of the binding affinity of  $T_3$  to hepatic TRs, but only a minor affinity (1.3% of  $T_3$ ) to cardiac TRs (Underwood et al. 1986; Leeson et al. 1988). This organ-selective thyromimetic has been reported to lower plasma cholesterol levels in experimental animals at doses that do not exhibit cardiotoxic side-effects. Very recently, another organ-selective compound with lipid-lowering properties was described, namely N-(4-{3-[(4-fluorophenyl)hydroxymethyl]-4-hydroxyphenoxy}-3,5-dimethylphenyl) malonic acid sodium (T-0681, formerly KAT-681; Tancevski et al. 2007).

### 10.2.2.2 TR $\beta$ -Selective TH Analogs

In the late 1980s, different TR isoforms (TR $\alpha$ , TR $\beta$ ) were cloned (Sap et al. 1986) and their tissue-specific expression characterized (reviewed by Apriletti et al. 1998; Lazar 1993). These findings led to the design of isoform-specific, TR $\beta$ 1-selective thyromimetics, such as N-[3,5-dimethyl-4-(4'-hydroxy-3'-isopropylphenoxy)-phenyl] oxamic acid (CGS23425), 3,5-dimethyl-4[(4'-hydroxy-3'-isopropylbenzyl)-phenoxy] acetic acid (GC-1), 3,5-dichloro-4[(4-hydroxy-3-isopropylphenoxy)-phenyl] acetic acid (KB-141), 3-{3,5-dibromo-4-[4-hydroxy-3-(1-methylethyl)-phenoxy]-phenyl}-amino-3-oxopropanoic acid (KB2115) and 3,5-diiodothyropropionic acid (DITPA; for structures, please see Fig. 10.2a, b).

TR $\beta$ 1-selectivity of CGS23425 and GC-1 seems to rely on the fact that both have methyl groups in place of iodides on the inner ring and that in the outer ring the single iodide of  $T_3$  has been replaced by an isopropyl group (Moreno et al. 2008). It is of interest that the strong binding of GC-1 and CGS23425 to TR $\beta$ 1 contradicts the usual substituent binding preference for the TR at the 3 and 5 positions on their inner ring (namely I>Br>methyl), according to which these compounds should bind with low affinity. However, as suggested by Moreno, the 3, 5 and 1 substituents of the inner ring should not be regarded independently, but as coupled moieties, when considering ligand-binding affinity (Moreno et al. 2008).

In summary, the past 20 years saw the development of either organ-selective or TR $\beta$ 1-selective TH analogs, all of which lowered plasma cholesterol without deleterious effects on the heart.

## 10.2.3 Selective Thyromimetics as Hypolipidemic Drugs

### 10.2.3.1 Increased LDL Clearance

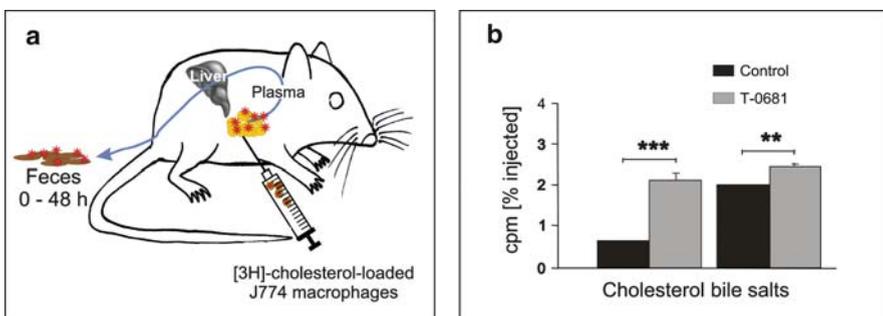
All of the above-mentioned thyromimetic compounds were demonstrated in preclinical studies to lower both plasma cholesterol and triglycerides (Underwood et al. 1986; Taylor et al. 1997; Grover et al. 2003, 2004; Tancevski et al. 2007). This effect

is thought to be mainly brought about by an increased LDL-C plasma clearance through an increase in LDL-R expression in liver, similar to that described for TH action (Staels et al. 1990; Salter et al. 1991; Bakker et al. 1998). In cynomolgus monkeys, both GC-1 and KB-141 were reported to lower Lp(a) by up to 50% (Grover et al. 2003, 2004). First reports on clinical studies with DITPA (Morkin et al. 2002) and KB2115 (Berkenstam et al. 2008), respectively, showed TR $\beta$ 1-selective thymimetics to lower plasma LDL cholesterol and triglycerides also in humans, without untoward cardiac effects.

### 10.2.3.2 Promotion of Reverse Cholesterol Transport

Very recently, both GC-1 and T-0681 were reported to stimulate the expression of key players of reverse cholesterol transport (RCT; Johansson et al. 2005; Tancevski et al. 2007). They markedly induce hepatic SR-BI levels, stimulate the activity of cholesterol 7 $\alpha$ -hydroxylase and induce the expression of hepatic cholesterol transporters ABCG5 and ABCG8. As a consequence, treated mice have a significant decrease in plasma HDL-C, with increased fecal excretion of bile acids and cholesterol. Interestingly, mice treated with T-0681 display reduced intestinal absorption of dietary sterols, most likely due to competition with sterols of biliary origin.

The hypothesis of promotion of RCT by a TM was tested by measuring RCT from macrophages to feces in mice treated with T-0681, according to the method developed by Rader and coworkers (Zhang et al. 2003). At 48 h after intraperitoneal injection of cholesterol-loaded, [ $^3$ H]-labelled J774 macrophages, T-0681-treated animals displayed a significant increase of both, fecal [ $^3$ H]-bile acids and [ $^3$ H]-cholesterol (Fig. 10.3; Tancevski et al. 2007). Thus, employment of TM may promote RCT from atherosclerotic plaque macrophages to the liver for fecal excretion.



**Fig. 10.3** Influence of thymimetic T0-681 on in vivo macrophage reverse cholesterol transport in mice. (a) Schematic drawing of experimental approach. (b) Increased fecal [ $^3$ H]-bile acids and [ $^3$ H]-cholesterol after intraperitoneal injection of [ $^3$ H]-labelled J774 macrophages in control and T-0681 treated animals

RCT in humans is profoundly different from that found in rodents in that cholesterol from plaque macrophages can be transported to the liver either directly via HDL particles, or – after transfer to VLDL and LDL mediated by CETP – via apoB-containing lipoproteins (Ritsch et al. 2003). Interestingly, CETP-transgenic mice require hepatic expression of LDL-R to counterbalance accumulation of apoB-containing lipoproteins in plasma. In line with these findings, adenoviral over-expression of SR-BI in rabbits, which naturally express CETP in plasma, led to accumulation of VLDL and LDL cholesterol (Tancevski et al. 2005). Thus, hepatic stimulation of SR-BI expression necessitates a concomitant, appropriate clearance of apoB-lipoproteins to guarantee maintenance of RCT in CETP-expressing species, like humans. Simultaneous upregulation of hepatic SR-BI and LDL-R may represent a rational approach to direct excessive cholesterol from the periphery to the liver in humans; and selective TM may prove useful to promote this mechanism.

### **10.2.3.3 Prevention of Atherosclerosis**

First evidence that accelerated clearance of LDL cholesterol and promotion of RCT by a TM may constitute a powerful approach to prevent the development of atherosclerosis came from studies in cholesterol-fed NZW rabbits treated with T-0681 (Tancevski et al. 2007). T-0681 reportedly decreased plasma cholesterol by 60%, triglyceride levels by more than 80% and hepatic expression of SR-BI and LDL-R were found increased 2-fold. Lipid staining of rabbit aortas revealed a 60% decrease in atherosclerotic lesion area, when compared to placebo-treated controls.

In summary, current data heavily suggest that selective thyromimetic compounds have great clinical potential as agents to treat hyperlipidemia and to protect from atherosclerosis and its clinical sequelae.

## ***10.2.4 Potential Additional Applications***

### **10.2.4.1 Obesity**

Besides their lipid-lowering properties, both GC-1 and KB-141 were shown to increase the metabolic rate, to increase oxygen consumption and to reduce body-weight in primates (Grover et al. 2003, 2004). These effects were not accompanied by deleterious effects on heart, skeletal muscle and bone mass. TR $\beta$ 1-selective compounds may therefore have potential to be used as therapeutic agents for the treatment of obesity. However, first studies with KB2115 in humans failed to influence the body-weight of probands (Berkenstam et al. 2008), probably due to the smaller doses employed when compared to previous animal studies (Grover et al. 2003, 2004; Berkenstam et al. 2008).

#### 10.2.4.2 Angiogenesis

GC-1 was shown to promote angiogenesis in the chick chorioallantoic membrane model (Mousa et al. 2005); and DITPA was reported to be angiogenic in the postinfarction rat heart (Tomanek et al. 1998), suggesting the employment of a TR $\beta$ 1-selective thyromimetic as proangiogenic agents in coronary artery disease, e.g. by administration via the coating of a coronary stent (Mousa et al. 2005).

#### 10.2.4.3 Congestive Heart Failure

In hypothyroid rats, DITPA increased cardiac performance with approximately half the chronotropic effect and less metabolic stimulation than L-T<sub>4</sub> (Barker et al. 1951). DITPA also improved left ventricular performance in rabbit and rat postinfarction heart failure models (Mahaffey et al. 1995). In primates, DITPA enhanced the in vivo force–frequency and relaxation–frequency relationships of the heart without increasing heart rate (Khoury et al. 1996; Hoit et al. 1997). In a phase I clinical study, DITPA did not affect the heart rate or blood pressure of healthy volunteers, while total plasma cholesterol and triglycerides were decreased significantly (Morkin et al. 2002). In heart-failure patients receiving this drug for 4 weeks, cardiac index was increased on an average to 17%, systemic vascular resistance index was decreased and diastolic function was improved. Total serum cholesterol and triglycerides were significantly decreased. These results indicated that DITPA could represent a useful agent to treat congestive heart failure and dyslipidemia.

#### 10.2.4.4 Chemoprevention for Hepatocellular Carcinoma

Interestingly, both T-0681 and GC-1 were found to stimulate rat hepatocyte proliferation in vivo (Hayashi et al. 2004; Hayashi et al. 2005; Columbano et al. 2006). Of note, T-0681 inhibited the development of hepatocellular carcinoma in rats induced by 2-acetylaminofluorene and partial hepatectomy after diethylnitrosamine initiation (Hayashi et al. 2004, 2005). Together, these data suggest the potential for TM as chemopreventive agents for hepatocarcinogenesis.

### 10.2.5 *Off-Target Toxicity of Selective Thyromimetics*

There are at least four main untoward effects of selective TM to be mentioned: (i) **relative hypothyroidism**, (ii) elevation of liver enzymes, (iii) cardiotoxicity and (iv) disturbed bone metabolism. Ideally, a selective TR agonist would cause modest increase in metabolic rate without tachycardia but would not reduce TSH and/or T<sub>4</sub>, as observed with all of the selective analogs at therapeutic doses. TR $\beta$ -selective TM lead to feedback-inhibition at the hypothalamus/pituitary, which reduces TSH and T<sub>4</sub> levels, thereby

causing a paradoxical hypothyroidism in some tissues. However, so far there are no data available on the consequences of such a relative hypothyroidism.

Both KB2115 and T-0681 were reported to induce an **elevation of liver enzymes**, when administered in high doses (Tancevski et al. 2007; Berkenstam et al. 2008). Either through TR $\beta$ 1-selectivity of KB2115 or through organ-selectivity of T-0681, both kinds of compounds may accumulate in hepatocytes and lead to the observed hepatotoxicity at high dosages.

Another important side-effect, observed solely at highest doses, is **tachycardia**, which may be explained by a loss of selectivity in such oversaturated settings. However, the use of TR $\beta$ 1-selective thyromimetics is likely to be safe, as GC-1 was reported to have a relative selectivity for cholesterol-lowering versus tachycardia of 18-fold (Grover et al. 2003) when compared to T<sub>3</sub>. KB-141 was shown to be approximately 27-fold more selective for cholesterol-lowering when normalized for T<sub>3</sub> (Grover et al. 2004).

TR $\beta$  is active in bone, and OHyper is well known to induce **osteoporosis**. Thus, skeletal safety of TR $\beta$ -selective TM needs to be demonstrated.

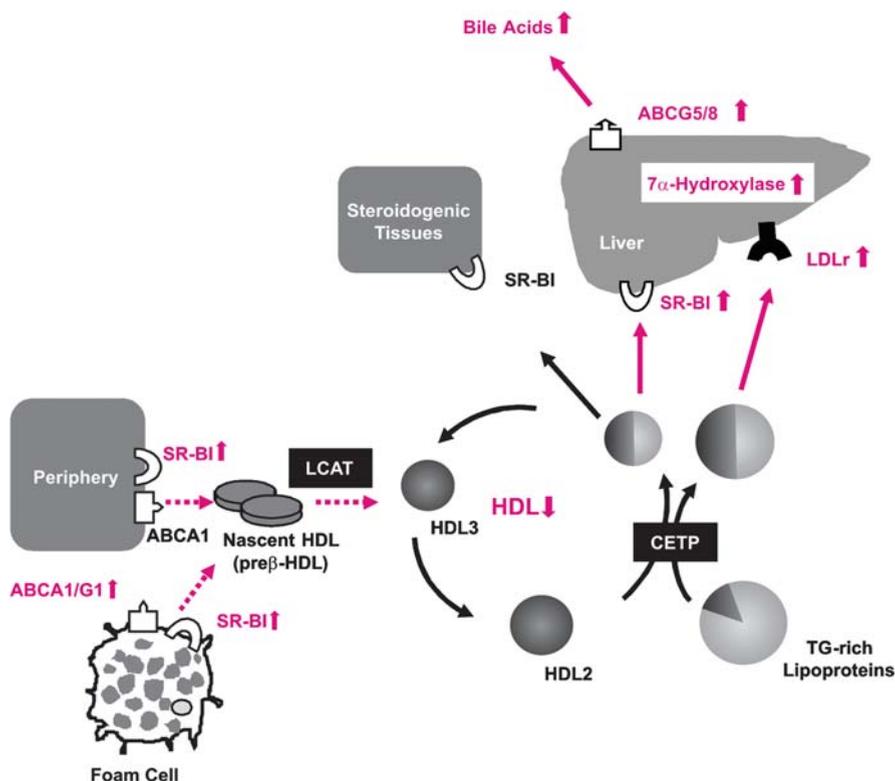


Fig. 10.4 Influence of thyromimetic T-0681 on reverse cholesterol transport

In conclusion, efforts spanning more than 50 years led to the development of selective thyromimetic compounds, a new drug class for the treatment of hypercholesterolemia and for prevention of atherosclerosis. The influence of thyromimetic compounds on lipid metabolism, i.e. on the reverse cholesterol transport, is illustrated in Fig. 10.4. Drugs of this type may be useful in treating patients who are intolerant to statins, or who do not respond to this medication. They also may be used to treat obesity or congestive heart failure. However, current studies do not provide sufficient data on the central question as to whether the observed relative hypothyroidism may have deleterious effects on the human organism. Thus, further studies on the mechanism of negative feedback on the thyroid axis induced by both TR $\beta$ 1-selective and organ-selective TH analogs are awaited to increase the potential of this novel drug class for the prevention of cardiovascular diseases.

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# Chapter 11

## Adipokines: Regulators of Lipid Metabolism

Oreste Gualillo and Francisca Lago

**Abstract** Obesity has reached epidemic dimensions, affecting millions of individuals; and it is the major contributor to type 2 diabetes, cardiovascular diseases and certain cancer types. The discovery in the mid-1990s of leptin, the first paradigmatic adipokine, renewed dynamic interest in the research of “forgotten” adipose tissue, until then believed to be only the body energy reservoir. During recent years several hormones, most of them with cytokine-like activity, have been isolated and characterized from white adipose tissue. Adipokines have been widely viewed, both in the context of obesity and its associated metabolic syndrome and insulin resistance, but also as modulators of the inflammatory state and immune response. However, the secretory endocrine function of adipose tissue cannot be separated from intracellular lipid storage and handling; hence, the goal of this chapter is to give the reader an overview of the signals evoked by the most relevant adipokines in the context of adipose tissue primordial physiology (i.e. lipid metabolism).

### 11.1 Introduction

A right upkeep of energy balance is a mandatory condition for the survival of all species, including man. Indeed, neither deficient nor massive nutrient intake is correct for energy homeostasis and this in turn results respectively in chronic starvation or obesity.

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It is generally conceived that starvation is a quickly detrimental process and, when it is prolonged up to a depletion of more than 30% (most of which is due to loss of fat stores) of body weight in a short timescale, it results in death. So, because of the fatal consequences of chronic starvation, human beings evolved along centuries in order to face various periods of famine and scarcity, by developing a greater capacity for storing energy as fat. Nowadays, this key characteristic for survival makes individuals more susceptible to obesity and its complications during times of abundance.

Obesity in adult age is a worldwide alarming situation, but more alarming is the increasing rate of obesity in children. Childhood obesity is particularly troubling because the extra pounds often start kids on the path to health problems that were once confined to adults, such as diabetes, high blood pressure and high cholesterol.

While the prevalence of obesity increases worldwide, the knowledge about its pathogenesis and metabolic consequences markedly advances.

Energy homeostasis requires a fine regulation of food intake which must be matched by an appropriate equilibrium among nutrient absorption, energy storage and fuel expenditure.

Appetite represents a key regulator of food intake which precedes intestinal carbohydrate and lipid uptake, whose consequent metabolic changeover to triacylglycerides provides the energy substrate that is stored within fat depots. The integration of these signals occurs mostly in the central nervous system, which orchestrates afferent signals and their transduction into homeostatic adjustments and balancing body economy.

Although the initial suggestion of the existence of a soluble factor circulating in blood that reflected the amount of fat store was made in the 1950s (Kennedy 1953), after a silence of more than 35 years, a renaissance in the interest about white adipose tissue occurred in the late 1980s when studies using obese mutant mice in parabiotic experiments gave evidence for the relevance of this hypothetical but still elusive circulating substance. The mystery was solved, at least in terms of compound identification, in 1994 when Zhang and collaborators identified the product of the gene *ob* and named it leptin (Greek, *leptos* = thin) due to its anorexigenic action. After the discovery of leptin, the past 16 years witnessed a revolution regarding the role of white adipose tissue. Indeed, more than 50 adipocyte-derived products, most of them with cytokine-like activity (which correctly have been named adipokines) have been identified.

This chapter focuses on the metabolic activities of the most relevant adipokines, with a special emphasis on lipid metabolism.

## 11.2 Regulation of Lipid Metabolism by Adipokines

### 11.2.1 *Leptin*

Leptin is a 16-kDa non-glycosylated peptide hormone encoded by the gene *obese* (*ob*), the mouse homologue of the human gene *LEP* (Zhang et al. 1994). Structurally, it belongs to the class I cytokine superfamily, consisting of a bundle of four

$\alpha$ -helices. It is mainly produced by adipocytes; and circulating leptin levels are directly correlated with white adipose tissue (WAT) mass. It decreases food intake and increases energy consumption by acting on hypothalamic cell populations (Ahima et al. 1996; Chan et al. 2003), inducing anorexigenic factors (cocaine- and amphetamine-related transcript, proopiomelanocortin) and inhibiting orexigenic neuropeptides (neuropeptide Y, Agouti-related protein, orexin); and leptin levels are correlated negatively with glucocorticoids (Zakrzewska et al. 1997) and positively with insulin (Boden et al. 1997). Its own synthesis is mainly regulated by food intake and eating-related hormones, but also depends on energy status, sex hormones (being inhibited by testosterone and increased by ovarian sex steroids) and a wide range of inflammation mediators (Sarraf et al. 1997; Gualillo et al. 2000), being increased or suppressed by pro-inflammatory cytokines depending on whether their action is acute or chronic. Through the mediation of these latter agents, leptin synthesis is increased by acute infection and sepsis. As a result of the effects of sex hormones, leptin levels are higher in women than in men even when adjusted for body mass index (BMI), which may be relevant to the influence of sex on the development or frequency of certain diseases (Blum et al. 1997). Thus, leptin appears to act not only as an adipostatin, the function in relation to which it was discovered, but also as a general signal of energy reserves (Otero et al. 2005) that is involved in a wide variety of other functions, including glucose metabolism, synthesis of glucocorticoids, proliferation of CD4+ T lymphocytes, cytokine secretion, phagocytosis, regulation of the hypothalamic–pituitary–adrenal axis, reproduction and angiogenesis (Otero et al. 2006). It can accordingly be described as a cytokine-like hormone with pleiotropic actions. For pathological conditions to which dysregulated leptin activity contributes (see below), the blockade/activation of this activity in specific target tissues may be a useful therapeutic strategy in the near future. Leptin exerts its biological actions by binding to its receptors. These are encoded by the gene *diabetes (db)* and belong to the class I cytokine receptor superfamily, which includes receptors for IL6, LIF, CNTF, OSM, G-CSF and gp130. Alternative splicings of *db* give rise to six receptor isoforms:

- A. The soluble form Ob-Re, which lacks a cytoplasmic domain,
- B. Four forms with short cytoplasmic domains (Ob-Ra, Ob-Rc, Ob-Rd, Ob-Rf),
- C. The long form Ob-Rb, which is found in almost all tissues and appears to be the only form capable of transducing the leptin signal (Frühbeck 2006).

As in the case of other class I cytokine receptors, the main routes by which Ob-Rb appears to transmit the extracellular signal it receives are JAK-STAT pathways (Frühbeck 2006), which involve JAK2 phosphorylating tyrosines in the cytoplasmic domain of the receptor. In particular, mutation of the intracellular tyrosine Y1138 of mouse Ob-Rb prevents STAT3 activation and results in hyperphagia, obesity and impaired thermoregulation; and replacing Y1138 with a serine residue likewise causes pronounced obesity in knock-in mice. However, since Y1138S knock-in mice do not exhibit other defects of *db/db* mice, such as infertility, the role of leptin in the processes that are disrupted in these latter conditions must be independent of STAT3 (Bates et al. 2003). Indeed, the other two cytoplasmic

tyrosines of mouse Ob-Rb (Y985, Y1077) have been shown to bind other intracellular signalling molecules (Gualillo et al. 2002; Prodi and Obici 2006). The early studies of leptin focused on its anorexigenic action. Both in humans and rodents, leptin levels are closely correlated with BMI; and defects of the genes encoding for leptin and its receptors give rise to severe obesity and diabetes (Lago et al. 2007). Treating leptin-deficient mice with leptin induces a reduction in food intake, accompanied by an increase in metabolic rate and weight loss. Mutations of these genes in humans appear to be rare, but the cases that are known have occurred in families with a high prevalence of morbid obesity; again, leptin administration has ameliorated all the problems associated with leptin deficiency (Lago et al. 2007). As noted in previous sections, leptin participates in the control of food intake by acting on an intricate neuronal circuit involving hypothalamic and brainstem nuclei, where it integrates a variety of different orexigenic and anorexigenic signals (Ahima and Lazar. 2008). Leptin therapy is not an effective treatment for morbid obesity that is not due to congenital deficiency of leptin or leptin receptors. In these non-congenital types of obesity, leptin concentrations are already high as a consequence of increased fat mass. The persistence of obesity in spite of high leptin levels suggests that high leptin levels can induce leptin resistance. This may occur due to a leptin-induced increase of SOCS3, which blocks intracellular transmission of the leptin signal (Bjorbaek et al. 1999), but our understanding of leptin resistance is still limited.

Lipid metabolism is the balance between lipid synthesis and degradation that in turn determines fat mass. Almost the totality of energy stores is accumulated in adipocytes as triacylglycerol that can be hydrolyzed (lipolysis) following specific stimulations to release free fatty acids. Fatty acids have two possible fates:  $\beta$ -oxidation to produce ATP or reesterification back into triacylglycerol. A number of experiments have shown that leptin has a direct autocrine or paracrine mode of action on the rates of synthesis and degradation of lipids; however, before describing these effects it is important to keep in mind that these experiments primarily used in vitro cell or tissue preparations. Therefore, caution should be taken when extrapolating these results to in vivo conditions, where many other regulating factors exist. An auto- or paracrine mode of action was initially demonstrated by measuring leptin-induced changes in the lipolytic rates of cultured adipose tissue (Frühbeck et al. 1997). Isolated lean wild-type mouse adipocytes had, respectively, a 34% and 40% higher rate of lipolysis than *ob/ob* and *db/db* mice, even before leptin treatment. When adipocytes from lean mice were treated with leptin, the lipolytic rate increased by 28%, whereas the *ob/ob* mouse lipolytic rate increased to 123%. No change occurred in *db/db* mouse adipocytes that lacked a functional leptin receptor. Since leptin-driven increase in lipolytic rate was measured on an in vitro preparation, it is likely that the lipolytic effect of leptin on adipocytes is not dependent upon hypothalamic, neural or adrenergic control, although these components certainly modify in vivo leptin effects. A similar study was carried out to measure the lipolytic rates of ex vivo adipocytes of wild-type and *fal/fa* rats with or without leptin treatment (*fal/fa* rats do not possess a functional leptin receptor). Similarly to mice, adipocytes of the wild-type rats increased their lipolytic rate upon leptin

stimulation, whereas no change was observed in the adipocytes lacking a functional receptor (Siegrist-Kaiser et al. 1997). In another study, lean, *ob/ob* and *db/db* mice were treated with three different doses of leptin before isolating adipocytes and measuring lipolytic rates. As expected, no effect of leptin treatment was observed in the *db/db* mice. In addition, the two highest doses induced a change in the *ob/ob* mice and only the highest dose caused a significant increase in lipolysis in the wild-type mice (Frühbeck et al. 1998). The fact that the lipolytic rates of the *ob/ob* adipocytes were much more strongly affected by leptin than the wild-type adipocytes in both their experiments, suggests that, in the absence of a functional leptin protein as is the case in *ob/ob* mice, the receptor is strongly up-regulated.

Leptin likely mediates fatty acid metabolism by changing specific enzyme mRNA levels. For instance, the presence of leptin inhibits the expression of acetyl CoA carboxylase in adipocytes (Bai et al. 1996). This is the rate-limiting enzyme for long-chain fatty acid synthesis and it is essential for the conversion of carbohydrate to fatty acids and caloric storage as triacylglycerol. Under basal conditions (serum-free starved cells), cultured differentiating adipocytes expressing the *ob* gene have lower acetyl CoA carboxylase and fatty acid and triacylglycerol synthesis compared with cells that do not express the leptin gene. Long-term treatment of wild-type mice with elevated doses of leptin increased mRNA expression of hormone-sensitive lipase, the key lipolytic enzyme, while causing a decrease in mRNA expression of the lipogenic enzyme, fatty acid synthase (Sarmiento et al. 1997). Therefore, it is most likely that leptin actually regulates lipolysis by controlling the activity of hormone-sensitive lipase. This is an enzyme which is controlled by cellular levels of cyclic AMP (cAMP), and although the regulation of lipolysis by leptin at the molecular level has not yet been fully described, preliminary evidence suggests that leptin, like glucagon and catecholamines, stimulates lipolysis by increasing cAMP concentrations (Takekoshi et al. 1999). Leptin may also work via the central nervous system to reduce adipose tissue mass by inducing adipocyte apoptosis. Due to the fact that leptin treatment induced a rapid loss of fat mass with a very slow reversal of this process when treatment was terminated, it has been hypothesized that the delayed recovery was due to the deletion of some adipocytes (Qian et al. 1998). Indeed, histological analysis of adipose tissue and quantification of DNA fragmentation of the adipocytes from leptin-treated rats compared with the controls provided some evidence that apoptotic events occurred following leptin injection. Further research is certainly needed to confirm whether this interesting effect is a normal occurrence that takes place under physiological conditions. Leptin-driven modifications in lipid metabolism have also been measured in other tissues that store triacylglycerol. Leptin treatment of isolated pancreatic islets of rats causes an increase in fatty acid oxidation and a decrease in esterification, leading to a reduction in intracellular triacylglycerol content (Shimabukuro et al. 1997). Modifications in triacylglycerol content, fatty acid oxidation or esterification has been reported in rats with the *falfa* mutation (Heo et al. 2002). Pancreatic islets of *falfa* rats contain as much as 20× the amount of triacylglycerol found in lean rats (Lee et al. 1997). Rats lacking a functional leptin receptor exhibit significantly higher expression of acyl-CoA synthetase

and glycerol-3-PO<sub>4</sub> acyltransferase (two enzymes required for lipogenesis) and reduced expression of acyl CoA oxidase and carnitine palmitoyl transferase I (two enzymes involved in fatty acid oxidation). Because of these differences in enzyme expression, as well as the much higher lipid content of *falfa* non-adipocytes, it has been hypothesized that one of the functions of leptin is to keep the triacylglycerol content of non-adipocyte cells low, essentially limiting triacylglycerol storage to adipocytes (Walder et al. 1997). A physiologic role of the hyperleptinemia of caloric excess could be to protect non-adipocytes from steatosis and lipotoxicity by preventing the up-regulation of lipogenesis and increasing fatty acid oxidation (Lee et al. 2001). Leptin-induced increases in insulin sensitivity are well established and may be related to the effects of leptin on lipid metabolism: insulin resistance induced by an acute lipid infusion is prevented by hyperleptinemia (Dube et al. 2007). Leptin also mediates lipid metabolism indirectly by reducing the lipogenic effects of insulin. Addition of insulin to cultured leptin-deficient adipocytes induces an increase in synthesis of acetyl CoA carboxylase, fatty acids and triacylglycerol than in adipocytes that do produce leptin (Bai et al. 1996). This may be due in part because leptin might inhibit binding of insulin to adipocytes (Walder et al. 1997). In skeletal muscle the situation is almost similar; indeed, it has been demonstrated that both the rates of fatty acid oxidation and fatty acid incorporation in triacylglycerol are modulated upon stimulation of leptin, insulin, or both into isolated mouse skeletal muscle (Muoio et al. 1997). They found that fatty acid oxidation was increased in the presence of leptin. Conversely, insulin had the opposite effect, depressing fatty acid oxidation and increasing triacylglycerol synthesis. However, when insulin and leptin were administered together, leptin decreased insulin's effects on lipid oxidation and synthesis. The inhibitory effect of leptin on insulin action in both adipocytes and muscle suggest that high leptin levels in vivo probably depress the temporary postprandial lipogenic effect of insulin. This is relevant because it means that, when circulating levels of leptin are high (i.e. obesity), triacylglycerol synthesis is not fully activated, even when lipogenic hormones are present. Also, even though leptin is able to greatly decrease insulin-induced changes in lipid metabolism, it appears that leptin does not modulate the effects of insulin on glycogen synthesis, glucose oxidation or lactate production in non-adipocytes (Muoio et al. 1997). Therefore, although leptin can prevent full activation of insulin-induced lipogenesis, it probably does not interfere with insulin's primary function: the reduction of high circulating glucose levels by upregulation of muscle and liver glycogenesis. In adipocytes, however, leptin has been shown to impair many aspects of insulin's effects on glucose metabolism, including stimulation of glucose transport and glycogen synthase activity (Müller et al. 1997). A number of feedback loops exist among leptin and other hormones involved in lipid metabolism. Indeed, leptin has been implicated in promoting the production of T<sub>3</sub> (Legradi et al. 1997), whereas high T<sub>3</sub> levels have been shown to reduce circulating leptin levels (Escobar Morreale et al. 1997). Growth hormone (GH) also appears to have a negative feedback loop with leptin. Indeed, obese subjects exhibit a marked decrease in plasma GH levels (Luke and Kineman 2006). However, the mechanisms by which increased adiposity

leads to an impairment of GH secretion are poorly understood. It has been suggested that the adipose tissue can markedly influence GH secretion via two different signals, namely free fatty acids (FFA) and leptin (Diéguez et al. 2000). FFA appear to inhibit GH secretion mainly by acting directly at the pituitary level. Interestingly, reduction in circulating FFA levels in obese subjects leads to a marked increase in GH responses to different GH secretagogues. This indicates that FFAs exert a tonic inhibitory effect that contributes to blunted GH secretion in obese subjects. Recent data have shown that leptin is a metabolic signal that regulates GH secretion, since the administration of leptin antiserum to adult rats leads to a marked decrease in spontaneous GH secretion. However, leptin prevents the inhibitory effect exerted by fasting on plasma GH levels. The effect of leptin in adult rats appears to be exerted at hypothalamic level by regulating growth hormone releasing hormone (GHRH), somatostatin and neuropeptide Y (NPY)-producing neurones. In addition, during foetal life or following the development of pituitary tumors, leptin can also act directly at the anterior pituitary (Diéguez et al. 2000). It is notable that prolactin, another lactogenic hormone, is able to stimulate leptin secretion by white adipose tissue. Intriguingly, the effect of prolactin on leptin expression is likely gender-dependent (Gualillo et al. 1999).

Tumor necrosis factor alpha (TNF- $\alpha$ ), a classic cytokine with lipolytic activity, also has leptin-modulating properties. Leptin and TNF- $\alpha$  are both secreted by adipocytes and play a role in controlling lipolysis. In vitro treatment of rodent and human adipocytes with TNF has been shown to inhibit leptin secretion and mRNA expression (Yamaguchi et al. 1998) whereas in vivo treatment causes an increase (Kirchgessner et al. 1997). Supporting this in vivo effect is the fact that TNF-deficient mice have circulating leptin levels that are lower than those found in the wild types. Finally, it appears that leptin mRNA expression is also regulated by adipocyte transcription factors involved in cell differentiation. These transcription factors are peroxisome proliferator-activated receptors (PPARs) and CCAAT/enhancer binding proteins (C/EBPs). PPAR- $\alpha$  has been involved in the regulation of fatty acid oxidation enzymes (Leone et al. 1999) and UCP3 expression (Brun et al. 1999), while both PPAR- $\alpha$  and PPAR- $\gamma$  upregulate fatty acid-binding protein expression. Leptin has been found to up-regulate mRNA expression of both PPAR- $\alpha$  and PPAR- $\gamma$  (Ahima et al. 1996). It is still not clear, however, whether PPARs are able to regulate leptin gene expression, since not all studies reached a clear consensus about this topic. Finally, the relationship between leptin and lipid metabolism during fasting is particularly interesting. Independent of changes in weight loss, circulating leptin levels actually decrease with fasting or caloric restriction (Ahima et al. 1996), while rates of lipolysis and fatty oxidation increase (Klein et al. 1993). It is not yet known what triggers the decrease in leptin that is observed during fasting; however, a decrease in plasma insulin concentration and increases in FFA and corticosterone levels precede the drop in leptin. In addition, it appears that this drop is initially caused by a decrease in leptin secretion rather than mRNA expression (Dallman et al. 1999). The decrease in leptin concentration observed with fasting obviously does not act to depress lipolysis and fatty acid oxidation; therefore, other hormones or factors

involved in controlling lipid metabolism must take control. Another interesting aspect of fasting metabolism is the decline in energy expenditure that is usually concurrently observed with a fast or caloric restriction. This decline in metabolic rate is most likely mediated through the fall in  $T_3$  levels that occurs under these circumstances. It is possible that the decrease in leptin concentration observed with fasting contributes to this decrease in energy expenditure through its effects on thyroid function and uncoupling proteins (Legradi et al. 1997). However, this has still not been entirely clarified.

### ***11.2.2 Adiponectin***

Adiponectin [also called gelatin-binding protein 28 (GBP28), adipose most abundant gene transcript 1 (apM1) and 30-kDa adipocyte complement-related protein (Acrp30, AdipoQ)] is a 244-residue protein that, as far as is known, is produced prevalently by white adipose tissue (WAT). It increases fatty acid oxidation and reduces the synthesis of glucose in the liver (Berg and Scherer 2005). Ablation of the adiponectin gene has no dramatic effect on knock-out mice on a normal diet, but when placed on a high-fat, high-sucrose diet, they develop severe insulin resistance and exhibit lipid accumulation in muscles (Whitehead et al. 2006). Circulating adiponectin levels tend to be low in morbidly obese patients and increase both with weight loss and with the use of thiazolidinediones, which enhance sensitivity to insulin (Maeda et al. 2001). Adiponectin acts mainly via two recently described receptors, one (AdipoR1) found mainly in skeletal muscle and the other (AdipoR2) in liver (for a third route, see Sect. 11.2.3). Transduction of the adiponectin signal by AdipoR1 and AdipoR2 involves the activation of AMPK, PPAR (both  $\alpha$  and  $\gamma$ ) and presumably other signaling molecules also. Adiponectin exhibits structural homology with collagen VIII and X and complement factor C1q and circulates in the blood in relatively large amounts in oligomeric forms (mainly trimers and hexamers, but also a 12- to 18-mer form; Kadowaki et al. 2006), constituting about 0.01% of total plasma protein. It is somewhat controversial whether the various oligomers have different activities, which would make the effect of adiponectin controllable through its oligomerization state; and this may depend on target cell type. Although authors working with myocytes reported that trimers activated AMP-activated protein kinase (AMPK) whereas higher oligomers activated  $\text{NF}\kappa\beta$ , it has also been reported that 12- to 18-mers promote AMPK in hepatocytes (Lago et al. 2007).

Increasing evidences showed that adiponectin is involved in the regulation of both lipid and carbohydrate metabolism. Adiponectin likely has direct and indirect actions that would be related to a protective effect against cardiovascular disease (Gualillo et al. 2007). Indeed, it has been hypothesized that reduced adiponectin concentrations observed in obese patients (Arita et al. 1999) are involved in the development of atherosclerosis and cardiovascular diseases (Funahashi et al. 1999; Matsuzawa et al. 1999). Decreased adiponectin levels have been linked to small dense LDL and high apoB and triglyceride levels (Kazumi

et al. 2002). Some reports have shown that adiponectin has direct actions on vascular endothelium that would protect against cardiovascular disease in part by suppressing lipid accumulation in human macrophages (Okamoto et al. 2000; Ouchi et al. 2001; Tian et al. 2008). More evidence comes from studies with adiponectin knock-out mice which showed that these animals exhibited an increase in inflammatory response to vascular injury (Kubota et al. 2002). In addition, the fact that adiponectin administration prevented atherosclerosis in apoE-deficient mice (Okamoto et al. 2002; Yamauchi et al. 2003) provides further support to the concept that adiponectin protects against cardiovascular disease. Several genes linked to circulating adiponectin levels have pleiotropic genetic effects on serum HDL and triglyceride levels (Havel 2004). In addition, data from two large cross-sectional studies indicate that, after adjusting for gender and body adiposity, circulating adiponectin concentrations are negatively correlated with triglyceride levels and strongly positively correlated with plasma HDL concentrations (Cnop et al. 2003; Tschritter et al. 2003).

### ***11.2.3 Other Relevant Adipokines Contributing to Lipid Metabolism***

#### **11.2.3.1 Tumor Necrosis Factor Alpha and Other Pro-Inflammatory Cytokines**

White adipose tissue secrete a multitude of inflammatory cytokines including TNF- $\alpha$ , and several interleukins (e.g. IL-2, IL-6) which adversely affect both glucose and lipid metabolism. TNF- $\alpha$  is a paradigmatic proinflammatory cytokine produced by numerous cells, but mainly macrophages and lymphocytes. Adipocytes significantly contribute to synthesize TNF- $\alpha$  in rodents, and in lower amounts in humans. In rodents, TNF- $\alpha$  is involved in the pathophysiology of insulin resistance (Hotamisligil et al. 1993). However, as above-mentioned, TNF- $\alpha$  is poorly expressed in human adipose tissue and its role in alterations of dyslipidemia in humans is not completely understood. It is likely that the TNF contribution to alterations in lipid metabolism comes from an active cross-talk between invading stromal mononuclear cells and adipocytes. Interestingly, the infiltrating macrophages constitute the major source of inflammatory mediators within the adipose tissue and likely act synergistically with adipocytes amplifying local and systemic inflammation. TNF- $\alpha$  acts at several levels on adipocyte lipid metabolism. First, TNF- $\alpha$  inhibits the uptake of free fatty acids through a mechanism that likely involves down-regulation of fatty acid transport protein (FATP), fatty acid translocase (FAT) and fatty acid-binding protein FABP4/aP2. TNF- $\alpha$  also regulates lipoprotein lipase (LPL) expression, but studies investigating the role of TNF- $\alpha$  on LPL in human adipose tissue have reported conflicting results (Cawthorn et al. 2008). In addition to suppressing gene expression of key proteins of fatty acid uptake, TNF- $\alpha$  reduces the transcript levels and expression of many proteins involved in glyceroneogenesis, de novo fatty acid synthesis

and esterification. This leads to impaired triglyceride storage in adipose tissue. Notably, most of these genes are regulated by PPAR $\gamma$  activity, so it could mediate those effects primarily through the inhibition of PPAR $\gamma$  activity and expression. TNF- $\alpha$  also induces lypolysis by a complex mechanism in absence of insulin, suggesting the importance of nutritional state in this process. Although the precise mechanism by which TNF- $\alpha$  promotes lypolysis is far to be completely defined, it is clear that activation of TNF receptor type 1 by TNF binding involves downstream signals such as ERK1/2, JNK, AMPK, IKK and PKA. In addition, other transient mechanisms (i.e. controlled at transcriptional regulation level) are at play. The past 10 years has unveiled many novel aspects of TNF- $\alpha$  besides its classic effects as oncolytic and cachectic factor. Indeed, TNF- $\alpha$  significantly alters lipid storage and oxidative capacity of WAT. (For a review, see Cawthorn et al. 2008).

### 11.2.3.2 Acylation Stimulating Protein

Acylation-simulating protein (ASP) is a complement-derived protein which is an adipokine that both increases triglycerides clearance and triacylglycerol storage and enhances insulin sensitivity by increasing glucose transport into adipocytes. ASP also inhibits lipolysis by decreasing the hormone-sensitive lipase (Van Harmelen et al. 1999) and is increased in human obesity, being a marker for this condition much the same as is leptin (Cianflone et al. 2003).

### 11.2.3.3 Apelin

Apelin is a recently discovered adipokine widely increased during adipocyte differentiation and whose levels are elevated in obesity, particularly when it is associated with insulin resistance (Boucher et al. 2005). It is also a mediator of endothelial vasodilation that exerts beneficial cardiovascular effects and lowers blood pressure by a nitric oxide stimulation-dependent mechanism (Ashley et al. 2005). It is an antagonist to angiotensin II since it decreases vascular tone and angiogenesis as an anti-inflammatory secretagogue (Kasai et al. 2004). Apelin appears to regulate adiposity and lipid metabolism in both lean and obese mice. Additionally, apelin treatment increased mRNA expression of uncoupling protein 1 (UCP1; a marker of peripheral energy expenditure in brown adipose tissue; BAT) and of UCP3, a regulator of fatty acid export, in skeletal muscle (Higuchi et al. 2007).

Other secreted proteins indicated to be involved in energy metabolism (Sethi and Vidal-Puig 2007) are rather obscure regarding their source, function and relevance to humans.

### 11.2.3.4 Visfatin

Visfatin is a recently intensively studied new “adipokine,” because it was shown to mimic insulin effects (Kasai et al. 2004) and as such might have linked obesity and insulin resistance. However, subsequent studies in human subjects

reported conflicting results regarding its relation with adiposity, with subcutaneous or visceral fat distribution and with insulin resistance (Berndt et al. 2005; Chen et al. 2006; Pagano et al. 2006), suggesting that the role of this protein in the development of obesity and insulin resistance is unclear. In addition, other authors could not repeat the insulin-mimic effect *in vivo* and *in vitro* (Revollo et al. 2007). Visfatin is a rediscovered cytokine which is identical to Pre-B-cell colony-enhancing factor 1 and is functionally characterized as a cytosolic nicotinamide phosphoribosyltransferase involved in the biosynthesis of nicotinamide adenine dinucleotide (NAD), a vital factor in cell survival and in the regulation of insulin secretion in  $\beta$ -cells. Although visfatin has been regarded as an adipose tissue-related hormone, it is ubiquitously expressed in the body. Among different human tissues, the maximum gene expression was found in liver and peripheral blood leukocytes. Apparently, adipocytes and adipose tissue are not the major contributor to the high concentration of visfatin in human circulation (10–40 ng/ml). The lack of taking account of other tissues and organs, such as liver and immune system, may explain the inconsistent conclusions with respect to the relation of circulating visfatin to insulin resistance and adiposity. Recent studies found that circulating visfatin is associated with HDL-cholesterol in humans (Smith et al. 2006; Wang et al. 2007). The relation between visfatin and lipid metabolism may be explained by its function in the biosynthesis of NAD, because one of the NAD precursors, nicotinic acid, is able to increase HDL-cholesterol considerably (Carlson 2005). This suggests that visfatin is a novel modulator of HDL metabolism. Interestingly, early studies showed that visfatin is strongly induced in white blood cells by cytokines and lipopolysaccharides in experimental inflammation and clinical sepsis. It is known that lipopolysaccharides and sepsis affect a wide range of apolipoproteins, plasma enzymes, lipid transfer factors and receptors that are involved in HDL metabolism.

#### 11.2.3.5 Omentin

Omentin is another recently rediscovered protein which was found to be related to the visceral adipose depot and was suggested to regulate insulin action (Yang et al. 2006). It has been identified before as intelectin 1, a protein that is involved in body defense by binding to galactofuranoses on bacteria (Tsuji et al. 2001). Although omentin is related to adipose tissue, it is not secreted from adipocytes, but from stromal vascular cells. Also, omentin has been found expressed abundantly in human tissues other than adipose tissue, such as vasculature, small intestine, colon, heart and thymus. Recent studies focused on its role in the response to infections and only a few studies investigated its role in obesity and insulin action. Although it is neither clear nor confirmed whether this protein contributes to the development of obesity and insulin resistance, it remains of interest to understand how omentin contributes to the physiological difference between visceral and subcutaneous adipose tissue and, very probably, to the regulation of lipid metabolism.

### 11.2.3.6 Chemerin

Chemerin is an adipokine identified in 2007 (Bozaoglu et al. 2007; Goralski et al. 2007) whose plasma levels are strongly associated with several key aspects of metabolic syndrome. Chemerin also influences adipose cell function and, importantly, it is able to induce lipolysis in adipocytes (Roh et al. 2007) acting through its receptor. The chemerin receptor, now termed chemerinR, was isolated and found to be an orphan G protein-coupled receptor known to stimulate intracellular calcium release, phosphorylate extracellular signal-regulated kinase-1 and -2 (ERK 1/2) and to inhibit cAMP accumulation through its binding to Gi-coupled heterotrimeric G proteins. Now, it has also been demonstrated also that chemerin enhances insulin signaling and potentiates insulin-stimulated glucose uptake in adipocytes (Takahashi et al. 2008) and probably in the near future the role played by this adipokine in lipid metabolism regulation will be clarified.

## 11.3 Conclusions

A large number of other adipocyte-derived factors have also been recently identified, including among these vaspin or hepcidin; however, they have as yet not been studied in terms of their ability to modulate lipid metabolism in either health or disease. The discovery of leptin nearly 15 years ago enlightened a new epoch in research as it was eventually demonstrated that this adipocyte-derived peptide not only regulates energy production but also exerts peripheral effects on a large number of tissues. The relevance of adipokines to lipid metabolic function in either physiology or pathology is still emerging; and studies addressing this question represent an exciting area of research not only with respect to obesity, since adipokine production is generally related to adiposity, but also with respect to the central issue of the potential role of adipose tissue as an endocrine organ regulating overall metabolic function in health and disease. There are numerous challenges facing researchers in this field. Important among these is the fundamental question of precisely how leptin, adiponectin and other adipokines affect metabolic diseases. This task will undoubtedly be made easier and hastened by the development of new pharmacological tools targeting specific adipokine systems. A second major challenge is to understand how the various adipokines interact with each other since several adipokines with diverse biological properties can be released simultaneously and the net effect of increased adipokine production may not reflect the actions of a single individual substance. This remains a challenge for future investigations which are important, not only for a full understanding of the role of adipokines in the metabolic regulation of lipids, but also in terms of the potential to develop novel therapeutic targets.

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# Chapter 12

## Cellular Cholesterol Transport – Microdomains, Molecular Acceptors and Mechanisms

Christopher J. Fielding

**Abstract** “Reverse” cholesterol transport (RCT) from peripheral tissues to the liver is believed to play a major role in preventing accumulation of this lipid locally. Lipid-poor (prebeta-migrating) high-density lipoprotein (prebeta-HDL) plays a key and probably rate-limiting role in RCT, even though only a small proportion of the cholesterol content of circulating HDL originates from RCT. Normal RCT is explained here on the basis of a two-compartment recycling model. Prebeta-HDL is lipidated in interstitial fluid and lymph by ATP-dependent lipid transporters. These particles are then passed to the plasma compartment, where they become lipid-filled under the influence of the lecithin:cholesterol acyltransferase (LCAT) reaction without further input from transporters. In atherosclerosis, where activated macrophages are uniquely in contact with plasma, lipid transporters can directly stimulate RCT driven by LCAT.

### 12.1 Overview

The cholesterol content of peripheral tissues is maintained by the balance between influx and efflux pathways. Preformed cholesterol is transported out of plasma to the peripheral tissues, mainly in the form of the low-density lipoproteins (LDL) that originate as triglyceride (TG)-rich particles from the liver. Some cholesterol is made locally. This synthesis is suppressed by LDL. The sum of these two fluxes is balanced: (i) in gonadal and adrenal tissues by the conversion of some cholesterol to steroid hormones, (ii) in skin via the secretion of cholesterol from the sebaceous glands by direct loss of cholesterol directly from the skin (Capponi 2002; Fielding and Fielding 2008; Nikkari et al. 1975) and (iii) mainly by centripetal or “reverse” cholesterol transport (RCT) that drives cholesterol net transport from peripheral tissues to the liver for catabolism (Fielding and Fielding 2007; Lewis and Rader 2005).

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RCT begins when a peripheral cell transfers “excess” cholesterol to acceptors in interstitial fluid (IF). Most of these cells divide rarely or not at all, and they have little use for any additional cholesterol. From there, via the lymphatic collecting ducts, the complex of cholesterol with its protein carrier moves into the plasma compartment. This complex, together with lipids transferred from circulating lipoprotein particles, transfers cholesterol to the liver which is then secreted into bile, partly as cholesterol itself and partly, after catabolism, as bile acid. In both IF and plasma, RCT uses high-density lipoprotein (HDL) for its major carrier. In IF, a lipid-poor HDL fraction with a prebeta-electrophoretic migration rate is also present (Nanjee et al. 2000). It contains about 10% w/w lipid, particularly phospholipids [PL; 2–3 mol mol<sup>-1</sup> apolipoprotein (apo)-A-I; Lee et al. 2004]. Prebeta-HDL makes up ~ 5–10% of HDL particles in normal plasma. The balance of HDL is present in alpha-migrating particles. Alpha-HDL contains ~ 50% of lipid. Apo-A-I is the major and diagnostic protein of all HDL, and is the only protein of its prebeta-HDL fraction.

Two origins for lipid-poor HDL are now recognized. Prebeta-HDL are generated at the surface of hepatocytes that secrete lipid-free apo-A-I. This combines extracellularly with small amounts of PL, mostly lecithin, to generate an efficient acceptor of cellular cholesterol. A little apo-A-I originates from intestinal cells (Krimbou et al. 2006). However, the apo-A-I in most circulating prebeta-HDL is not newly synthesized. It has been detached from larger, more lipid-rich HDL during remodeling of the particle surface in the plasma compartment. This process, still poorly understood, takes place when large HDL interact with plasma lipases and lipid transfer proteins (Hennessy et al. 1993). It is not known whether this “recycled” prebeta-HDL is released from alpha-HDL as lipid-free apo-A-I, or as a lipid-poor complex. The practical effect of apo-A-I recycling between plasma and IF is that each molecule can function multiple times to promote RCT. In this, apo A-I differs from other lipoprotein structural proteins. The apo-A-I polypeptide is exceptionally flexible and can refold into several different conformations. This is probably how apo-A-I adapts to changes in HDL volume. Nevertheless changes in the surface lipid composition of alpha-HDL, as well as HDL diameter, occur at the same time. It is not clear which of these properties is the major driving force in apo-A-I recycling.

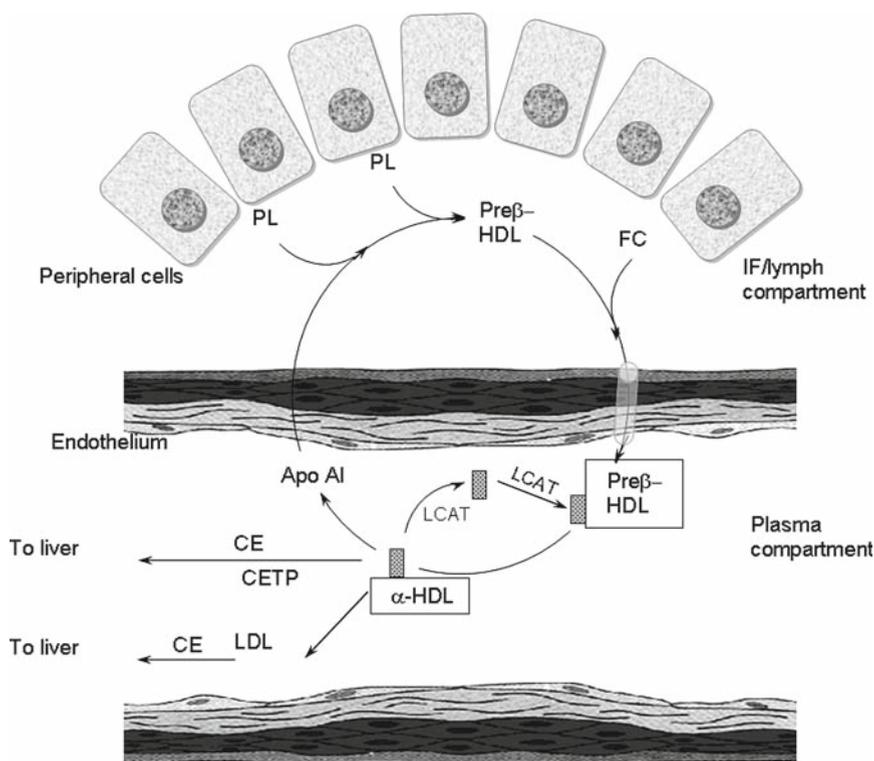
Almost all cholesterol in peripheral tissues is free (unesterified). Free cholesterol (FC) transfers spontaneously by simple diffusion through the aqueous phase, or by collision between lipid surfaces (cell membranes, lipoprotein particles). The unproductive exchange of FC between surfaces is much more rapid than observed rates of RCT. This means that the chemical potential of FC in all lipid surfaces would normally be at an equilibrium; and without input of metabolic energy, RCT would be zero.

Two different mechanisms have been identified to drive RCT. The effective concentration of FC in membranes is strongly influenced by their FC/PL molar ratio. Active transport of PL out of cells, driven by an ATP-dependent PL transporter, could promote the exodus of FC. This would continue until the chemical potential of FC in IF once more reached that of the donor membrane. ATP-dependent PL transporters are expressed by most peripheral and hepatic parenchymal cells (O’Connell et al. 2004). They are inactive, or nearly so, in vascular endothelial cells and the formed

elements of blood (Hassan et al. 2006; O’Connell et al. 2004). The role of transmembrane or extracellular proteins is emphasized by the observation that RCT to prebeta-HDL is blocked if living peripheral cells (fibroblasts, macrophages, vascular smooth muscle cells) pretreated with an extracellular protease (Kawano et al. 1993).

In plasma (but not IF or lymph), a second mechanism can drive RCT. FC exchanges readily between lipid surfaces while cholesteryl ester (CE) does not. Lecithin:cholesterol acyltransferase (LCAT) converts FC and lecithin (the major PL of both cell membranes and lipoproteins) into CE and lysolecithin. LCAT activity is restricted almost entirely to the plasma compartment (Clark and Norum, 1977). As a result of plasma LCAT activity, most (>75%) of the total plasma HDL cholesterol is esterified. Apo-A-I is cofactor for LCAT activity with HDL.

These data define a two-compartment model (Fig. 12.1). In the first (peripheral cells, IF, lymph) movement of FC out of cells is driven by lipid transporters. These generate prebeta-HDL which contain FC and PL but no CE. The second includes only the plasma. Lipid-poor HDL react with LCAT to form lipid-enriched, alpha-migrating particles. As part of this process additional apo-A-I molecules are recruited to the



**Fig. 12.1** Two-step model of RCT illustrating the lipidation of prebeta-HDL, the intravascular metabolism of prebeta-HDL and alpha-HDL formation via the LCAT reaction and recycling of apo-A-I as part of the surface remodeling of alpha-HDL. *CETP* Cholesteryl ester transfer protein

surface of each enlarging HDL (Nakamura et al. 2004). The detailed organization of apo-A-I at the HDL surface changes as they grow. This is indicated by modification in the pattern of apo-A-I epitopes recognized by monoclonal antibodies (Curtiss et al. 2000). This process involves changes in the composition of both HDL “core” (CE, triglyceride; TG) and surface (mainly FC, PL, apo-A-I). If CE is removed by selective transfer into the liver, a reduction of HDL particle size can lead to the release of apo-A-I. Part of this is filtered through the endothelium back to the IF.

Much of the current interest in RCT has been driven by the hope that a better understanding of the steps involved could lead to strategies that would reduce or prevent the cholesterol accumulation in the vascular bed which leads to atherosclerosis. It might be thought that details of the apo-A-I cycle would be by now well established. However, this is far from the case. Many aspects of the RCT reaction sequence and its regulation are poorly understood, in particular the mechanism of apo-A-I recycling. No assay is yet available to measure apo-A-I recycling in plasma. The rate-limiting step of the apo-A-I cycle is unknown. It is not clear whether the rate of apo-A-I recycling (a major contributor to the availability of prebeta-HDL for RCT) can be modified by drugs or diet. The sections that follow review recent research on each step of this complex reaction.

## 12.2 Structure and Properties of the Cell Surface

RCT begins at the plasma membrane of quiescent peripheral cells. Like most cells *in vivo*, these retain little newly synthesized sterol, or FC from internalized LDL. This situation contrasts with that seen in transformed and other continuous cell lines. Here much of the new FC helps form new membrane. The choice of an appropriate cell model to study RCT has bedeviled research in this field.

Quiescent cells have more cholesterol than transformed cells in surface FC-rich microdomains. Much of this cholesterol is in caveolae. These are membrane invaginations stabilized by the structural protein caveolin (Sens and Turner 2004). When primary cells do divide, much of their plasma membrane FC is withdrawn from caveolae and FC efflux is decreased. Caveolin at the cell surface is transferred to intracellular pools (Fielding et al. 1999). Synthesis of new caveolin is inhibited at the transcriptional level. Multiprotein complexes at the cell surface associated with FC microdomains dissociate (Zeidan et al. 2003). Each of these processes is reversed in a co-ordinated way when the cell re-enters the quiescent stage.

There is controversy as to whether FC-rich (caveolar) or FC-poor microdomains of the peripheral cell surface contribute most to the FC that is lost from peripheral cells by RCT. It has been argued, based on the structure of synthetic phospholipid vesicles and in particular from its condensing effect on PL acyl chains, that less energy would be needed to transfer FC from lipid-poor areas than from FC-rich domains of the plasma membrane (Rothblat et al. 1992). However it was recently shown that lipid packing is looser, not tighter, in caveolae compared to FC-poor domains in the same membranes (Hill et al. 2005). This probably reflects that distorting effects on caveolin on local lipid packing in caveolae.

When the FC content of caveolae was monitored with cholesterol oxidase, this pool was selectively depleted when the cells were incubated either with  $\beta$ -methyl cyclodextrin, a synthetic FC sequesterant (Parpal et al. 2001), or with prebeta-HDL in normal plasma (Fielding and Fielding 1995). It has been reported that, in detergent-treated membranes, PL transporters and caveolin appear in different fractions (Mendez et al. 2001; Sdrobnik et al. 2002). However it has since been recognized that this treatment can modify the composition of membranes (Ortegren et al. 2004). Most recently, confocal microscopy of intact plasma membranes has shown that lipid transporters and caveolae may be in the same microdomain in some intact cells (Lin et al. 2007).

There is also disagreement about the contributions of different molecular mechanisms to RCT. Passive FC exchange, not RCT, was found to predominate in some continuous cell lines (Adorni et al. 2007). In contrast, receptor-based mechanisms depending on lipid-poor HDL appeared to be most important in cultured primary cells, such as those from human aortic endothelium and smooth muscle (Fielding and Fielding 2001). These arguments emphasize the relevance of primary cells as models of RCT.

### 12.3 Role of Cell-Surface Lipid Transporters in RCT

In IF, LCAT is almost absent (Clark and Norum 1977). A major role for plasma membrane PL transporters there is indicated. Contributions by several members of the ATP-binding cassette (ABC) family of lipid transporters have been suggested. One of these, ABCA1, is now broadly accepted to play a key role (Singaraja et al. 2006). In human Tangier disease, where ABCA1 is genetically defective, no normal HDL are formed, despite normal synthesis and secretion rates for apo-A-I. The low circulating concentration of apo-A-I in the plasma of Tangier patients (1–2% of normal) is secondary, reflecting an inability of Tangier cells to transfer PL to apo-A-I to initiate RCT. Also, the lipid-free polypeptide is rapidly filtered by the kidney. A high proportion of apo-A-I in Tangier plasma circulates as a proprotein that retains a 6-amino-acid propeptide. Proapo-A-I in Tangier disease plasma reflects the high proportion of newly secreted protein in this fraction. Newly secreted proapo-A-I bound PL poorly compared to the mature protein (Chau et al. 2006). We recently showed proapo-A-I to be cleaved extracellularly by a metallic proteinase, bone morphogenetic protein-1 (BMP-1) (Chau et al. 2007). These data confirm that ABCA1 plays a key role in the early synthesis of normal HDL for RCT.

Other studies suggest that ABCA1 plays additional roles. Transport of proteins through the Golgi secretion pathway is inhibited in ABCA1  $-/-$  cells (Zha et al. 2003). The transporter may play a role in regulating the PL composition of Golgi transport vesicles. A second activity supported by ABCA1 is that of a molecular chaperone, refolding apo-A-I at the surface of hepatocytes, prior to its PL lipidation by ABCA1. Transfer of PL mediated by ABCA1 follows, and depends on, this earlier refolding step. The chaperone and PL transporter roles of ABCA1 can be distinguished by the sensitivity of only the latter function to the ATPase inhibitor glyburide (Chau et al. 2006).

There has been controversy as to whether FC is carried to apo-A-I by primary active transport of ABCA1. Because of the complex three-dimensional structure of

this transporter and a lack of information about its mechanism, protein binding sites that might be directly involved in PL and FC transport have not yet been identified. However, several studies throw additional light on this question. PL transfer to the exofacial leaflet of the membrane bilayer by ABCA1 is not dependent on the presence of apo-A-I or other extracellular acceptors. Cells expressing ABCA1 that are incubated in the absence of apo-A-I accumulate “blebs” on their exofacial surface that represent “excess” PL (Wang et al. 2000). Some Tangier cell lines with mutations within the extracellular loops of ABCA1 lack PL transfer activity, though they retain the ability to bind apo-A-I (Singaraja et al. 2006). Transfer of PL to apo-A-I does not depend on co-transport of FC, since this lipid can be transferred to preformed prebeta-HDL by cells without functional ABCA1 (Fielding et al. 2000). These data argue that direct (primary) FC transport is not a necessary, and possibly, not even a significant function of this transporter.

ABCA1 may play one additional role, by mediating PL transfer from a cell surface to the plasma phospholipid transfer protein (PLTP; Oram et al. 2003). PLTP actively redistributes PL between lipoprotein and PL vesicles. PLTP deficiency reduced FC efflux promoted by ABCA1 in macrophage foam cells (Lee-Rueckert et al. 2006). Part of the PL leaving the cell under the influence of ABCA1 may be directed to lipoproteins other than prebeta-HDL (alpha-HDL is not an acceptor for ABCA1-derived lipids). It is not known whether either lipid-free apo-A-I or prebeta-HDL could accept PL directly from PLTP.

## 12.4 Cholesterol Efflux and the LCAT Reaction

Newly synthesized apo-A-I of hepatic origin and recycled apo-A-I can both be converted to lipid-poor, prebeta-migrating HDL that initiates RCT. It was widely accepted until recently that prebeta-HDL could grow in IF and/or lymph by additional of further PL and FC from surrounding cells, in the absence of LCAT, to generate a discoidal product resembling that formed *in vitro* from isolated lipid-free apo-A-I and PL. These discoidal HDL could then be converted to spherical alpha-HDL by the action of LCAT. This model seemed to be supported by the observation that in congenital and acquired LCAT deficiency, discoidal lipoproteins were a prominent feature of the lipoprotein spectrum in plasma and lymph.

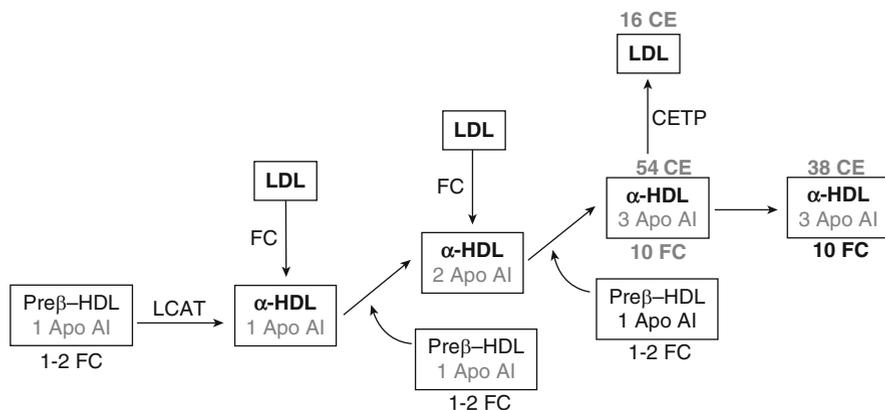
More recently, it seemed likely that this otherwise attractive scenario does not fit experimental observations on the properties and composition of lymph HDL and on the substrate specificity of LCAT. Discoidal HDL are almost absent from normal human lymph (Kujiraoka et al. 2003). Discoidal HDL identified in plasma and lymph in human LCAT deficiency were found to contain bound apo-E, but very little apo-A-I. In fact, prebeta-HDL of apparently normal properties were prominent there, in co-existence with the apo-E discs (Mitchell et al. 1980). Further, in cultures of human hepatocytes, even extended incubation of prebeta-HDL did not lead to the appearance of apo-A-I discoidal HDL or other PL-rich forms larger than prebeta-HDL (Chau et al. 2006, 2007). These data suggest that discoidal HDL might not, after all, be an obligatory intermediate in the conversion of prebeta- to alpha-HDL.

By studying synthetic lipid-poor recombinants of apo-A-I, PL and FC with a composition similar to that of prebeta-HDL, Sparks et al. (1999) showed that LCAT was very reactive with apo-A-I monomers containing as few as 2–3 moles of PL and FC. The  $V_{\max}$  of LCAT with these lipid-poor apo-A-I particles was greater than with native alpha-HDL. These observations were extended by our laboratory. We studied the metabolism of lipid-poor (prebeta-)HDL in native plasma (Nakamura et al. 2004). These particles not only efficiently bound  $^3\text{H}$ -FC newly transferred from equilibrium-labeled cultured cell monolayers. They also reacted more efficiently with LCAT than the rest of HDL in plasma. The significance of this observation is that a pathway by which cell-derived FC was a preferential substrate for LCAT would significantly increase the efficiency of RCT. CE accumulated in the labeled alpha-HDL particle. Of interest, these particles became enlarged by fusion with additional apo-A-I units. These data strongly support a model in which prebeta-HDL, released into the plasma from lymph, is directly a substrate for LCAT. No evidence was found for an intermediate discoidal HDL. As the lipid content of unreacted prebeta-HDL is very small, we were unable to determine whether lipid-free apo-A-I or prebeta-HDL bound to the enlarging complex initiated by prebeta-HDL and LCAT. These data suggest a model in which prebeta-HDL, first formed in IF by binding cell-derived PL and FC, is converted to alpha-HDL in plasma by LCAT. It is possible that apo-A-I recycles from alpha-HDL with a small amount of PL. The evidence is not yet available.

There is an important consequence of these findings in RCT *in vivo*. It is that most of the FC supplied to alpha-HDL over its life-time must originate not by RCT from peripheral cells, but by spontaneous transfer from other lipoproteins (and perhaps from red blood cells) in the plasma compartment. This means that the rate of LCAT activity and that of other parameters reflecting cholesterol metabolism in the plasma compartment such as HDL cholesterol (HDL-C) levels, need not reflect the rate of RCT. The rate-limiting step of RCT is likely to be determined by events in the IF/lymphatic pool – the activity of PL transporter ABCA1, or the availability of its PL acceptor (newly secreted or recycled apo-A-I).

LCAT converts PL and FC in equal numbers to CE. About 30% of the CE formed on the reaction product of LCAT and prebeta-HDL was transferred away to other plasma lipoprotein particles (Nakamura et al. 2004). Some CE is likely lost to the liver via receptor CLA-1 (the human analog of SR-BI, though the proportions of HDL-CE uptake by this and endocytosis pathways are not yet established and its expression in human liver is lower than in steroid-forming tissues; Cao et al. 1997). We can first estimate how many molecules of FC and PL are needed to convert prebeta-HDL in IF to alpha-HDL in plasma (Fig. 12.2).

Each prebeta-HDL would contribute 1–2 mol of FC, or a maximum of 3–6 mol for an alpha-HDL particle containing three apo-A-I. The CE content of the alpha-HDL can be shown to contain ~38 mol of CE, representing 70% of the CE formed there (54 mol of CE) formed by LCAT from the same number of FC. The HDL contains 10 mol of FC. Formation of an alpha-HDL particle from three prebeta-HDL requires roughly 64 FC, of which no more than three to six would have originated, by RCT, from peripheral cell membranes. The balance (58–61 FC) would have been transferred by simple diffusion or collision from other plasma lipoprotein particles, mostly LDL. The LCAT activity in plasma is ~20  $\mu\text{g}$  FC esterified  $\text{ml}^{-1}$  plasma  $\text{h}^{-1}$  or about 2.4  $\text{g}$  day $^{-1}$



**Fig. 12.2** Phospholipid (PL), free cholesterol (FC) and cholesteryl ester (CE) fluxes on HDL during RCT

for a human subject of 75 kg and a plasma volume of 5 l. Applying the ratio calculated above, this suggests that RCT in this subject is 0.12–0.24 g FC day<sup>-1</sup>. If RCT contributes only a small proportion of FC required for LCAT activity, this could explain why plasma parameters such as LCAT and CETP activities, and HDL cholesterol itself, are normally not sensitive indicators of RCT (Dietschy and Turley 2002).

## 12.5 Significance of ABCG1

A key role in RCT has been ascribed to a second cell surface transporter, ABCG1 (Cavelier et al. 2006). This promotes FC from cell surfaces to alpha-migrating HDL and PL vesicles, but not to lipid-free apo-A-I. In normal mice in which ABCG1 had been knocked out, the plasma cholesterol level, including that of HDL cholesterol, remained within normal limits. The HDL in these animals also had a normal composition. The main pathological finding was an accumulation of PL and FC in lung macrophages. Although ABCG1 was widely expressed, levels of FC and CE were not increased in most cells in ABCG1 <sup>-/-</sup> mice (Kennedy et al. 2005). Homozygous ABCG1 mutation in human subjects has not been reported, so whether this would be linked to disease is not known. ABCG1, though present, was inactive in endothelium in promoting FC efflux to HDL (O'Connell et al. 2004). In view of the large contribution of plasma FC and PL to the enlargement of alpha-HDL, it would be surprising if ABCG1 played an obligatory role in normal HDL metabolism.

## 12.6 Recycling of apo-A-I

It is widely agreed that the stability of the hydrophobic core of plasma lipoprotein particles depends on the presence of a monomolecular film of polar lipids and protein. Alpha-HDL are subject to a number of different reactions that affect their

core/surface ratio. CE is transferred from the core of HDL to acceptor tissues (adrenals, gonads, liver) via scavenger receptor B-I. Independently, part of HDL-CE is exchanged for TG on other lipoprotein particles via the activity of CETP. Unlike the reaction with SR-BI, this has little direct effect on HDL core volume, though a TG molecule is somewhat larger than a CE. However the exchange of CE for TG makes these HDL a substrate by plasma lipases, whose product, unesterified fatty acids, is transferred away to albumin.

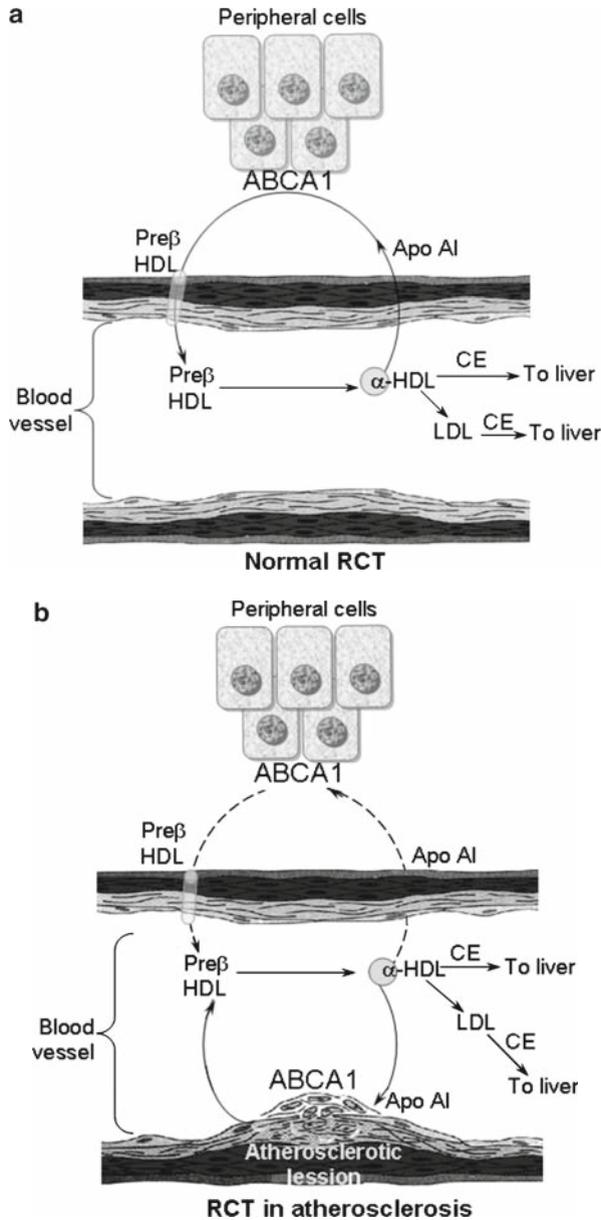
A net decrease in core can lead to one of two effects: further CE formation and storage under the influence of LCAT, or loss of “surface” components (PL, FC, protein, especially apo-A-I). The equilibrium between these processes appears to be one major factor that stabilizes the distribution of HDL species in the plasma compartment. Other changes affect the composition of HDL without changing the ratio of surface to volume. For example, another protein could binding to HDL, displacing apo-A-I and promoting recycling and RCT. It was recently found that when a newly identified HDL protein, apolipoprotein M (apo-M) was knocked out in mice, prebeta-HDL entirely disappeared from plasma. If apo-M was over-expressed, prebeta-HDL levels were increased (Wolfrum et al. 2005). These data suggest that an equilibrium between apo-M and apo-A-I could determine recycling. However other interpretations are possible. When LCAT activity is blocked in plasma, prebeta-HDL levels increase. When LCAT acts in plasma in the absence of nucleated cells, prebeta-HDL disappears (Kawano et al. 1993). Apo-M could mediate its observed effects not by promoting apo-A-I recycling, but by regulating LCAT activity. Apo-M could stimulate PLTP activity to promote prebeta-HDL production indirectly. A systematic study of the effects of apo-M on the metabolic rates of lipid factors in native plasma will be needed to unravel this skein of connected reactions.

A second unresolved question is the composition of the apo-A-I that dissociates from alpha-HDL. Is this lipid-poor HDL with the composition of prebeta-HDL in plasma? Or is it lipid-free apo-A-I? If so, can this apo-A-I immediately bind PL from plasma lipoproteins, or does it need to interact first in IF with ABCA1? This is an important issue, because it frames whether ABCA1 has a continuing role to play in activating apo-A-I for RCT, or this is limited to the activation of newly secreted apo-A-I. We recently presented data indicating that new apo-A-I was monomeric not dimeric. The same seems likely for recycled apo-A-I. Otherwise, co-existence of alpha-HDL with 2, 3 and 4 apo-A-I/particle would be difficult to explain.

## 12.7 RCT from Activated Macrophages

RCT from extrahepatic tissues is important in determining the FC content of their cells. The ideal response of cells to increased cholesterol uptake local synthesis of FC, would be to increase RCT via the two-compartment pathway described above by increasing the activity of ABCA1 or concentration of prebeta-HDL in IF. However another mechanism may come into play in atherosclerosis. In this condition, the physical separation of the earlier and later steps of RCT breaks down. Vascular

lesions (plaques) containing lipid-filled activated macrophages (foam cells) are exposed at the endothelial surface of the large vessels. These cells express high levels of ABCA1 and ABCG1 (Out et al. 2008) and these activities are directly



**Fig. 12.3** Comparison of RCT between normal peripheral cells (a) and vascular lesion-activated macrophages (b)

accessible to both prebeta-HDL and alpha-HDL in the plasma (Fig. 12.3). If this model is correct, the expression of lipid transporters in macrophage/foam cells may represent a defense (though ultimately not successful) against increased FC levels in the vascular bed, using increased levels of apo-A-I recycling, equilibrium prebeta-HDL and RCT.

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# Chapter 13

## The Ins and Outs of Adipose Tissue

Thomas Olivecrona and Gunilla Olivecrona

**Abstract** The aim of this chapter is to discuss what mechanisms are available to rapidly modulate fatty acid uptake/mobilization in adipose tissue. The major pathway for net uptake is lipoprotein lipase (LPL)-mediated hydrolysis of lipoprotein lipids. There are several mechanisms for control and they all serve to suppress LPL activity on a time-scale of hours in the setting of essentially unchanged LPL mRNA and mass. A protein complex that specifically binds to LPL mRNA can block synthesis of new enzyme. The  $\text{Ca}^{2+}$  milieu, and perhaps other conditions in the ER, can partition more of the enzyme towards intracellular degradation and less for export. After secretion from the adipocytes, active LPL can be converted into inactive monomers through interaction with angiopoietin-like proteins. At the vascular endothelium, product control may balance LPL action. If fatty acids accumulate at sites of lipolysis they eliminate the effect of apolipoprotein CII, which is a necessary activator for LPL. Intracellular lipolysis is initiated by adipose tissue triglyceride lipase (ATGL) which hydrolyzes triglycerides to diglycerides. These can either be re-esterified by a diacylglycerol acyl transferase (DGAT) enzyme or further hydrolyzed by hormone-sensitive lipase (HSL). The system is controlled by phosphorylation mediated by protein kinase A, and perhaps other protein kinases, as well as protein phosphatases. The prime target is perilipin, a lipid droplet protein which in its unphosphorylated form suppresses the activity of both ATGL and HSL. The two lipase systems are modulated by different mechanisms and on different time-scales. Both systems seem to operate at levels that generate an excess of fatty acids. The overriding control of how much gets deposited in the tissue as triglyceride and how much spills over into blood as albumin-bound fatty acids (NEFA) is exerted by the rate of glyceride synthesis. Recent studies show that glycerol-3-phosphate for this is generated mainly through glyceroneogenesis from citric acid cycle intermediates.

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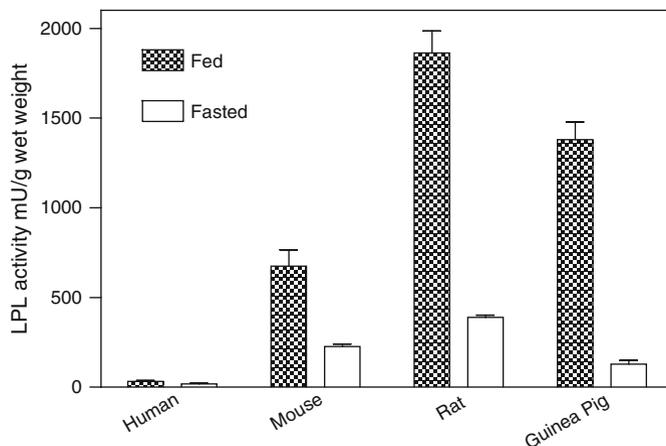
**Abbreviations** Angptl, angiopoietin-like protein; Apo, apolipoprotein; AKAP, A kinase-binding protein; AQP, aquaporin; ATGL, Adipose triglyceride lipase; CETP, cholesteryl ester transfer protein; CGI-58, comparative gene identification 58; cld, combined lipase deficiency; CoA, coenzyme A; DGAT, diacylglycerol acyl transferase; DG, diglyceride; EM, electron microscopy; ER, endoplasmatic reticulum; FATP, fatty acid transport protein; GPI, glycerolphosphatidylinositol; GPIHBP, glycerolphosphatidylinositol-linked high-density binding protein; HDL, high-density lipoprotein; HSL, hormone-sensitive lipase; HSPG, heparan sulfate proteoglycan; LDL, low-density lipoprotein; Lmf, lipase maturation factor; LPL, lipoprotein lipase; LPS, lipopolysaccharide; LRP, low-density lipoprotein receptor-related protein; MAGH, monoacylglycerol hydrolase; MG, monoglyceride; MGAT, monoacylglycerol acyl transferase; NEFA, non-esterified fatty acids (also called albumin-bound free fatty acids); PTH, parathyroid hormone; PKA, protein kinase A; RAP, receptor-associated protein; SR-B1, scavenger receptor type B1; SREBP, steroid regulatory element binding protein; TG, triglyceride; UTR, untranslated region; VLDL, very low-density lipoprotein

### 13.1 Introduction

The major chemical form in which the body stores energy substrate is the triglyceride. This is a smart molecule for the purpose. Triglycerides can be stored in very compact form as lipid droplets (Chap. 1). When mobilized during starvation, the triglyceride molecule provides fatty acids as a direct energy source for most tissues and a substrate for production of ketone bodies which, together with glucose produced by gluconeogenesis from the glycerol released, can provide an energy substrate for the nervous system. On a day to day basis, the main function of adipose tissue is to serve as a buffer for daily lipid flux (Frayn 2002), with net uptake of fatty acids in the postprandial state and net release between meals. The tissue also sends out a number of signal molecules that participate in the regulation of energy metabolism, substrate selection and appetite (Chap. 12).

The triglyceride molecule cannot be efficiently moved across cell membranes and extracellular spaces. For this it has to be split into fatty acids and monoglycerides which move more readily. Therefore, lipases are central for both uptake and release of lipids from adipose tissue. This chapter focuses on the properties of these lipases and what mechanisms are available to control their action in harmony with overall energy metabolism in the body. Our objective is not to cover the physiology, endocrinology or pathology of these processes. There are several excellent recent reviews on these subjects (Arner and Langin 2007; Brasaemle 2007; Granneman and Moore 2008; Jaworski et al. 2007; Mead et al. 2002; Merkel et al. 2002a; Otarod and Goldberg 2004; Preiss-Landl et al. 2002; Pulinilkunnil and Rodrigues 2006; Stein and Stein 2003; Watt and Steinberg 2008; Zechner et al. 2000, 2005, 2008).

A word of caution is in place before we begin our discussion. Most of the data we discuss come from experiments on rodents and are not necessarily directly applicable

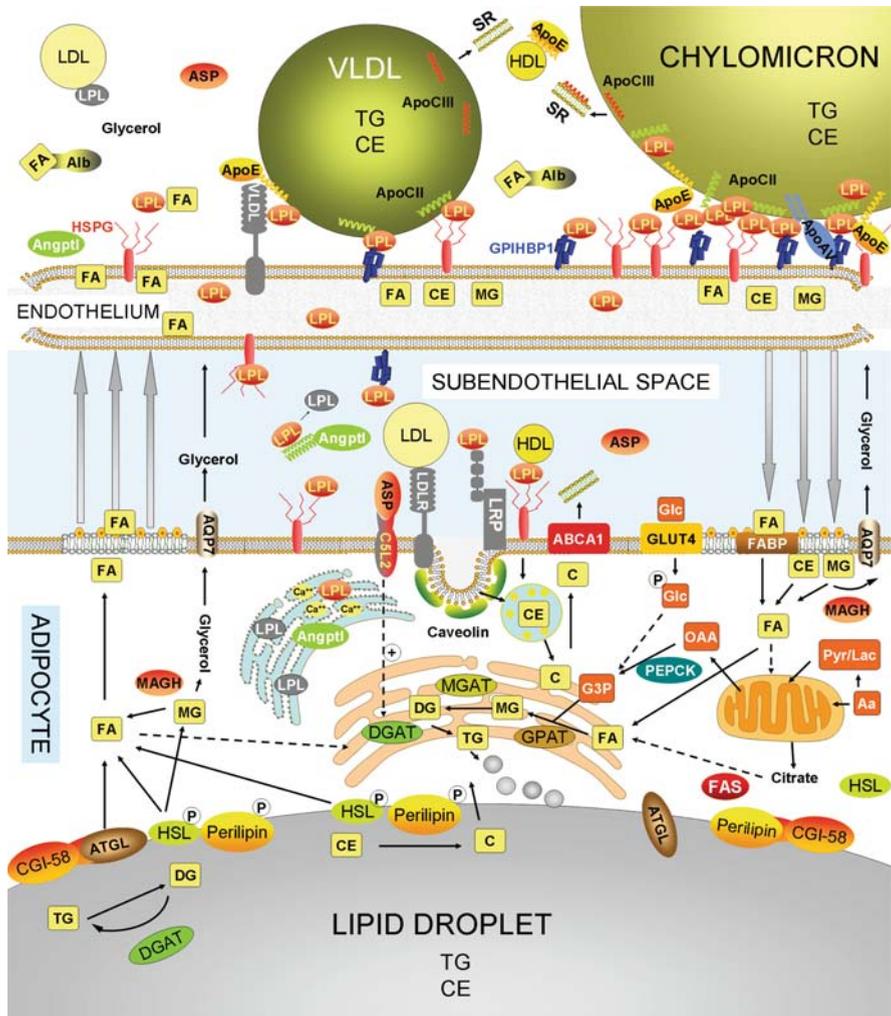


**Fig. 13.1** Changes of adipose tissue LPL activity with nutritional state. The data are from previous publications from our laboratory (Bergo et al. 1996; Ruge et al. 2004, 2005; Semb and Olivecrona 1986) and represent total LPL activity per gram tissue before and after fasting overnight. For rats it is known that the activities change with age and body weight. The same is probably true for guinea pigs and mice, so that the activities in the fed state in these three species should be considered as in the same range. The ability to down-regulate LPL activity on fasting appears to be more pronounced in guinea pigs than in rats or mice. The most marked species difference is, however, the much lower adipose tissue LPL activity in humans compared to the other three species, both fed and fasted

to human physiology. On the contrary, we know that there are major differences in the lipid transport systems. Compared to humans, rats and mice have low LDL and high HDL, they have no CETP in their blood and they produce chylomicron-like apoB48-lipoproteins in their livers. In mice the hepatic lipase circulates in plasma, whereas in humans (and rats) it is confined to the liver. Important to the current topic is the large difference in adipose tissue lipoprotein lipase (LPL) activity between rodents and humans (Fig. 13.1). Also important for interpretation of the data are the regional differences between different localities of adipose tissue, subcutaneous, visceral, pericardial, mammary, etc (Santosa and Jensen 2008). A further complexity is the differences between small adipocytes from young/lean animals compared to enlarged adipocytes in older/obese animals (Hartman 1977). We do not address these variations between species and between anatomic locations in any systematic manner since the objective of our review is to discuss the mechanisms involved in lipid uptake and release from a biochemical/molecular biological perspective.

### 13.2 Sources of Lipids for Deposition in Adipose Tissue

In principle, fatty acids can be deposited in adipose tissue by net uptake from blood or by local synthesis in the tissue (Fig. 13.2). The adipocytes have the enzymes required for fatty acid synthesis, but under most conditions *de novo* fatty acid synthesis in



**Fig. 13.2** The metabolic networks involved in lipid transport into and out from adipocytes. The *left* part shows pathways involved in mobilization. Perilipin is phosphorylated and has brought HSL (which is also phosphorylated) to the lipid droplet. ATGL forms a complex with its activator (CGI-58) and hydrolyzes TG to DG which are either re-esterified to TG by DGAT, or further hydrolyzed to MG by HSL and to glycerol by MAGH. The glycerol moves out from the adipocytes through the AQP7 channel. FA from all three steps of hydrolysis of CE probably mix and move out from the adipocyte, perhaps primarily through raft areas of the plasma membrane. After crossing (or moving around in the membrane of) the endothelial cells the fatty acids bind to albumin and leave the tissue. LPL is synthesized in RER (still inactive, *gray* symbol) and matures with the aid of calcium ions ( $Ca^{2+}$ ), into active dimers (*red* symbol) which are secreted and bind to heparan sulfate proteoglycans (HSPG), or other LPL-binding molecules, at the surface of the adipocytes. In the fasting state, production of angptl4 in the adipocytes is up-regulated and the protein converts most of the LPL dimers to inactive monomers, probably outside the adipocyte. Some LPL molecules escape this and move along cell surfaces to reach the luminal surface of blood vessels. High affinity binding to GPIHBP1 may cause a gradient (and/or in vesicles through the endothelial cells) that drives the movement of active LPL.

the tissue is of small magnitude (Chong et al. 2007). The main source of fatty acids is the lipoproteins in blood. This is true in man as well as in rats and mice. One should be aware that the conditions may be different in other animal species. For instance, in ruminants, a major pathway for lipid deposition is de novo synthesis from acetate.

There are two potential sources of fatty acids for uptake from blood, plasma non-esterified fatty acids (NEFA) and fatty acids released by LPL-mediated hydrolysis of lipoprotein-triglycerides. Bragdon and Gordon (1958) compared the uptake of labeled fatty acids into adipose tissue from chylomicrons and from NEFA and found that a much larger fraction was taken up from chylomicrons. This observation has been repeated many times in animals. Recently, Bickerton et al. (2007)

**Fig. 13.2** (continued) At the endothelium the enzyme can engage VLDL particles and hydrolyze their TG. Several other molecules take part in formation of such a “binding-lipolysis” site, apoCII which activates LPL, the VLDL receptor which can bind LPL and perhaps escort the enzyme (and lipoproteins) across the endothelial cells. The VLDL receptor may also bind apolipoproteins B (not shown) and E on the lipoprotein. ApoAV promotes the lipolysis in some yet undefined way. The FA generated by LPL bind to albumin and join the FA from intracellular lipolysis into the circulating blood. The MG move into the adipocytes, perhaps mix with MG from intracellular lipolysis and are hydrolyzed by MAGH or possibly re-esterified by MGAT. When the particle loses core volume by triglyceride hydrolysis, excess surface is shed as “surface remnants” (SR) in the form of cholesterol (C)-phospholipid-apolipoprotein discs which leave the tissue and VLDL are remodeled into HDL. Excess FA may displace some LPL from the cell surface into blood. Some of the LPL rendered inactive by interaction with angptl4 also leaves the tissue with blood to be taken up and degraded in the liver. In the fed state (*right* part of the figure) there are chylomicrons in blood and insulin suppresses production of angptl4. Most of the LPL molecules now make it to the vascular endothelium in active form. Large “binding-lipolysis” sites are generated where many LPL molecules act simultaneously on the chylomicron and cause the very rapid delipidation characteristic for chylomicron metabolism. Some of the FA generated spill over into blood, but most of the FA move into the tissue, driven by a gradient generated by active synthesis of TG. The MG also move into the adipocyte and are hydrolyzed by MAGH or perhaps are directly esterified by MGAT to DG and then by DGAT to TG. De novo synthesis of TG is stimulated by ASP which binds to its receptor CSL2 on the adipocyte membrane. The glycerol-3-phosphate (G3P) needed for GPAT may come in part from glycolysis, but most is probably generated by glyceroneogenesis by way of oxaloacetic acid (OOA) from citric acid cycle intermediates and from pyruvate/lactate (Pyr/Lac) that the adipocyte may have taken up from blood. Some of the glycerol from hydrolysis of MG may be phosphorylated to G3P but most of the glycerol returns to blood by way of the AQP7 channel. Glucose (Glc), transported into the adipocyte by GLUT4, may also serve as a substrate for de novo synthesis of FA (not shown in detail). In this nutritional state perilipin and HSL are dephosphorylated. Perilipin now captures CGI-58 and ATGL displays low activity as it is deprived of its activator. Some basal lipolysis continues and generates DG but most of these are re-esterified by DGAT since HSL has been displaced from the droplets into the cytoplasm. In both nutritional states (*center* of figure) lipoproteins can bind to receptors (LDL-receptor, LRP) on the adipocytes, be endocytosed and delivered to lysosomes (blue circle with yellow border). Some CE may move into the adipocyte by selective transfer of core lipids from lipoproteins mediated by the bridging function of LPL. The cholesterol may either be esterified into CE that are deposited in the lipid droplets, be included in intracellular membranes, or be transferred to the plasma membrane and pumped out of the cell by the ABCA1 transporters which use precursor HDL discs as acceptors. Several important steps are omitted so as not to overcrowd the figure, for instance lmf1 and other chaperons needed for correct folding of LPL in the ER, conversion of FA to CoA esters, etc

addressed the question of what the sources are for uptake of lipids into adipose tissue in humans. They studied arteriovenous differences in subjects given a combination of a test meal containing [ $^{13}\text{C}$ ]palmitate to label chylomicrons and an intravenous infusion of [ $^2\text{H}$ ]palmitate to label plasma NEFA, and from them VLDL. The results showed a clear preference for uptake of fatty acids from lipoprotein triglycerides compared to from plasma NEFA. In addition, the study showed a greater fractional extraction of fatty acids from chylomicrons than from VLDL (Bickerton et al. 2007).

LPL is necessary for normal lipoprotein metabolism. Deficiency of the enzyme or its activator, apolipoprotein (apo)CII, leads to massive hypertriglyceridemia. Mouse models in which the endogenous LPL gene was inactivated and a construct was inserted expressing human LPL demonstrated that expression of LPL in a single tissue is enough to rescue nearly normal lipoprotein metabolism. This is true for expression in cardiac muscle (Levak-Frank et al. 1999) skeletal muscle (Levak-Frank et al. 1997) and even in the liver (Merkel et al. 1998), a tissue which usually does not express LPL. Growth and body mass composition was similar among all groups, including essentially normal adipose tissue mass. In line with this, humans (Brun et al. 1989; Ullrich et al. 2001), cats (Veltri et al. 2006) and mink (Christophersen et al. 1997) with LPL deficiency are not pathologically lean. However, ob/ob mice rendered deficient in adipose tissue LPL could not store lipid as rapidly as normal ob/ob mice (Weinstock et al. 1997).

The lipid composition of adipose tissue in LPL deficiency shows a marked decrease in polyunsaturated fatty acids in humans (Ullrich et al. 2001), cats (Veltri et al. 2006) and in mouse genetic models (Levak-Frank et al. 1999; Levak-Frank et al. 1997). There appears to be an SREBP-1 driven induction of a number of enzymes involved in fatty acid and triglyceride biosynthesis (Wagner et al. 2004). Furthermore, adipose tissue from mice that lacked LPL expressed large amounts of mRNA for endothelial lipase, which is not expressed by adipocytes in wild-type mice (Kratky et al. 2005). This suggests that endothelial lipase, which acts primarily on phospholipids in HDL and is structurally closely related to LPL, provided a source of long-chain polyunsaturated fatty acids for the adipose tissue. Another source of essential fatty acids may be net uptake from circulating NEFA. Hence, even though LPL hydrolysis of triglycerides in plasma lipoproteins is the main source of fatty acids for the adipose tissue under normal conditions, there are compensatory mechanisms when LPL-mediated fatty acid delivery does not keep up.

It was early recognized that the nutritional state had a large effect on the uptake of lipids into adipose tissue (Bragdon and Gordon 1958). More chylomicron fatty acids were taken up in fed compared to fasted rats. In contrast, the fraction of fatty acids administered as NEFA that was taken up into adipose tissue did not differ much with the nutritional state. Robinson and his coworkers demonstrated an almost linear correlation over a more than tenfold range between the LPL activity in adipose tissue and the uptake of labeled fatty acids from injected chylomicrons (Cryer et al. 1976). This shows that the LPL activity has a strong, directive effect on the uptake of lipoprotein-derived fatty acids into adipose tissue (Fielding and Frayn 1998; Hartman 1977). This concept is supported by recent studies in mouse models where uptake of lipids into different tissues correlated with the expression

of LPL (van Bennekum et al. 1999). The concept is also illustrated by a number of studies on the relation between LPL expression in heart and heart function (Lee and Goldberg 2007; Park et al. 2007). Knock-out of the enzyme in cardiomyocytes leads to a compensatory increase of glucose utilization and cardiac dysfunction with age (Augustus et al. 2004), while overexpression leads to increased fatty acid oxidation, signs of lipotoxicity and cardiomyopathy (Yokoyama et al. 2004).

## 13.3 Lipoprotein Lipase

### 13.3.1 *Molecular Properties*

Active LPL is a non-covalent dimer of two identical subunits. The human LPL subunit has 448 amino acid residues and two asparagine-linked oligosaccharide chains giving a total molecular mass of about 55,000 (Mead et al. 2002; Merkel et al. 2002a; Olivecrona and Olivecrona 1999; Stein and Stein 2003; Zechner et al. 2000). The subunits are probably arranged in a head to tail orientation (Wong et al. 1997). The enzyme is member of a gene family that also comprises endothelial lipase (Rader and Jaye 2000), hepatic lipase and pancreatic lipase (Kirchgessner et al. 1987). Modelling on the basis of X-ray structures of pancreatic lipase suggests that the subunit in LPL has a similar structure, with two independently folded domains (van Tilbeurgh et al. 1994). This is supported by biochemical evidence from limited proteolysis (Bengtsson-Olivecrona et al. 1986) and by the fact that functional chimeric enzymes have been produced by genetic engineering joining the N-terminal domain of LPL with the C-terminal domain of the related hepatic lipase and vice versa (Davis et al. 1992). The larger N-terminal domain contains the active site and the site for interaction with apoCII, whereas the C-terminal domain contains sites for interaction with lipoproteins, receptors and heparin (Kobayashi et al. 2002; Lookene et al. 2000).

It is a common experience that LPL is unstable under physiological conditions (pH, ionic concentrations, temperature). Loss of activity was traced to a slight change in structure as evidenced by the CD spectrum, and was associated with dissociation of active LPL dimers into monomers (Osborne et al. 1985). Further studies showed that there is rapid subunit exchange between the active dimers, but the intermediate active monomer is prone to change structure with loss of catalytic activity (Lookene et al. 2004). It is concluded that the enzyme is spring-loaded and endowed with a built-in mechanism to self-destruct (Osborne et al. 1985). This is an important property for an enzyme that acts extracellularly but whose activity needs to be rapidly regulated.

#### 13.3.1.1 **Mode of Action**

There are two steps in the action of a lipase (Verger 1976). First the enzyme must adsorb to the lipid–water interface of its insoluble substrate and be properly aligned at the surface so that single substrate molecules can enter the active site. The second

event is the actual chemical steps of catalysis. Because of this sequence of events, the kinetic parameters associated with “substrate affinity” take on a different meaning than for enzymes acting on water-soluble substrates. The dependency of the overall reaction on substrate concentration reflects the propensity of the lipase to bind to the lipid–water interface. LPL can hydrolyze lipids in all types of lipoproteins, chylomicrons, VLDL, LDL and HDL, but when presented with a mixture, as in plasma, the enzyme hydrolyzes almost exclusively lipids in the triglyceride-rich lipoproteins. This is because it prefers to bind to that type of lipid structure. In contrast, hepatic lipase (Bengtsson and Olivecrona 1980a) and endothelial lipase (Rader and Jaye 2000) prefer HDL.

The active site is that of a typical serine hydrolase (Faustinella et al. 1992). The first catalytic event is that a fatty acid transfers from the substrate (e.g. a triglyceride) to the active site serine with formation of an acyl–enzyme intermediate. This is then cleaved by water with release of the fatty acid. The active site is located at the bottom of a hydrophobic pocket in the lipase molecule. A loop of 22 amino acids covers the opening to this pocket (Faustinella et al. 1992). Analogy to pancreatic lipase suggests that, when the lid is closed, the enzyme exposes a hydrophilic surface towards the surrounding aqueous phase but when the lipase adsorbs to a lipid interface, for instance on a lipoprotein, the loop swings around a hinge formed by a disulfide bond. What used to be the inner surface of the loop now becomes part of a hydrophobic lipid-binding site, and the entrance to the active site opens for substrate molecules to enter (Santamarina-Fojo and Dugi 1994).

Rojas et al. (1991) questioned how substrate molecules enter and products leave the active site of LPL. To approach this they compared hydrolysis of triglycerides in liposomes and in emulsion droplets, made up of the same phospholipids and triglycerides. This approach was based on data of Hamilton and Small, who showed that about 3 mol% triolein can be accommodated in phosphatidylcholine bilayers and suggested, on the basis of thermodynamic considerations, that the same applies to the surface structure of a phospholipid–triglyceride emulsion droplet and that of lipoproteins (Hamilton et al. 1983). Rojas et al. (1991) found that the relation between the rate of hydrolysis and the surface area was similar for liposomes containing 3% triolein and for emulsion droplets. Hence the lipid binding site(s) on the lipase did not detect any marked difference between the surfaces of the two types of particles. If each hydrolytic event was followed by dissociation of the lipase into the aqueous phase, one would expect to see the same ratio of phospholipid to triglyceride hydrolysis and similar kinetics with liposomes and emulsion droplets. The ratios were, however, quite different. With liposomes LPL hydrolyzed more phospholipid than triglyceride, whereas with emulsion droplets triglyceride hydrolysis was much faster. Furthermore, the maximal rate of triglyceride hydrolysis was more than 40× higher with the emulsion droplets than with the liposomes. These results show that, when LPL binds to the substrate particle, it stays for several rounds of lipolysis, i.e. the action is processive. Furthermore, the results suggest that when the products leave the active site it is easier for a new substrate molecule to come in from below, i.e. the core of the particle, than from the side, i.e. the surface layer. This seems to fit the proposed three-dimensional structure of LPL, which

shows the active site at the bottom of a hydrophobic pocket (van Tilbeurgh et al. 1994), after the lid has been turned away as presumably happens on binding to a lipid–water interface.

The main initial product formed by LPL action on a triglyceride is a *sn*-2,3-diglyceride (Morley and Kuksis 1972), which is then hydrolyzed further to a 2-monoglyceride. Diglycerides do not accumulate during the hydrolysis of triglycerides (Nilsson-Ehle et al. 1973; Scow and Olivecrona 1977). This is in striking contrast to what is observed with the intracellular adipose tissue triglyceride lipase (ATGL; Zechner et al. 2005) and indicates that diglycerides are a preferred substrate for LPL. It is likely that, when a triglyceride enters the active site of LPL from the core of the lipid particle (see above), it is hydrolyzed to a diglyceride, which tends to stay in the active site and be further hydrolyzed to a monoglyceride before a new triglyceride can enter.

#### 13.3.1.1.1 ApoCII

ApoCII is a necessary activator for LPL (LaRosa et al. 1970; Shen et al. 2002). Individuals with a genetic deficiency of apoCII have massive hypertriglyceridemia and the same clinical symptoms as patients with LPL deficiency (Fojo and Brewer 1992). When LPL is added to chylomicrons from a CII-deficient individual, no hydrolysis occurs. When apoCII is added, hydrolysis immediately starts (Olivecrona and Beisiegel 1997). The detailed mechanism of the activation is not known. LPL displays high activity without apoCII against some model substrates, e.g. the short-chain triglyceride tributyrin (Rapp and Olivecrona 1978). It is only with long-chain triglycerides, phospholipids and lipoproteins as substrate that the enzyme requires its activator (Bengtsson and Olivecrona 1980c).

It was early shown that peptide fragments corresponding to the C-terminal one-third of apoCII could activate LPL in some model systems (Kinnunen et al. 1977). The N-terminal two-thirds were assumed to be responsible for binding the apolipoprotein to the lipoprotein surface. With chylomicrons from an apoCII-deficient individual, the lipid-binding N-terminal part of apoCII was required; a C-terminal fragment that could activate LPL in model systems had no effect with natural lipoproteins (Olivecrona and Beisiegel 1997).

Comparative studies of apoCII sequences from widely different vertebrates (fish, birds, a number of mammals) showed that, in the C-terminal part of the molecule, seven amino acids are strictly conserved (Shen et al. 2000). Mutagenesis confirmed the functional importance of these residues (Shen et al. 2002). Combining this with the 3-D structure (Zdunek et al. 2003) revealed that some of these amino acids are in a helical segment and form a common surface. This is presumably the structure in apoCII that interacts with LPL. In contrast to the strict conservation of structure in this part of the molecule, the N-terminal part showed widely different structures (Shen et al. 2000). In human apoCII this part of the molecule forms two amphipathic helices that anchor the protein in the lipoprotein surface (Zdunek et al. 2003).

It is not entirely clear what property apoCII imparts to the LPL reaction. ApoCII is provided as an integral part of the substrate lipoprotein. There is no evidence for LPL–apoCII complexes at the endothelium, in the absence of lipoproteins. It should be noted, however, that apoCII is expressed in adipose tissue (Gonzales and Orlando 2007). There is also no evidence that the amount of apoCII is limiting for LPL action *in vivo*. The related pancreatic lipase needs a small protein cofactor, colipase, to allow the enzyme to bind to lipid droplets in the presence of bile salts which remove most proteins from the droplets (Borgstrom et al. 1979; Lowe 2002). This is a problem that LPL does not have to overcome; and there is no sequence or structural similarity whatsoever between apoCII and pancreatic colipase. ApoCII does not seem to be important for the binding of LPL to lipoproteins (Olivecrona and Beisiegel 1997) or model substrate droplets (Bengtsson and Olivecrona 1980c), but probably orients the enzyme in the correct manner at the interface (Bengtsson and Olivecrona 1980c; Shirai et al. 1983). The role of apoCII in the product control of LPL action is revisited in Sect. 13.3.1.2.

#### 13.3.1.1.2 Other Apolipoproteins

An important parameter in lipase action is the “quality of the interface” (Verger 1976) which is a function of the lipid and protein composition of the lipid–water interface. LPL action can be severely inhibited by a variety of synthetic peptides that have no sequence homology to native apolipoproteins (Chung et al. 1996). Hence, when one considers reports on effects of apolipoproteins on the LPL reaction, one has to bear in mind that the effect may be relatively non-specific.

#### 13.3.1.1.3 Apolipoprotein CIII

Apolipoprotein CIII is the most abundant C-apolipoprotein in humans. There are many reports that apoCIII inhibits LPL, both with model substrates and with lipoproteins (Jong et al. 1999). ApoCIII has also been shown to enhance binding of triglyceride-rich lipoproteins to SR-B1 (Zannis et al. 2006) and impede binding to receptors in the LDL-R family (Narayanaswami et al. 2004). One mechanism by which apoCIII may do this is by displacing apoE from the lipoprotein surface (Narayanaswami et al. 2004). Likewise, apoCIII may displace apoCII from the surface, explaining in part its inhibition of LPL action (Jong et al. 1999). Overexpression of apoCIII in mice causes hypertriglyceridemia, which appears to be due to impeded lipolysis of triglyceride-rich lipoproteins (Ito et al. 1990). The expression of apoCIII in the liver is decreased by fibrates and other PPAR- $\alpha$  agonists and this is accompanied by decreased plasma triglyceride levels (Kolovou et al. 2008). There is a correlation between apoCIII expression and triglyceride levels in plasma (Fredenrich 1998). Mutations in the apoCIII gene are associated with deranged triglyceride metabolism (Talmud et al. 2002). Hence, there is strong evidence that apoCIII impedes LPL action *in vivo* but the mechanism has not been revealed. The 3-D structure for apoCIII at a lipid–water interface was recently reported (Gangabadage

et al. 2008). ApoCIII is arranged in the interface as six amphipathic helices (each approx. ten residues long), which are connected via semiflexible hinges. Several structural motifs along the solvent-exposed face of apoCIII are highly conserved among mammals. The authors propose molecular mechanisms for some of the multifaceted actions of apoCIII in lipoprotein metabolism (Gangabadage et al. 2008).

#### 13.3.1.1.4 Apolipoprotein CI

Apolipoprotein CI is less abundant in human plasma than apoCIII or apoCII. It has been suggested that a major function of apoCI is to aid in the innate immune defense system (Berbee et al. 2006). The protein contains a highly conserved consensus KVKEKLLK binding motif for lipopolysaccharide (LPS), an outer-membrane component of gram-negative bacteria. ApoCI may bind LPS and present it to macrophages. It was recently shown that HDL from patients with sepsis are almost depleted from apoCI, indicating that the apolipoprotein has been consumed in the defense reaction.

Mice overexpressing human apoCI display gross hypertriglyceridemia (Berbee et al. 2005). This goes along with a function in the innate immune system, since triglyceride-rich lipoproteins bind LPS. The hypertriglyceridemia is not due to interference with binding to hepatic receptors (LDL-R, VLDL-R or LRP) and cannot be due to interference of apoCI with the functions of apoCIII or apoE, since it is seen also in mice deficient in these apolipoproteins (van der Hoogt et al. 2006). In vitro apoCI directly inhibits LPL, indicating that this is a factor in the development of hypertriglyceridemia. These effects may also explain why mice overexpressing apoCI are protected against obesity (Berbee et al. 2005).

#### 13.3.1.1.5 Apolipoprotein AV

Apolipoprotein AV is present in very low amounts in plasma compared to the other apolipoproteins. ApoAV was therefore not discovered until the year 2001, although the gene is located in the *APOA1/C3/A4* gene complex (Kluger et al. 2008; Talmud 2007; Wong and Ryan 2007). ApoAV is a 40-kDa protein with low solubility due to long stretches of hydrophobic and amphipathic sequences (Beckstead et al. 2003). ApoAV is produced mainly (or only) in the liver, but in chickens it is also found in brain, kidney and ovarian follicles (Dichlberger et al. 2007). When overexpressed in hepatoma cells (Hep3B) it accumulates around intracellular lipid droplets but does not affect secretion of apoB-containing lipoproteins (Shu et al. 2007). In plasma, apoAV is found predominantly on triglyceride-rich lipoproteins and on HDL, just like apoCIII, but the levels of apoCIII are about 300-fold higher. Mice lacking apoAV have fourfold increased plasma triglyceride levels (Pennacchio et al. 2001), while overexpression of apoAV results in decreased plasma triglycerides due to faster catabolism of chylomicrons and VLDL (Kluger et al. 2008). From experiments by several groups the current view is that apoAV does not stimulate LPL activity in vitro, but there is strong evidence that LPL action is markedly stimulated in vivo

(Kluger et al. 2008). Stimulation of LPL activity against VLDL was demonstrated with LPL attached to heparan sulfate-covered wells of microtiter plates (Dorfmeister et al. 2008; Merkel et al. 2005). ApoAV has high affinity for heparin and was shown to mediate binding of chylomicrons to heparin-covered surfaces (Lookene et al. 2005). ApoAV also binds to receptors in the LDL-R family (LRP, SorLA; Nilsson et al. 2007) and in the Vsp10p domain families (SorLA and sortilin; Nilsson et al. 2008). Binding of apoAV to these receptors leads to endocytosis of the complex (Nilsson et al. 2008). Similar data were reported with chicken apoAV and the major avian LDL receptor (Dichlberger et al. 2007). A puzzling fact is that the levels of apoAV in human plasma are positively correlated with plasma triglyceride levels (just like apoCIII; Kluger et al. 2008; Talmud 2007). From detailed studies on obese and non-obese subjects, Wolfgang Patch's group concluded that the expression of *APOA5*, and the plasma levels of apoAV, are intimately linked to hepatic lipid metabolism (Hahne et al. 2008). At this moment the mechanism for stimulating triglyceride clearance by apoAV remains in the dark.

### 13.3.1.2 Fatty Acids – an Unusual Mechanism for Product Control

For the LPL hydrolysis of triglycerides to proceed, a fatty acid acceptor must be present. Otherwise the reaction comes to near stop when only a few percent of the triglycerides have been hydrolyzed (Bengtsson and Olivecrona 1980b). Inhibition by fatty acids is seen with other lipases, but is much more pronounced with LPL. The related pancreatic lipase is even activated by fatty acids (van Kuiken and Behnke 1994). Hence, some special mechanism must be at play to cause the very strong inhibition of LPL. Detailed studies of this revealed that there are three factors that contribute (Bengtsson and Olivecrona 1980b).

One mechanism is general for lipases with the serine hydrolase type of reaction mechanisms. The fatty acids and monoglycerides formed on hydrolysis locate at the lipid–water interface and, since they are substrates for the reaction, act as competitive inhibitors of triglyceride hydrolysis. This “reverse reaction” is widely used by the chemical industry, where lipases are used to synthesize various types of esters from fatty acids in water-free or low-water conditions. In the LPL system, however, this type of mechanism explains only a small part of the fatty acid inhibition.

Another, more important mechanism is that LPL forms complexes with fatty acids (Bengtsson and Olivecrona 1979a, b, 1980b). Therefore, as fatty acids accumulate in the system, the enzyme is sequestered into enzyme–fatty acid complexes. This impedes further lipolysis and can break the binding of LPL to heparan sulfate (Saxena and Goldberg 1990) and cause the lipase to dissociate from the capillary wall into the blood. Albumin can prevent the formation of such complexes since it has a higher affinity than the enzyme for fatty acids. The cut-off point when albumin can no longer prevent the formation of LPL–fatty acid complexes is when the fatty acid to albumin ratio exceeds 7–8 (Bengtsson and Olivecrona 1980b; Scow and Olivecrona 1977).

The third and most important component to the inhibition is that, when fatty acids accumulate, apoCII no longer activates the lipase (Bengtsson and Olivecrona 1979a, 1980b; Saxena and Goldberg 1990). This may be why evolution has endowed LPL with a strict dependence on an activator: to be able to stop the reaction when products start to accumulate. The question on what role this may have for control of the LPL system in vivo is tackled in Sect. 13.3.4.

### 13.3.1.3 Action on Lipoproteins

LPL is an efficient enzyme, with a turnover number for triglyceride hydrolysis of about  $1000\text{ s}^{-1}$  (Scow and Olivecrona 1977). A typical chylomicron contains millions of triglyceride molecules. Hydrolysis of most of these to fatty acids and monoglycerides would require several million hydrolytic events and it would take a single LPL molecule hours to accomplish this. The metabolism of chylomicrons is much quicker. It follows that several lipase molecules must act simultaneously on the particle. We return to this concept when we discuss LPL at the endothelium (Sect. 13.3.4).

LPL hydrolyzes both triglycerides and phospholipids. Deckelbaum and associates (1992) studied the relation between these processes by measuring the hydrolysis of [ $^{14}\text{C}$ ]triolein and [ $^3\text{H}$ ]dipalmitoylphosphatidylcholine incorporated into lipoproteins by lipid transfer processes. The relation between phospholipid and triglyceride hydrolysis was generally linear until at least half of the particle triglyceride had been hydrolyzed. Phospholipid hydrolysis, relative to triglyceride hydrolysis, was most efficient in VLDL, but could not fully account for the loss of surface phospholipid that accompanies triglyceride hydrolysis and decreasing core volume. Thus, shedding phospholipid molecules from the VLDL particle, presumably as phospholipid–apolipoprotein discs, must be a major mechanism for losing excess surface as large lipoprotein particles are converted to smaller particles. The discs are precursors for HDL formation, by remodeling processes in blood (Chap. 9). This creates a link between efficient LPL action and HDL levels in blood (Patsch et al. 1978).

### 13.3.1.4 Polyanion Binding Site

LPL was early found to bind to heparin and it was suggested that this is how the enzyme is anchored to the vascular endothelium via heparan sulfate proteoglycans (Olivecrona et al. 1977). This is an attractive model since it positions the enzyme at a distance from the membrane, in the periphery of the glycocalyx. This should facilitate the capture of and action on lipoproteins from the circulating blood. The model also explains how heparin injection releases the enzyme into the circulating blood. Studies in model systems showed that LPL is catalytically active when bound to heparin (Bengtsson and Olivecrona 1981).

Several segments in the LPL molecule have been shown to contribute to heparin binding. A main heparin-binding site is probably in a cleft between the N- and

C-terminal folding units (van Tilbeurgh et al. 1994). Within this segment (residues 260–306 in human LPL) there are 14 positively charged residues. Structures in the C-terminal domain and at the C-terminal end of the molecule also contribute to heparin binding (Lookene et al. 2000; Sendak and Bensadoun 1998).

Lookene et al. (1996) studied the interaction of LPL with heparan sulfate and with size-fractionated fragments of heparin by several approaches. Heparin decasaccharides formed a 1:1 complex with dimeric LPL and were the shortest heparin fragments which could completely satisfy the heparin-binding regions. Equimolar concentrations of octasaccharides also stabilized dimeric LPL, while shorter fragments (hexa-, tetrasaccharides) were less efficient. The number of ionic interactions between LPL and high-affinity decasaccharides was estimated to be ten. Binding of heparin did not induce major rearrangements in the conformation of LPL, indicating that the heparin-binding region is preformed in the native structure. Using a model system with immobilized heparan sulfate bound to a biosensor chip, it was shown that LPL moves rapidly between individual heparan sulfate chains within the layer (Lookene et al. 1996). The overall dissociation constant is very low. This creates a high concentration of LPL along the surface layer of heparan sulfate chains, but the lipase is free to move within the layer.

Early studies showed that several structurally different linear polyanions, e.g. dextrane sulfate and polynucleotides bind LPL avidly, so it was clear that a relatively non-specific electrostatic interaction is enough for binding of LPL (Olivecrona and Bengtsson 1978). There have been several searches for a structure in heparan sulfate specific for binding of LPL, but most of these studies have returned the result that the more sulfated the polysaccharide chain is the higher is the affinity for LPL (Bengtsson et al. 1980; Larnkjaer et al. 1995; Parthasarathy et al. 1994). The most recent study came to the conclusion that LPL, “containing several clusters of positive charges on each subunit, may constitute an ideal structure for a protein that needs to bind with reasonable affinity to a variety of modestly sulfated sequences of the type that is abundant in heparan sulfate chains” (Spillmann et al. 2006).

Heparan sulfate is a component of the outer glycocalyx on virtually all cells (Bishop et al. 2007). The high-affinity binding of LPL to heparan sulfate can explain why the enzyme avidly binds to cells, but is hardly specific enough to fully explain why the enzyme gets concentrated on the luminal surface of endothelial cells.

### **13.3.1.5 LPL as a Ligand – Binding to Receptors and Cell Surfaces**

In experiments on the binding of lipoproteins to cultured cells, for instance to study binding to the LDL receptor, it is usually only a small fraction of the lipoproteins that bind. If LPL is added, binding increases many-fold (Eisenberg et al. 1992). This demonstrates the so-called bridging function of the lipase. Dimeric LPL presumably binds with one of its subunits to the lipoprotein and with the other subunit to the cell surface. This binding can be to cell surface heparan sulfate but it can also be to receptors. That LPL is not only an enzyme, but also a

receptor ligand was first suggested by Felts et al. (1975). Experimental proof for this hypothesis came in 1991 when Beisiegel and her collaborators demonstrated that LPL binds with high affinity to LRP (the low-density lipoprotein receptor-related protein; Beisiegel et al. 1991). This has been extended to most members of the LDL receptor family (Gliemann 1998) and to the SorLA (Jacobsen et al. 2001) and Sortilin receptors (Nielsen et al. 1999). The binding site for the receptors has been localized to the surface loop on the C-terminal domain in LPL (Nykjaer et al. 1993, 1994). This is close to and perhaps partly overlapping an important site for binding to lipid–water interfaces and to lipoproteins. Therefore one LPL subunit can only bind to either a lipoprotein or a receptor. Bridging of lipoproteins to receptors requires dimeric LPL.

A common feature for all the receptors we have mentioned is that their interactions with ligands are blocked by the receptor associated protein (RAP). This is true also for their interaction with LPL. RAP is a 39-kDa resident ER protein which is thought to act as a chaperone/escort protein (Willnow 1998). RAP binds tightly to LDL receptor family members in the endoplasmic reticulum (ER). After escorting them to the Golgi, RAP dissociates from the receptors (Lee et al. 2006). It has been suggested that the function of RAP is to prevent premature binding of ligands to the receptors during their passage through the secretory pathway.

RAP is thought to block binding of ligands, e.g. lipoproteins, by binding to the receptors. Surprisingly, RAP also binds directly to LPL forming a stable complex between monomeric LPL and RAP (Page et al. 2006; van Vlijmen et al. 1999). Overexpression of RAP in the liver of mice that lack both LRP and the LDL receptor caused a marked hypertriglyceridemia in addition to the pre-existing hypercholesterolemia in these animals and also caused a sevenfold increase of circulating, but largely inactive, LPL (van Vlijmen et al. 1999). RAP-deficient adipocytes secreted mostly inactive LPL (Page et al. 2006). These studies suggest that RAP may act as a chaperone/escort protein also for LPL, as for some of the receptors. This may prevent premature interaction of LPL in the secretory pathway with other molecules that it could potentially bind, e.g. LRP and other receptors, angiopoietin-like proteins and heparan sulfate proteoglycans.

### ***13.3.2 Synthesis, Maturation and Transport of LPL***

LPL is expressed in many cell types (Mead et al. 2002; Preiss-Landl et al. 2002). A common theme is that the enzyme is synthesized in parenchymal, not in endothelial cells. Therefore LPL must be secreted and transported to the luminal side of the vascular endothelium where it can engage lipoproteins from blood and hydrolyze their triglycerides. In adipose tissue the enzyme is produced in adipocytes and preadipocytes. It is also produced by macrophages, but in the adipose tissue this makes only a small contribution to overall tissue LPL activity.

The LPL transcript starts with a signal sequence which targets it for vectorial synthesis into the ER. The enzyme has been visualized by electron microscopy

(EM) in vesicles of the secretory pathway (Blanchette-Mackie et al. 1989). There are reports that LPL is localized in a special type of secretory vesicles, different from those used by leptin in adipocytes (Roh et al. 2001) or by insulin in beta cells (Cruz et al. 2005).

### 13.3.2.1 Folding into Active LPL

It has been debated at what stage of assembly and processing LPL becomes catalytically active. Zhang et al. (2005) studied the folding of LPL *in vitro*. The first step was rapid and resulted in an inactive monomer with a completely folded C-terminal domain, whereas the N-terminal domain was in a molten globule state. The second step converted the monomers to a more tightly folded state that rapidly formed dimers. The second step was slow and it appears that proline isomerization (rather than dimerization as such) is rate-limiting.

Interestingly, the second step was dependent on  $\text{Ca}^{2+}$  (Zhang et al. 2005). There are several other pieces of evidence that  $\text{Ca}^{2+}$  may affect LPL activity. In an early study it was shown that incubation of rat adipocytes in the absence of extracellular calcium produced a rapid decline of LPL activity in the cells (Soma et al. 1989). The enzyme could be rapidly reactivated by the addition of calcium. The degree of reactivation was proportional to the concentration of extracellular calcium. Others reported that parathyroid hormone (PTH), which causes a rise in cytosolic calcium, suppressed LPL activity in rat adipocytes by a post-translational mechanism, and this could be prevented by addition of the calcium channel blocker verapamil (Querfeld et al. 1999). The  $\text{Ca}^{2+}$ -dependence of LPL folding and secretion is so pronounced that Osibow et al. (2006) used the LPL activity state to monitor the impact of ER  $\text{Ca}^{2+}$  dynamics on protein folding. These studies raise the possibility that  $\text{Ca}^{2+}$ -dependent control of LPL dimerization might be involved in post-translational regulation of LPL activity.

In studies of folding of transfected human LPL in CHO cells, it was found that most of the cellular LPL was in the ER, while only a small fraction passed to or through the Golgi, as evidenced by the pattern of glycosylation (Ben-Zeev et al. 2002). In the ER there were two fractions of LPL: (a) properly folded, active dimers and (b) disulfide-linked aggregated LPL proteins. The aggregates were formed directly during synthesis. They could not be recruited into active LPL, nor were they formed from the active dimers. LPL molecules in the Golgi and released into the medium were in the form of active dimers. Hence, it appears that only a fraction of newly synthesized LPL subunits fold correctly are approved by ER quality control mechanisms and are passed to the Golgi for further transfer to the cell surface and, in tissues, to the vascular endothelium. A relatively large fraction of newly synthesized LPL subunits do not attain a proper folding but form large, irreversible aggregates which are retained in the ER and degraded there. It had previously been noted that a large fraction of LPL in 3T3-L1 cells is inactive and mostly insoluble (Olivecrona et al. 1987). In rat adipocytes, isolated after collagenase digestion, only about one-third of the LPL protein was in the active, dimeric form (Wu et al. 2003).

Two-thirds was catalytically inactive and probably corresponded to the LPL aggregates characterized by Ben-Zeev et al. (2002). The pattern was the same in adipocytes from both fed and fasted rats (Wu et al. 2003).

Folding of LPL requires ER molecular chaperones. This was evidenced in attempts to express LPL in large scale using *Baculovirus*-infected insect cells (Zhang et al. 2003). The cells expressed and secreted LPL protein, but virtually no LPL activity. Co-infection of the insect cells with mammalian molecular chaperones, in particular with calreticulin, endowed them with the ability to form increased amounts of active LPL dimers (Zhang et al. 2003). Ben-Zeev et al. (2002) demonstrated interaction of LPL with several chaperones by co-immunoprecipitation, mainly with calnexin and BiP. Another molecule that may have a role in the maturation of LPL is RAP, which has been shown to bind tightly to LPL (Sect. 13.3.1.5).

Mice homozygous for the combined lipase deficiency (*cld/cld*) mutation show impaired maturation of LPL and hepatic lipase in the ER. Tissues and cells from mutant mice express only a few percent of normal lipase activity, even though they produce relatively normal amounts of lipase protein (Masuno et al. 1990; Olivecrona et al. 1985). The defective lipase molecules are retained in the ER (Masuno et al. 1990). The gene containing the *cld* mutation was recently identified and given the name lipase maturation factor 1, *Lmf1* (Peterfy et al. 2007). It encodes a transmembrane protein which localizes in the ER. *Lmf1* consists of an N-terminal part that is predicted to have five transmembrane segments which presumably anchor the protein in the ER membrane. The C-terminal domain is conserved among species and the *cld* mutation is located in this part. In further support of the importance of *Lmf1*, a patient homozygous for a mutation causing loss of part of the C-terminal domain had severe hypertriglyceridemia (Peterfy et al. 2007). *Lmf1* is expressed in virtually all tissues in the mouse, including all major LPL-producing tissues. However, the highest abundance of *Lmf1* mRNA is in kidney and testes, tissues that have low LPL. This suggests that *Lmf1* may have additional functions.

Taken together, these studies demonstrate that the folding of LPL into its active form is not a simple, spontaneous process, but is a major bottle-neck that requires molecular chaperones, a special lipase maturation factor and a suitable  $\text{Ca}^{2+}$  concentration within the ER. This is not surprising since the lipase has to fold into a metastable conformation to form active dimers that are spring-loaded and prone to reorganize into a more stable, catalytically inactive monomeric state (Zhang et al. 2005). What role, if any, the folding process has in tissue-specific regulation of LPL activity is not clear.

### 13.3.2.2 The Role of Glycosylation

During the 1980s it was noted that, when glycosylation was blocked by tunicamycin, cells synthesized a non-glycosylated, inactive form of LPL that was not secreted (Chajek-Shaul et al. 1985; Olivecrona et al. 1987; Ong and Kern 1989b). Along the same line, when adipocytes were cultured in a medium devoid of glucose, more than 90% of the LPL was unglycosylated, inactive and not secreted (Ong and Kern 1989b).

A glucose concentration of  $1 \text{ mg ml}^{-1}$  in the medium, much below the physiological range, was enough to restore glycosylation, catalytic activity and secretion. LPL from most animal species has two oligosaccharide chains, but only one of these, attached to Asn43 in the human sequence, must be glycosylated for the enzyme to fold correctly (Ben-Zeev et al. 1994). It was debated whether core glycosylation was enough for LPL to fold into its active form or whether the oligosaccharide chains must be processed from their initial high mannose form into more complex oligosaccharide structures (Chajek-Shaul et al. 1985; Masuno et al. 1991; Vannier and Ailhaud 1989). Isolation of LPL containing only high-mannose oligosaccharides by lectin affinity chromatography from homogenates of guinea-pig adipocytes showed that these LPL molecules were catalytically active and could be secreted (Semb and Olivecrona 1989a). Others reached the same conclusion (Ben-Zeev et al. 1992, 2002). It thus appears that processing of the oligosaccharide chains indicate that the enzyme subunits have been approved by the quality control mechanisms in the ER (Ellgaard and Helenius 2003) and have been passed on to the Golgi, but trimming and processing as such is not necessary for transport or for activity (Ben-Zeev et al. 2002).

### 13.3.2.3 Secretion

The intracellular movement and metabolism of LPL has been traced in pulse-chase experiments by several groups. The enzyme begins to appear in the medium within 30 min, and secretion is largely complete by 1 h. These times are compatible with unrestricted movement through the default secretory pathway. When collagenase-isolated adipocytes are incubated in medium without heparin, only a relatively small fraction appears in the medium and much of the lipase is degraded. Less than 20% of pulse-labeled LPL was secreted into the medium with guinea pig adipocytes and nearly 40% was degraded within 1 h (Semb and Olivecrona 1987). In experiments with cultured 3T3-F442 cells, less than 20% of the pulse-labeled LPL was secreted and about 70% was degraded in 70 min (Vannier and Ailhaud 1989). Only 2% of the labeled lipase was secreted and about 50% was degraded in 2 h with adipocytes from fasted rats (Lee et al. 1998). Similar figures were obtained in experiments where protein synthesis was blocked by cycloheximide (Ben-Zeev et al. 2002; Semb and Olivecrona 1987). Hence, without heparin in the medium, the predominant fate of newly synthesized LPL is intracellular degradation. When heparin is added, much more lipase is secreted. With guinea pig adipocytes the secretion increased from 20% to 50% (Semb and Olivecrona 1987); with 3T3-F442 cells secretion increased from less than 20% to 90% (Vannier and Ailhaud 1989). The increased secretion was balanced by decreased degradation in these studies. The amount of labeled lipase remaining in the cells was about the same. These data can be reinterpreted in light of more recent data on the maturation of the lipase (Ben-Zeev et al. 2002). If a lipase molecule folds properly to active dimers, it traverses the secretory path and arrives at the cell surface where it initially binds, perhaps mainly to heparan sulfate chains. If a high-affinity ligand is available (e.g. heparin) the lipase detaches from the cell surface. In the absence of a ligand, the lipase recycles into the cells and is degraded.

Perhaps it goes through several rounds of recycling as has been observed with LPL-transfected CHO cells (Berryman and Bensadoun 1995).

#### 13.3.2.4 Transport

It is not known in detail how LPL finds its way to the vascular endothelium. A picture of this was obtained in a study where LPL was immunolocalized by EM in mouse hearts (Blanchette-Mackie et al. 1989). Within the myocytes, LPL was found in ER, Golgi and in secretory vesicles. In the extracellular space, the enzyme was found near the orifice of secretory vesicles of the myocytes, along all cell surfaces and crossing the endothelium in vesicles or intracellular channels. This is in concert with the observations by Goldberg and his associates that LPL undergoes bidirectional transport over layers of cultured endothelial cells (Obunike et al. 2001) and that the VLDL receptor appears to be involved in the transcytosis of active LPL across endothelial cells (Obunike et al. 2001). The EM study showed that the endothelial surface had processes that extended into the lumen of the capillaries. The density of immunogold particles over these projections, which comprised about 10% of the total luminal surface, was 2.4× that over the rest of the luminal surface. In fasted mice, the amount of immunoreactive LPL at the luminal projections increased by a factor of five (Blanchette-Mackie et al. 1989).

This pattern of LPL suggests that the enzyme moves in the tissue by an essentially two-dimensional route along cell surfaces. In this process, the enzyme may move from one heparan sulfate chain to the next (Lookene et al. 1996; Obunike et al. 2001). Interaction with such chains may also help the enzyme traverse the basal lamina. To impart directionality to the movement of the lipase, there must be higher-affinity binding sites at the luminal surface of the endothelial cells, particularly at the projections. An interesting possibility is that such high-affinity binding sites are provided by the newly discovered GPIHBP1 protein (Young et al. 2007). This protein, which binds to both LPL and chylomicrons, is expressed on the luminal surface of capillaries in several tissues, notably adipose tissue, heart and skeletal muscle. Mice lacking GPIHBP1 manifest chylomicronemia, demonstrating that the protein is of crucial importance for normal lipoprotein catabolism. The mice display an abnormal pattern of LPL release after injection of heparin. The rapid component is lacking, indicating that the amount of LPL at the endothelial surface is much reduced, supporting the view that GPIHBP1 is required for binding LPL at the endothelium (Weinstein et al. 2008). The N-terminal domain of GPIHBP1 has a highly negatively charged stretch of residues (17 of 25 residues in the mouse sequence and 21 of 25 in the human are glutamates and aspartates). The C-terminal domain shows homology to the lymphocyte antigen 6 (Ly-6) motif, which is defined by a distinct disulfide-bonding pattern between eight or ten cysteine residues (Ploug et al. 1993). Many members of the large Ly-6 gene family are receptors or have other protein–protein binding properties and, like GPIHBP1, have a glycosylphosphatidylinositol anchor that tethers them to membranes. It seems likely that the acidic stretch of residues in the N-terminal domain engages the heparin-binding

sites on the lipase molecule, whereas the C-terminal Ly-6 domain interacts in a more specific way with some other part of the lipase molecule. The expression of GPIHBP1 is regulated by dietary factors and by PPAR- $\gamma$  (Davies et al. 2008).

In the EM study it was estimated that, in mouse heart, about 80% of immunoreactive LPL protein was in myocytes and most of the remainder was associated with endothelial cells (Blanchette-Mackie et al. 1989). In adipose tissue much more of the enzyme is extracellular. Less than one-third of the LPL protein was recovered with adipocytes after digestion of rat adipose tissue with collagenase (Wu et al. 2003). The rest was presumably extracellular. This includes both active and inactive forms of the lipase. Considering only active LPL, about 10% was released from the adipose tissue of fed rats within 5 min after an intravenous injection of heparin (Wu et al. 2003). This presumably represents easily accessible LPL at or close to the endothelial surface. About 30% of the LPL activity was recovered with the adipocytes. The rest, about 60%, must have been in extracellular locations other than the endothelial surface. Hence, LPL is not restricted to the lipase-producing cells and the luminal aspect of the endothelial cells, but there is LPL throughout the extracellular spaces of the tissue.

### 13.3.2.5 Turnover of Extracellular LPL

LPL turns over rapidly. Several studies in which synthesis of new protein has been blocked by puromycin (Schotz and Garfinkel 1965) or cycloheximide (Semb and Olivecrona 1987; Wing et al. 1967; Wu et al. 2003) have indicated that the half-life measured as LPL activity is 2 h or less. Inactive LPL protein turns over at a similar rate (Wu et al. 2003) or even faster (Ben-Zeev et al. 2002). How does this occur? Binding, endocytosis and degradation of LPL has been demonstrated with several cell types, including adipocytes (Berryman and Bensadoun 1995; Obunike et al. 1996; Olivecrona et al. 1987). Thus, degradation by cells within the tissue is a clear possibility. Friedman et al. (1982) compared the ability of different cell types to bind, internalize and degrade the enzyme. Fibroblasts and endothelial cells, which do not synthesize LPL, showed a low ratio of degradation to surface binding. In contrast, cardiomyocytes and preadipocytes, which synthesize LPL, showed a much higher ratio of degradation to binding. The authors speculated that LPL-producing cells have a prominent role in re-uptake and degradation of extracellular LPL. Another possibility is extracellular degradation by matrix metalloproteinases, as has been demonstrated in explants of adipose tissue (Wu et al. 2005).

A third, interesting, possibility is that the enzyme is released into the blood, followed by uptake and degradation in the liver (Vilaro et al. 1988). There are both active and inactive LPL in blood. A typical value for LPL activity in human plasma is  $1.7 \pm 1.1$  mU ml<sup>-1</sup> (1 mU is 1 nmol min<sup>-1</sup> of fatty acid released; Tornvall et al. 1995). This corresponds to less than 1% of the activity in post-heparin plasma and could account for hydrolysis of less than 5% of all plasma triglycerides per hour. Active LPL in plasma is bound mainly to triglyceride-rich lipoproteins, VLDL and chylomicrons (Zambon et al. 1996) and it has been suggested that the enzyme

enhances hepatic binding and uptake of the lipoproteins, particularly chylomicron remnants (Heeren et al. 2002). In line with this, the amount of active LPL in plasma increases after a lipid meal (Karpe et al. 1998) or after infusion of a fat emulsion (Hultin et al. 1992; Nordenstrom et al. 2006; Peterson et al. 1990). It has been shown that the addition of triglyceride-rich lipoproteins causes release of LPL from the surface of endothelial cells (Sasaki and Goldberg 1992; Saxena et al. 1989).

Active LPL is rapidly cleared from the blood and more than half is taken up by the liver (Vilaro et al. 1988), where it initially localizes along sinusoids (Neuger et al. 2004). With time, the immunostaining for LPL shifts to the hepatocytes, becomes granular and then fades, indicating internalization and degradation. Hepatocytes in livers of adult rats do not express LPL (Peinado-Onsurbe et al. 1992). Nonetheless, the liver contains substantial amounts of LPL, most of which is inactive (Neuger et al. 2004), in accord with the hypothesis that there is continuous transport of LPL from adipose and other tissues to the liver, where the enzyme is degraded. In further support of this, Chajek-Shaul et al. (1988) perfused rat livers with LPL-containing media and found that the enzyme lost its catalytic activity soon after binding/uptake. It is not known whether active LPL binds to heparan sulfate or to receptors in the liver, or perhaps first to heparan sulfate and then transfers to receptors; but the binding is impeded by injection of heparin (Neuger et al. 2004). Overall clearance of the lipase becomes much slower and the initial immunostaining shifts from the sinusoids to the Kupffer cells (Neuger et al. 2004).

There is much more inactive than active LPL in plasma (Tornvall et al. 1995). The nature and metabolic significance of inactive LPL is not known. Western blot analysis shows that it is full length and not bound in disulphide-linked aggregates (Olivecrona et al. 1995). On heparin-agarose it elutes in the position expected for monomeric LPL (Olivecrona et al. 1995). It is bound to lipoproteins, mainly LDL, corresponding to about one lipase molecule for every thousand LDL particles in human plasma (Vilella et al. 1993). Inactive LPL enhances the binding of LDL to macrophages, but is much less efficient than active LPL (Makoveichuk et al. 2004). When labeled LPL monomers were injected to rats, they disappeared even more rapidly from plasma than active LPL does (Neuger et al. 2004). Virtually all label ended up in the liver. In contrast to active LPL, most of the inactive lipase was taken up by Kupffer cells (Neuger et al. 2004).

Karpe and his associates have studied the arteriovenous gradients of plasma LPL activity and mass across adipose tissue and skeletal muscle in humans (Karpe et al. 1998). Adipose tissue releases relatively large quantities of inactive LPL, whilst at the same time extracting small amounts of active LPL. In contrast, skeletal muscle releases less LPL overall, and this is mostly active LPL. From these data, and some assumptions, it was estimated that transport away in blood can account for a large fraction of LPL turnover (Karpe et al. 1998). In a more recent paper, a group of patients with diabetes type 2 were treated for 12 weeks with rosiglitazone (Tan et al. 2006). The release of LPL protein from adipose tissue increased after treatment and this correlated to an overall increase of inactive LPL in plasma. The authors conclude that the adipose tissue is a major source of the inactive LPL in plasma. An observation that goes along with this was made on a group of patients who

underwent major elective surgery. Three days after this trauma, which would be expected to decrease adipose tissue LPL production, LPL mass in plasma had decreased by about 50% (Thorne et al. 2005).

A study of pre- and post-heparin LPL in survivors of myocardial infarction and controls showed no correlation between LPL mass in pre- and postheparin plasma, indicating that these parameters reflect different aspects of the LPL system. In contrast, there was a rather strong correlation between LPL mass in pre-heparin plasma and HDL cholesterol (Tornvall et al. 1995). This interesting finding was followed up in a number of studies, mainly by Japanese groups (Saiki et al. 2007). These studies confirmed the relation between LPL mass and HDL cholesterol and extended the correlation of LPL mass to a number of parameters characteristic for the metabolic syndrome, such as fasting plasma insulin and homeostasis model assessment of insulin resistance (HOMA-R). It thus appears that the level of inactive LPL mass in plasma is a marker of insulin resistance (Saiki et al. 2007). Taken together with information on the nature and turnover of inactive LPL in plasma, this suggests that the underlying correlation is between insulin resistance and LPL production rate in adipose tissue.

### ***13.3.3 LPL at the Endothelium***

This is where LPL exerts its well known physiological function (Fig. 13.2). Examples of other enzymatic reactions that occur at the endothelium are blood coagulation, fibrinolysis and complement activation. All are complex processes regulated by fine-tuned protein–protein interactions, rather than by gene expression or phosphorylation, as most intracellular processes are. LPL is extracellular and the substrate molecules are contained in large macromolecular lipoproteins. The products of the reaction are surface-active and, if allowed to accumulate, threaten to disrupt membrane organization in adjacent cells.

The notion that the lipase is anchored at the endothelium solely by electrostatic interaction with heparan sulfate chains (Olivecrona et al. 1977) is widely accepted but may be too simplistic. Esko and his collaborators studied mice in which the gene for an enzyme responsible for addition of sulfate to heparan sulfate chains (Ndst1) was inactivated either in hepatocytes (MacArthur et al. 2007) or on endothelial cells (Wang et al. 2005). Hepatocyte Ndst1 null mice developed hypertriglyceridemia due to accumulation of VLDL-like particles, while, surprisingly, endothelial Ndst1 null mice showed no effects on plasma triglyceride levels, suggesting that heparan sulfate proteoglycans present on extrahepatic cells may not interact significantly with lipoproteins or with LPL (Bishop et al. 2008; MacArthur et al. 2007). This argues against the hypothesis that it is only the interaction with the sulfate groups on endothelial heparan sulfate proteoglycans that anchors LPL to the vessel wall. An apo-B related protein was reported to play a role in the binding of LPL (Sivaram et al. 1994), but this has not been followed up. The VLDL receptor is likely to participate in transport of LPL to the endothelium and its action there

(Goudriaan et al. 2004; Obunike et al. 2001). The newly discovered GPIHBP1 protein probably has an important role (Sect. 13.3.2.4).

EM of heart (Blanchette-Mackie et al. 1989) and lactating mammary gland (Schoefl and French 1967) show chylomicrons attached to the luminal surface of the capillary endothelium. The chylomicrons are often partially enveloped by the endothelium, but they are never seen inside cells or penetrating the basal lamina. As discussed in Sect. 13.3.1.3, several LPL molecules must act simultaneously on a chylomicron to account for the rapid delipidation observed *in vivo*. In one of the EM studies, antibodies demonstrated clusters of LPL molecules over chylomicrons at endothelial projections (Blanchette-Mackie et al. 1989). A likely sequence of events is that chylomicrons initially are attracted to the endothelial surface through electrostatic interactions between cell surface heparan sulfate (and perhaps other polyanions) and the clusters of positively charged amino acid residues on apolipoproteins B and E on the particle. If the particle encounters a binding molecule, LPL, apoAV and/or GPIHBP1, or some other yet unknown molecule, the interaction is enhanced and the particle stays a little longer. LPL molecules move in by lateral diffusion along the endothelium, the interaction is further enhanced and the particle is retained at this “endothelial binding-lipolysis site” (Fig. 13.2). The picture that emerges is that of a large lipolytic complex. Several LPL molecules act on the lipoprotein and are themselves tethered to the endothelium via heparan sulfate chains and/or by interaction with lipase-binding proteins such as GPIHBP1. Important components of the lipolytic complex are apoCII (which binds to and activates the lipase), apoAV (which facilitates hydrolysis in a yet undefined manner) and apo-B and E (which help bind the particle to the cell surface by interacting with heparan sulfate and receptors). ApoCI and apoCIII may also have roles in modulating lipolysis (Sect. 13.3.1.1). There may be more, as yet undiscovered actors that participate in the lipolytic process. It is important to realize that lipolysis is not a single LPL molecule that acts in isolation on the lipoprotein; the process is much more complex. Lipolysis generates several different products (fatty acids, monoglycerides, lysophospholipids), all of which have different fates, as we discuss in Sect. 13.3.3.1. In addition, excess surface components (phospholipids, cholesterol, apolipoproteins) are shed from the lipoprotein and return to blood as a substrate for remodeling reactions that produce HDL (Chap. 10).

In such a lipolytic complex there are a multitude of interactions. The lipoprotein must stay at the same site for many rounds of lipolysis. There is not time for dissociation, movement to a new site and alignment of all necessary interactions, at least not during the rapid lipolysis of large chylomicron particles. One must then ask what terminates the lipolysis and causes the complex to dissociate. This may be a purely stochastic phenomenon, but an interesting possibility is that lipolysis goes on until the tissue and/or the available albumin gets saturated so that fatty acids start to accumulate at the site of lipolysis. As discussed in Sect. 13.3.1.2, experiments in model systems and with cultured cells show that the accumulation of fatty acids causes lipolysis to stop and lipase–lipoprotein as well as lipase–heparan sulfate interactions to dissociate (Bengtsson and Olivecrona 1980b; Saxena and Goldberg 1990; Saxena et al. 1989). This would make good sense in relation to control of

energy metabolism, but would also guard against dangerously high local concentrations of fatty acids that could endanger membrane integrity. In vitro studies have shown that albumin binds fatty acids more strongly than the lipase does (Bengtsson and Olivecrona 1980b). It is only when the fatty acid:albumin ratio exceeds 7–8 that lipase–fatty acid complexes are formed. These ratios are never reached in the circulating blood. It follows that modulation of lipase action by fatty acids is not caused by high amounts of NEFA in the blood, but is a local phenomenon at the endothelial lipolysis sites.

The model predicts that at any given moment a substantial fraction of chylomicron particles are “marginalized” at endothelial “binding-lipolysis sites”. Direct evidence for this was presented in experiments where humans were injected with retinyl palmitate-labeled chylomicrons (collected the day before from the same individual; Karpe et al. 1997). Shortly thereafter, a large bolus dose of a triglyceride emulsion was injected. Retinyl palmitate-containing lipoproteins in the Sf > 400 fraction rapidly appeared in plasma, indicating that marginalized chylomicrons had returned to the circulating blood as a consequence of being displaced from endothelial sites by the emulsion droplets.

Triglyceride-rich lipoproteins and their remnants have been observed to bind to endothelial cells in culture. Interestingly, this binding appears to occur predominantly in lipid-raft-type membrane microdomains (Wang et al. 2008). This would be in accord with GPIHBP1 having a role in the binding, since GPI-linked proteins tend to localize in raft areas. Addition of LPL with ensuing release of lipolysis products has a number of effects on endothelial cells in culture. Membrane lipids are re-organized via aggregation of raft microdomains (Eiselein et al. 2007). There are signs of endothelial cell inflammation (Wang et al. 2008) and apoptosis (Reinbold et al. 2008). Endothelial permeability increases (Rutledge et al. 1997). Monocyte adhesion increases (Saxena et al. 1992; Williams et al. 2004). A large number of genes are up- or down-regulated (Williams et al. 2004). It is apparent that lipolysis at the endothelium in vivo must be kept under control so that products do not accumulate in sufficient amounts to trigger some of these deleterious effects. Most of the experiments cited above were with cultured endothelial cells. In such systems there is no sink that can absorb the products of lipolysis. In vivo, utilization of fatty acids in the underlying cells and binding by albumin provides such a sink. A safety valve (in case the capacity of the sink is overwhelmed) is provided by fatty acid inhibition of LPL action.

### 13.3.3.1 Fate of the Products of Lipolysis

The main products of the LPL reaction are fatty acids and 2-monoglycerides. Scow and his associates perfused rat parametrial adipose tissue with chylomicrons labeled either with glycerol or fatty acids (Scow 1977). Of the chylomicron triglycerides perfused, 1.1% was removed and more than 95% of that taken up by the tissue was hydrolyzed to fatty acids and glycerol. Using tissue from fed rats about 55% of the fatty acids liberated from triglycerides were retained in the tissue as

newly synthesized triglycerides, about 45% returned to the perfusion medium as albumin-bound fatty acids. Several other studies also show that a large fraction of the fatty acids return to blood as albumin-bound free fatty acids (NEFA). Frayn and his associates have shown this directly, by measuring the arteriovenous difference over human subcutaneous adipose tissue (Bickerton et al. 2007). Mathematical modeling of data from experiments where labeled fat has been given as a fat meal or by injection of chylomicrons also show that a large fraction of the fatty acids returns to the blood in the NEFA pool (Hultin et al. 1996; Teusink et al. 2003).

In contrast to the escape of fatty acids into the effluent when chylomicrons were perfused through adipose tissue, all of the glycerol released returned to the perfusion medium. Retention of glycerol in the tissue was negligible (Scow 1977). Fatty acid release began immediately, while there was a time-lag of a minute before glycerol appeared. When perfusion was switched to medium without chylomicrons, release of fatty acids dropped quickly, whereas release of labeled glycerol continued for several minutes. The authors concluded that the chylomicron triglycerides were hydrolyzed by LPL at sites in near contact with the medium, whereas the monoglycerides probably moved into the adipocytes where they were hydrolyzed. In accord with this, plasma concentrations of mono- or diglycerides are low (Fielding et al. 1993) and in studies on rats only small amounts of labeled di- or monoglycerides appeared in the circulating blood during the clearing of injected labeled chylomicrons (Belfrage et al. 1965; Fielding et al. 1993; Morley et al. 1977).

LPL can hydrolyze monoglycerides, but this reaction is far too slow to keep pace with triglyceride hydrolysis (Scow and Olivecrona 1977). Hence, some other enzyme must step in to complete the hydrolysis. The obvious candidate is monoacylglycerol hydrolase (MAGH), the same enzyme that catalyzes the final step in hydrolysis of intracellular triglycerides (Karlsson et al. 1997). This is an intracellular enzyme present in many tissues and known to be present in adipocytes. It is not known if the enzyme is also present in endothelial cells. It would seem likely that the monoglycerides move to and are hydrolyzed in the adipocytes and that glycerol returns to the blood (Fig. 13.2). An interesting corollary is that, in situations where there is net flux of lipids from blood into adipose tissue, more than half of the fatty acids that are retained originate from monoglycerides. Using the data of Scow (1977), 45% of the chylomicron fatty acids return to the blood as albumin-bound NEFA, 30% move into the adipocytes as monoglycerides and only 25% move as fatty acids.

### 13.3.3.2 Transport of Fatty Acids and Monoglycerides Generated by LPL

The molecular mechanism for transport of fatty acids in tissues has been much debated. The driving force for their movement is disposal by metabolic reactions within the cells. In adipocytes the main pathway is synthesis of triglycerides. This creates a sink that generates a gradient along which the fatty acids flow, as long as the sink can accommodate them. If/when the capacity of the sink is overwhelmed by the rate of LPL-mediated lipolysis, fatty acids and other products of lipolysis start to accumulate at the endothelial sites and shut off the reaction, as discussed in

Sect. 13.3.1.2. It seems somewhat unlikely that the net direction of fatty acid flow would be determined by the transport process as such.

The fatty acids (and the monoglycerides) that are generated by LPL in the capillaries need to move across or around the endothelial cells and then move through the basal lamina before they can enter the adipocytes. Scow, Blanchette-Mackie and their coworkers suggested that the fatty acids and the monoglycerides move mainly by lateral diffusion in the endothelial cell plasma membrane (Scow and Blanchette-Mackie 1985). They also argued that there are enough membrane continuities that penetrate the basal lamina so that here too the fatty acids and monoglycerides can move in membranes rather than through aqueous spaces. From studies in model systems, it seems likely that these processes are rapid and not rate-limiting. The next step for the fatty acids and monoglycerides is to move across the adipocyte plasma membrane. How this occurs is the subject of much current debate (Hamilton 2007; Schaffer 2002). On the basis of physical studies, all three necessary steps for transmembrane movement of a fatty acid (adsorption of a fatty acid anion into the outer leaflet of a lipid bilayer, flip-flop over the membrane as a protonated fatty acid, desorption from the inner leaflet in the form of a fatty acid anion) are rapid enough so that they would not be rate-limiting for overall metabolism. Other investigators argue that transmembrane movement of fatty acids depends on specific transport proteins, analogous to the transport of other nutrients, such as glucose and amino acids. There are a number of proteins which have been suggested to facilitate the uptake of fatty acids by cells (Hamilton 2007; Schaffer 2002). Unfortunately, these studies do not consider how monoglycerides move across the membrane.

Fatty acid transport proteins (FATP) form a family of six related membrane proteins that enhance fatty acid import when expressed in cultured cells (Gimeno 2007). Members of this family are present in all tissues engaged in active fatty acid uptake and metabolism. All of the FATPs display a structural motif characteristic of ATP-dependent enzymes and several of the FATPs have been shown to catalyze the first step in the cellular metabolism of fatty acids, the formation of fatty acyl-CoA esters. The FATP that has received most interest in terms of adipose tissue function is FATP1 (Gimeno 2007). There is evidence that insulin causes FATP1 to translocate from intracellular membranes to the plasma membrane in adipocytes, analogously to the glucose transporter GLUT4. FATP1 knockout mice show no obvious abnormality on a normal diet, but are less prone to develop insulin resistance and obesity on a high-fat diet (Gimeno 2007). At least one study demonstrates that FATP1 enhances fatty acid uptake even when localized in intracellular membranes (Garcia-Martinez et al. 2005), suggesting that the protein may act primarily by catalyzing the formation of fatty acyl-CoA derivatives and thereby accelerate further metabolism of the fatty acid, rather than acting on the transmembrane movement as such.

FABPpm (plasma membrane fatty acid binding protein) was identified in brush border plasma membranes of the jejunum (Stremmel 1988). It has the same amino acid sequence as an aspartate aminotransferase (Stump et al. 1993). This is a protein that is synthesized on free polysomes in the cytosol and gets imported into mitochondria by virtue of an N-terminal targeting sequence. Overexpression of this protein has been reported to increase fatty acid uptake (Isola et al. 1995). What role, if any, this has in adipocyte lipid uptake is unclear.

Caveolin 1 is a peripheral membrane protein that associates with the cytoplasmic side of raft domains in the plasma membrane and causes bending and formation of invaginations of the membrane, named caveolae. Adipocytes are very rich in these structures, which have been suggested to have important roles in fatty acid flux across the plasma membrane (Pilch et al. 2007). Caveolin-1 null mice show hypertriglyceridemia and are resistant to diet-induced obesity (Razani et al. 2002). Hamilton (2007) has suggested a model for how caveolin 1 may facilitate fatty acid uptake and utilization. He notes that there are 14 basic residues in caveolin 1 which are close to the inner leaflet of the phospholipid bilayer. Fatty acids that flip-flop over the membrane and form anions on the cytosolic side may be sequestered by these positive charges on caveolin and favorably organized for further metabolism. In this respect it is of interest that triglyceride synthesis in adipocytes appears to be localized to the caveolae (Ost et al. 2005).

CD36 is a multifunctional membrane glycoprotein (Ibrahimi and Abumrad 2002). It has been described as a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation and lipid metabolism. CD36 binds a diverse list of ligands that includes, in addition to long-chain fatty acids, oxidized low-density lipoproteins, anionic phospholipids, as well as a number of proteins (e.g. thrombospondin, some collagens) and apoptotic cells. CD36 is expressed in many tissues, with high expression in cells with active fatty acid transport such as intestinal fat-absorbing cells, adipocytes and oxidative fiber myocytes. In adipocytes the expression is modulated by a number of factors that go along with a function in lipid assimilation, including PPAR- $\gamma$  agonists (Ibrahimi and Abumrad 2002). CD36 null mice show increased levels of triglycerides and NEFA in blood (Goudriaan et al. 2005) and defects in fatty acid uptake/utilization in heart (Park et al. 2007). It has been suggested that the increased plasma triglyceride levels are caused by decreased LPL-mediated hydrolysis of triglyceride-rich lipoproteins resulting from fatty acid inhibition of LPL action (Goudriaan et al. 2005).

A mutation that introduces a premature stop in the CD36 gene in Asian and African populations is thought to cause some forms of heart hypertrophy (Ibrahimi and Abumrad 2002). CD36 was also shown to be the defective gene in a strain of rats with spontaneous hypertension (SHR rats; Ibrahimi and Abumrad 2002). These rats display hypertriglyceridemia and insulin resistance which is supposed to be secondary to deranged fatty acid metabolism. Interestingly, CD36 has been shown to be preferentially located in caveolae (Zhang et al. 2008). Perhaps this is a clue to how the protein facilitates fatty acid uptake, as discussed above for caveolin 1.

### **13.3.3.3 LPL Facilitates the Movement of Cholesterol and Other Lipids into Cells**

Scow and his associates found that, when adipose tissue or mammary gland was perfused with chylomicrons, not only fatty acids but also cholesterol and other lipids moved into the tissue (Scow 1977). This uptake was related to the LPL activity in the tissue. The mammary gland of a normal, lactating rat had high LPL activity and

removed about 15% of the chylomicron cholesterol infused, whereas the tissue of a hypophysectomized rat had low LPL activity and removed less than 1%. Others found that rat hearts took up substantial amounts of cholesteryl esters when perfused with chylomicrons (Fielding 1978) and that LPL catalyzed the transfer of tocopherol from lipoproteins to fibroblasts (Traber et al. 1985). The Steins and their collaborators demonstrated that addition of LPL markedly enhanced transfer of cholesteryl esters from chylomicrons to cultured cells (Friedman et al. 1981). Release of surface-bound endogenous or exogenous LPL by heparin was accompanied by almost complete elimination of uptake of cholesteryl esters, even though rapid hydrolysis of triglycerides continued in the medium (Chajek-Shaul et al. 1982). They concluded that LPL must bind to the cell surface to catalyze the uptake of cholesteryl esters. Transfer of cholesteryl esters did not require that they were presented in lipoproteins, LPL-catalyzed transfer was also observed with phospholipid liposomes. The transfer did not require any lipid hydrolysis, but occurred with non-hydrolyzable diether phospholipid analogues and cholesteryl ethers. A likely mechanism for the transfer is that the lipase binds on the one hand to the lipoprotein particle/liposome and on the other hand to the cell surface, thereby creating an apposition that favors exchange and/or net transfer of lipids.

Goldberg, Deckelbaum and their associates showed that, in a system where labeled LDL was incubated with LDL receptor-negative fibroblasts, LPL mediated a greater uptake of [<sup>3</sup>H]-cholesteryloleoyl ether than of [<sup>125</sup>I]-LDL protein (Seo et al. 2000). This result indicated selective lipid uptake, as in the experiments from the Steins' laboratory, described above. Selective lipid uptake was not affected by tetrahydrolipstatin (which inhibits LPL hydrolysis) but was nearly abolished by heparin, monoclonal anti-LPL antibodies and by chlorate treatment of cells (which inhibits sulfation of proteoglycans) and was not found using CHO cells deficient in heparan sulfate proteoglycans. Similar results were obtained with HDL and it appears likely that LPL can facilitate selective uptake of lipids from all types of lipoproteins and model lipid particles, such as liposomes and emulsion droplets. In continued experiments, this group used a mutated form of LPL where exchange of one of the critical residues in the active site rendered the LPL catalytically inactive (Merkel et al. 2002b). Transgenic expression of this mutated inactive LPL enhanced selective transfer of cholesteryl esters both in cell systems and in vivo.

#### 13.3.3.4 LPL Mediates Particle Uptake

LPL enhances the binding of lipoproteins to cells. This is a necessary feature for its function as an enzyme that hydrolyzes lipoprotein triglycerides at the surface of endothelial cells. In this case, the binding is reversible and the particles return to the circulating blood after a round of lipolysis. In other cases, the binding can result in endocytosis (Casaroli-Marano et al. 1998; Eisenberg et al. 1992; Fernandez-Borja et al. 1996). For this function, the lipase can bind to proteoglycans in the same way as

when it functions as an enzyme, but LPL also binds to several cell surface receptors (Sect. 13.3.1.5). It has been suggested that chylomicron remnants bring some LPL with them when they leave the sites of lipolysis in adipose and other tissues and that this facilitates the binding and uptake of remnants in the liver (Felts et al. 1975; Heeren et al. 2002). It is of interest to note that the liver does not normally produce LPL. This may be a case when the non-enzymatic ligand function of the lipase dominates. It seems clear that most chylomicron and VLDL particles are metabolized by sequential binding-lipolysis in extrahepatic tissues, followed by binding-endocytosis in the liver, but some of the particles are cleared in extrahepatic tissues (Fielding 1978; Hultin et al. 1996; Merkel et al. 2002b) and this occurs mainly in LPL-rich tissues (van Bennekum et al. 1999; Yokoyama et al. 2007). It has also been suggested that LPL-mediated uptake of lipoproteins by macrophages can cause excessive lipid load on the cells and accelerate atherosclerosis (Babaev et al. 2000). Bridging by LPL can also contribute to retention of lipoproteins in the extracellular matrix (Edwards et al. 1993; Neuger et al. 2001; Pentikainen et al. 2002), a process that may also accelerate atherosclerosis (Mead et al. 1999; Stein and Stein 2003).

### ***13.3.4 Regulation/Modulation of Tissue LPL Activity***

LPL activities change rapidly and profoundly in a tissue-specific manner (Scow et al. 1977). Several-fold changes over one or a few hours have been reported both for heart (Pedersen and Schotz 1980; Pulinilkunnil and Rodrigues 2006) and for adipose tissue LPL activity (Semb and Olivecrona 1986). Thus, LPL activity is one of the most, if not the most, dynamic parameters in lipoprotein metabolism. The LPL reaction is the first step in lipoprotein catabolism and it is a common feature of biochemical pathways that the initial step is the main site of control. We discuss the regulation of LPL action from an adipose tissue perspective and we focus on the nature of the mechanisms involved.

#### **13.3.4.1 LPL Turns Over Rapidly**

A number of studies *in vivo*, in tissue explants or in adipocytes have shown that LPL decays rapidly when protein synthesis is blocked (Bergo et al. 2002; Cryer et al. 1973). The turnover time for the lipase protein, calculated from these data is in the order of 2–3 h. All information available indicates that modulation of tissue LPL activity occurs by active or inactive LPL molecules flowing through the system, or by the conversion of active LPL to inactive. There is no evidence for reversible shifts of long-lived lipase molecules between active and inactive states.

#### **13.3.4.2 Transcriptional Control is Important But Too Slow to Explain the Rapid Modulation of LPL Activity**

There are several examples of major changes in LPL expression (in rats or mice): (a) increase in mammary gland at the onset of lactation (Jensen et al. 1994) with concomitant decrease of LPL expression in adipose tissue (Jensen et al. 1994; Ling et al. 2003; Martin-Hidalgo et al. 1994), (b) increase in brown adipose tissue on cold exposure (Carneheim et al. 1988), (c) induction of expression on development of preadipocytes into adipocytes (Dani et al. 1990), (d) suppression of expression in liver in the neonatal period (Peinado-Onsurbe et al. 1992), (e) induction of LPL expression on activation of monocytes to macrophages (Auwerx et al. 1988). Insulin (Ong et al. 1988), cortisol (Ong et al. 1992b), catecholamines, cellular  $\text{Ca}^{2+}$  signaling (Querfeld et al. 1999), PPAR $\gamma$  agonists (Laplante et al. 2008), statins (Saiki et al. 2005) and other hormones/agents have been reported to change LPL mRNA abundance in adipocytes, but results vary between laboratories. In general these effects are slow with time-scales of days, indicating that they mediate long-term adaptations to the environment, rather than rapid modulation in response to meals, exercise and other rapid/transient events. This is not to say that gene expression is not an important aspect of the LPL system (Enerback and Gimble 1993). On the contrary, LPL gene expression has been suggested to be a risk factor for development of obesity (Chen et al. 2008), insulin resistance (Holzl et al. 2002) and dyslipidemia (Sprecher et al. 1996). Most impressive are two recent large studies that used advanced genetic techniques to identify networks of genes of importance for metabolic diseases (Chen et al. 2008), which identified LPL as a member of such a network with impact on obesity. In-depth discussions of the LPL gene, the potential regulatory sites that it contains and the hormones/agents that regulate its expression can be found in recent reviews (Auwerx et al. 1996; Enerback and Gimble 1993; Mead et al. 2002; Merkel et al. 2002a).

#### **13.3.4.3 Rapid Modulation of LPL Activity is (Mainly) Post-Transcriptional**

Adipose tissue LPL activity changes rapidly (within one or a few hours) in response to changes in nutritional state (Doolittle et al. 1990; Semb and Olivecrona 1989b) and other changes, e.g. physical activity versus inactivity (Bey and Hamilton 2003). In contrast, LPL mRNA in (rat) adipose tissue turns over relatively slowly; a half-life of 17 h has been reported for white adipose tissue (Bergo et al. 2002) and 20–30 h in brown adipose tissue (Carneheim et al. 1988). This is far too slow to account for the rapid changes in LPL activity. In further support of post-transcriptional mechanisms, the changes in LPL activity are much larger than the changes in LPL mass (Bergo et al. 1996; Ong and Kern 1989a), at least in experimental animals. In humans, LPL transcription may be modulated on a relatively rapid time-scale in parallel with LPL activity and mass (Ruge et al. 2005).

#### 13.3.4.4 Translation of LPL mRNA into Protein can be Controlled

A highly specific mechanism to suppress LPL translation, without effects on translation of other proteins, has been described. Stimulation of the PKA system in cultured adipocytes causes a decrease in LPL synthesis (Ball et al. 1986; Ong et al. 1992a). Kern and his associates demonstrated that this is mediated by a protein complex that binds to the 3' UTR of LPL mRNA and blocks its translation into LPL protein. The inhibiting protein complex consists of the catalytic subunit of PKA and the A kinase anchoring protein (AKAP) (Ranganathan et al. 2005). This protein, which is abundant in white adipocytes, is expressed at low levels or not at all in myocytes or brown adipocytes. Hence, this mechanism for control of LPL translation appears to be specific for white adipose tissue. Kern and his associates reported that, while the rate of LPL synthesis was decreased, the rate at which cell-associated LPL is degraded was also decreased (Ong et al. 1992a) so that LPL protein mass would not change much. Ball and her coworkers, in contrast, reported that the rate of LPL degradation was increased by  $\beta$ -adrenergic stimulation (Ball et al. 1986).

It was noted in several studies on the synthesis of LPL that the specific effects are compounded by a general anabolic effect of insulin on overall protein synthesis in adipocytes. In the system (rat fat pads) used by Ashby and Robinson (1980), insulin more than doubled the rate of incorporation of labeled amino acids into proteins. Others found a 40–50% decrease of protein synthesis in adipocytes of rats starved for 1 day (Lee et al. 1998) or found that methionine incorporation was only 30–60% in adipocytes from starved compared to fed guinea pigs (Semb and Olivecrona 1989b). Ong and Kern found that LPL synthetic rate in rat adipocytes was reduced by 80% in the absence of glucose in the medium (Ong and Kern 1989b). Synthesis increased directly with glucose concentration. Insulin doubled the synthetic rate at all glucose concentrations (Ong and Kern 1989b). Hence, studies on the effects of nutritional state on adipose LPL activity are likely to be confounded by this general effect on protein synthesis.

#### 13.3.4.5 Extracellular LPL Activity Changes more than Intracellular

This was first shown for rat heart. The technique used was to perfuse the heart with heparin-containing medium. LPL appears immediately, i.e. the first drop of perfusate that contains heparin also contains LPL. Within one or a few minutes a peak of LPL activity appears. This is interpreted to be the lipase that was at or near the vascular endothelium (Borensztajn and Robinson 1970; Pedersen and Schotz 1980). This peak is several-fold higher with hearts from fasted compared to fed rats, and the change can occur rapidly. In one early experiment, fasted rats were given glucose and this caused the heparin-releasable LPL activity in heart to decrease by 85% within 1 h (Pedersen and Schotz 1980). In contrast to this rapid change in heparin-releasable LPL, there is only a small change of total tissue activity, often statistically not significant. The amount of LPL released is typically in the order of 10–25% of tissue total so that even a large change in the endothelium-localized

LPL causes only a small change in the total. Rodrigues and his associates have shown directly that LPL activity within the cardiomyocytes does not change significantly (An et al. 2005). This protocol with brief heparin release of heart LPL has been used in several laboratories to study LPL turnover (Liu and Olivecrona 1991) and regulation (Pulinilkunnil and Rodrigues 2006).

The same type of observation has been made for adipose tissue. The technique used to separate intra- and extracellular LPL has been to digest the tissue with collagenase and isolate the adipocytes. When adipose tissue from fed rats was digested with collagenase, only about 20% of the LPL activity was recovered (Cunningham and Robinson 1969). With tissue from fasted rats, virtually all the LPL activity was recovered with the adipocytes. Moreover, the LPL activity in the adipocytes was the same irrespective of whether they came from fed or fasted animals. The overall conclusion is the same for adipose tissue as for heart: the rapid modulation of LPL activity in response to changes in nutrition engages only the amount of active extracellular LPL; the activity in the lipase-producing cells changes much less, or not at all.

Additional insight has come from studies of LPL mass. When adipose tissue of fed and fasted rats were compared, the LPL activity was 4.8× higher in adipose tissue from the fed rats, but there was no difference in LPL mass (Bergo et al. 1996). Separation on heparin-agarose confirmed that the difference lay in the proportion of inactive to active LPL. With tissue from fasted rats most of the lipase protein eluted in the position of inactive lipase, whereas with tissue from fed rats most of the lipase protein was in the position of active lipase. Hence it was not a change in the activity state of all lipase molecules (as could for instance have been brought about by a phosphorylation/dephosphorylation event), but it was the fraction of lipase molecules that were in the active state that changed. The specific activity (activity/mass) was the same for the active species whether it came from fed or fasted rats. Further studies showed that within the adipocytes there was no difference in LPL mRNA, protein mass or activity between fed and fasted rats. Separation on heparin-agarose showed the same distribution between active and inactive forms of the lipase (Wu et al. 2003). Hence, the difference lay in the activity state of lipase molecules located extracellularly. Studies with transcription blockers (actinomycin D,  $\alpha$ -amanitin) demonstrated that for this mechanism to operate in going from the fed to the fasted state, another gene(s), separate from the LPL gene has to be expressed to suppress LPL activity. When transcription is blocked in fasted rats, LPL activity rapidly increases in a manner similar to when the animals are given food (Bergo et al. 2002). Thus, a rapidly turning-over LPL-controller protein was implicated.

#### **13.3.4.6 Angiopoietin-Like Proteins can Convert Active LPL into Inactive Monomers**

A recently discovered family of proteins, the angiopoietin-like proteins (Angptl; Chap. 10), has at least three members (angptl 3, 4, 6) that inactivate LPL (Li 2006). Work in several laboratories showed that deficiency of either angptl3 or angptl4

was associated with high LPL activity and low plasma triglyceride levels. Conversely, overexpression resulted in low LPL activity and hypertriglyceridemia (Chap. 10). In vitro model studies showed that the C-terminal coiled-coil domain of angptl4 binds to active LPL dimers and converts them into inactive monomers (Sukonina et al. 2006). The turnover of the mRNA for angptl4 in rat adipose tissue was shown to be rapid enough to modulate LPL activity in response to feeding/fasting and the LPL activity levels in adipose tissue correlated to the angptl4 mRNA levels (Sukonina et al. 2006). Hence, the angptl proteins are likely candidates for the LPL-controlling protein inferred from the studies on effect of transcription blockade (Bergo et al. 2002). Angptl4 has affinity both for heparin (Sukonina et al. 2006) and components in the extracellular matrix (Cazes et al. 2006) and can thus be positioned to inactivate LPL on its travel from the adipocyte surface to the luminal side of the endothelium.

## 13.4 Intracellular Lipases

For a long time it was believed that hormone-sensitive lipase (HSL) was the only lipase hydrolyzing tri- and diglycerides in adipocytes and that regulation of its activity by phosphorylation of the lipase itself was the main rate-determining step in the mobilization of fatty acids. It was discovered that: (a) perilipin is a lipid droplet protein whose state of phosphorylation has a decisive influence on the rate of lipolysis (Greenberg et al. 1991) and then (b) Adipose triglyceride lipase (ATGL) is the enzyme that catalyzes the first step in overall lipolysis (Zechner et al. 2008); and these discoveries have generated an intense interest in intracellular lipolysis. We limit our discussion here to the properties of the main proteins that participate in intracellular lipolysis and the molecular mechanisms by which they are regulated as a background for a discussion of how their action is integrated with that of the LPL system and with triglyceride synthesis to enable a fine-tuned regulation of lipid deposition/mobilization over the entire spectrum of circumstances from starvation to caloric excess. For more detailed discussions of aspects of intracellular lipolysis, the reader is referred to several excellent reviews (Arner and Langin 2007; Brasaemle 2007; Duncan et al. 2007; Granneman and Moore 2008; Watt and Steinberg 2008; Zechner et al. 2008).

### 13.4.1 *Adipose Triglyceride Lipase*

This enzyme catalyzes the first step in the breakdown of stored triglycerides: their hydrolysis to diglycerides (Zechner et al. 2008). ATGL was discovered only recently, in 2004. This is surprising in view of the fundamental role the enzyme has. Apparently, its activity did not show up under the assay conditions used by the many groups that studied HSL or LPL in tissue extracts. ATGL is a 486-amino-acid protein. Related enzymes are found in all eucaryotes; vertebrates, flies, fungi and plants (Zechner

et al. 2008). Mammalian ATGL belongs to a gene family that is characterized by the presence of a patatain-like domain. This is named after the most abundant protein in potato tuber, which has lipase activity. There are several other members of this family in the human and/or mouse genomes. Some of these are expressed in adipocytes and some have lipase and/or phospholipase activity (Lake et al. 2005). The physiological roles of these proteins are unknown and we will not discuss them further. A comprehensive list of all the 23 putative lipases in mammalian tissues is given by Watt and Steinberg (2008). The combined action of ATGL and HSL accounts for more than 95% of adipose tissue lipolysis (Schweiger et al. 2006). ATGL shows a strong preference for triglycerides as substrate (Zimmermann et al. 2004). It hydrolyzes diglycerides and phospholipids only slowly, and does not hydrolyze cholesteryl esters or monoglycerides at all.

Most cell types in the body can produce lipid droplets as (a) a local buffer that ensures uninterrupted access to fatty acids for metabolism and (b) as a way to detoxify a temporary surplus of fatty acids. All these cells contain ATGL (Zechner et al. 2008), which is distributed between the cytosol and the surface of the lipid droplets. ATGL knockout mice show a severe phenotype, indicating gross disturbance of overall energy metabolism and decreased ability to respond to metabolic stress, e.g. cold acclimatization (Haemmerle et al. 2006). These mice accumulate triglycerides in virtually all organs. When fasted, they have low plasma NEFA, ketone bodies and triglycerides, indicating defective mobilization of fatty acids from the adipose tissue.

The catalytic activity of ATGL is regulated through interaction with proteins on the lipid droplets. Comparative gene identification 58 (CGI-58), also known as  $\alpha/\beta$  hydrolase fold domain 5 (ABHD5), acts as a specific activator. It is a 349-amino-acid protein that interacts with and enhances the activity of (mouse) ATGL about 20-fold (Lass et al. 2006). Mutations in CGI-58 cause the rare Chanarian–Dorfman syndrome characterized by ichthyosis and accumulation of triglycerides in most tissues (Lass et al. 2006).

### ***13.4.2 Hormone-Sensitive Lipase***

Hormone-sensitive lipase (HSL) was for a long time considered to be the only enzyme responsible for hydrolysis of triglycerides and cholesteryl esters in lipid droplets. It came as a surprise that HSL knockout mice displayed a rather mild phenotype (Haemmerle et al. 2002; Osuga et al. 2000; Wang et al. 2001). The animals had an apparently normal energy metabolism. In particular, they were not overweight. HSL-deficient adipocytes responded to stimulation by catecholamines and released fatty acids at an almost normal rate. A main difference compared to wild-type mice was that the HSL knockout mice accumulated diglycerides in several tissues, indicating that HSL was rate-limiting for diglyceride hydrolysis.

HSL has a complex gene structure with ten exons. Alternative exon usage generates mRNA transcripts and proteins of different sizes in a tissue-specific manner. Modeling of the 3-D structure indicates that the molecule is organized in two major structural domains, encoded by exons 1–4 and 5–9, respectively (Osterlund et al. 1999). The

C-terminal domain contains the active site and a loop region (amino acids 521–669) where all known phosphorylation sites are located. The enzyme hydrolyzes a wide variety of substrates in model systems. Of the substrates relevant in vivo, diglycerides are the best and triglycerides are the worst (Zechner et al. 2008).

HSL is present throughout the body. In most tissues its main role is probably, as in adipose tissue, to act in a co-ordinated manner with ATGL to hydrolyze tri- and diglycerides for metabolic use. While HSL knockout mice appear to be able to mobilize lipid at a sufficient rate during basal conditions, the enzyme is needed for accelerated lipolysis under stressed conditions, e.g. submaximal exercise (Fernandez et al. 2008a). HSL hydrolyzes not only tri- and diglycerides but also cholesteryl esters, a reaction that ATGL cannot carry out. The importance of this reaction is readily demonstrated in steroidogenic tissues, where HSL is needed for hormone synthesis both from stored cholesteryl esters and from cholesteryl esters taken up from lipoproteins by so-called selective transfer mediated by the SR-B1 receptor (Kraemer 2007). In a similar manner, HSL is probably needed to split cholesteryl esters in many cell types so that the cholesterol can be used by the cell or pumped out via the ABCA-1 transporter. Therefore, it is not surprising that HSL knockout mice show signs of disturbed cholesterol homeostasis (Fernandez et al. 2008b).

Under basal conditions, HSL is a cytoplasmic protein. The enzyme has several sites for phosphorylation by PKA and some other protein kinases (Zechner et al. 2008). Phosphorylation stimulates the enzyme activity modestly (twofold or less) in model systems, whereas adrenergic stimulation of adipocytes can increase the release of fatty acids by up to 100-fold. This dramatic stimulation is mediated by perilipin, as we discuss below.

### ***13.4.3 Monoacylglycerol Hydrolase***

The enzyme monoacylglycerol hydrolase (MAGH), which is a 302-amino-acid protein, is present in most tissues (Watt and Steinberg 2008). It catalyzes the last step in the hydrolysis of stored triglycerides in the adipocyte (Fig. 13.2). It is presumably the same enzyme that hydrolyzes the monoglycerides that emanate from LPL-mediated action, although it is not known if these monoglycerides have to move into the adipocytes or if they can be hydrolyzed already in the endothelial cells (Sect. 13.3.3.1). Another possibility is that some of the monoglycerides, from intracellular lipolysis or from LPL action, are re-esterified by the monoacylglycerol acyl transferase (MGAT), which has been demonstrated in adipose tissue (Schultz et al. 1971). The transmembrane movement and metabolism of monoglycerides must be efficient. There are no reports of conditions where these molecules accumulate.

MAGH has no activity against tri- or diglycerides but it has been shown to participate in the degradation of some endocannabinoids (Saario and Laitinen 2007). There is a large literature dealing with pharmacological inhibition of MAGH to target endocannabinoid metabolism (Viso et al. 2008). An interesting connection to our present topic is that one of the major endocannabinoids is 2-arachidonoylglycerol, a molecule that could derive from lipase action on triglycerides.

### 13.4.4 *Perilipin and the Orchestration of Lipolysis*

Perilipin is a lipid droplet associated protein that has important functions in modulating lipolysis in mammalian adipocytes (Fig. 13.2) (Brasaemle 2007; Ducharme and Bickel 2008). There are three isoforms of perilipin that arise from differential splicing but perilipin A, the largest protein (517 amino acids in mice), is the most abundant in adipocytes. There are six phosphorylation sites in perilipin. One of these, serine 517 appears to be a master switch (Miyoshi et al. 2007). What role phosphorylation of HSL itself has is at present not clear.

While perilipin has a pivotal role in regulating lipolysis, the details are not yet known. A current model suggests the following sequence of events (Granneman and Moore 2008). Under basal conditions perilipin binds CGI-58 at the surface of the lipid droplets and thereby denies ATGL access to its activator. HSL is mainly in the cytosol and overall lipolysis is slow. When the adipocytes are stimulated, for instance by a  $\beta$ -adrenergic agonist, a signaling relay causes protein kinase A (PKA) to phosphorylate perilipin. This abolishes the binding between perilipin and CGI-58 which now binds to and activates ATGL. At the same time the phosphorylated perilipin recruits (phosphorylated) HSL from the cytosol to the lipid droplets (Granneman and Moore 2008). Now the stage is set for rapid hydrolysis of triglycerides to diglycerides by ATGL and further hydrolysis of these to monoglycerides by HSL. MAGH can then finish the job and release a fatty acid and glycerol. Another effect of  $\beta$ -adrenergic stimulation of adipocytes is that the lipid droplets show a dramatic remodeling: a few large centrally located lipid droplets fragment into a myriad of tiny microlipid droplets that scatter throughout the cytoplasm (Brasaemle 2007), thus enlarging the surface at which the lipases can act (Chap. 1).

## 13.5 Triglyceride Synthesis

Net release of fatty acids is not a simple function of the rate of lipolysis in adipocytes, but reflects a balance between lipolysis and re-esterification, sometimes referred to as the triglyceride–fatty acid cycle. Current thinking is that lipolysis generates an excess of fatty acids and that it is the rate of re-esterification that determines the net outflow of fatty acids from the adipocytes (Fielding and Frayn 1998). The fatty acids from the LPL reaction must also be factored-in. During periods of nutrient excess there is net uptake of fatty acids from lipoprotein triglycerides. During starvation there is net release of fatty acids from the tissue. These fatty acids come both from intracellular lipolysis and from LPL-mediated hydrolysis of lipoprotein triglycerides (Fielding and Frayn 1998). If we assume that fatty acids from the two sources mix, then (re-)esterification becomes the overriding control of their fate (Fielding and Frayn 1998).

Glyceride synthesis requires that the fatty acids are presented as their coenzyme A (CoA) esters. Such esters can not pass over the cell membrane. Therefore conversion

to an acyl-CoA ester traps the fatty acid in the cell and favors triglyceride synthesis (Soupene and Kuypers 2008). In this respect it is of interest to note that some of the putative fatty acid transport proteins have acyl-CoA synthetase activity (Sect. 13.3.3.2). These proteins have been shown to enhance transport of fatty acids into cells, but the mechanism by which they do this is not entirely clear. Perhaps trapping is the way some of them work.

### ***13.5.1 A Triglyceride–Diglyceride Cycle?***

There are two diacylglycerol acyl transferase (DGAT) enzymes in adipocytes (Yen et al. 2008). They are located in the ER and perhaps also on the lipid droplets. In the adipocytes, diglycerides are continuously generated by ATGL action. Some of these can be further hydrolyzed by ATGL, but most are probably re-esterified by DGAT. When adipocytes are stimulated, ATGL action accelerates and HSL becomes active. In this situation there will be a competition for the diglycerides between re-esterification into triglycerides by DGAT and further hydrolysis by HSL. Diglycerides also arise as intermediates in de novo synthesis of triglycerides. The balance between HSL and DGAT activity probably determines whether diglycerides are hydrolyzed or esterified (Fig. 13.2).

There is much evidence to support that DGAT enzymes are regulated and have important roles for lipid deposition in adipose tissue. In cultured adipocytes, DGAT1 mRNA is increased by glucose and insulin (Meegalla et al. 2002) and by PPAR- $\gamma$  agonists (Payne et al. 2007). Overexpression of DGAT enzymes leads to triglyceride accumulation in cells (Chen et al. 2002). DGAT1-deficient mice are viable and lean, even on a high-fat diet (Smith et al. 2000). A synthetic small molecule inhibitor to DGAT1 reduced weight gain in mice on a high fat diet (Zhao et al. 2008).

### ***13.5.2 Reacylation of Monoglycerides***

Monoglycerides are generated both in intracellular lipolysis by HSL (Sect. 13.4.1.2) and in the LPL reaction (Sect. 13.3.1.1). We know that the monoglycerides from LPL must move into the tissue, since they do not appear in appreciable amounts in the blood (Belfrage et al. 1965; Fielding et al. 1993; Scow 1977). Whether monoglycerides from the two sources mix is not known. There is monoacylglycerol acyl transferase (MGAT) activity in adipocytes (Schultz et al. 1971) and one of the DGAT enzymes can use monoglycerides as substrate (Yen et al. 2008). Hence, the fate of monoglycerides must be determined by the relative activities of MAGH, leading to breakdown to fatty acids and glycerol, and of MGAT enzymes, leading to synthesis of triglycerides. How this balance is set under different conditions is not known, but reacylation of monoglycerides is probably a minor pathway.

### ***13.5.3 De Novo Synthesis of Triglycerides***

It was thought for a long time that the only source of glycerol-3-phosphate for triglyceride synthesis was glucose that the adipocyte took from blood by the insulin-regulated GLUT-4 transporter. This led to the view that insulin suppresses the net release of fatty acids both by decreasing lipolysis and by providing glycerol-3-phosphate for re-esterification. This view is now changing. The main source of glycerol-3-phosphate for triglyceride synthesis in adipocytes appears to be glyceroneogenesis from lactate/pyruvate or amino acids via the citric acid cycle, with glycolysis from glucose being less important (Nye et al. 2008a, b). The key regulatory enzyme for glyceroneogenesis is phosphoenolpyruvate kinase (PEPCK). Knockout of this enzyme from adipocytes resulted in lean mice, with 25% of the animals displaying lipodystrophy (Olswang et al. 2002). Overexpression resulted in obese mice (Franckhauser et al. 2002). In contrast, when GLUT-4 (which mediates insulin-stimulated glucose uptake in adipocytes) was knocked out, the mice had normal fat mass (Abel et al. 2001).

There is a glycerol channel (aquaporin 7; AQP7) in adipocytes (Fain et al. 2008) and in endothelial cells in adipose tissue (Skowronski et al. 2007). The expression of AQP7 is up-regulated in response to starvation and down-regulated by insulin (Kishida et al. 2001). Knockout mice showed impaired glycerol release from adipose tissue (Skowronski et al. 2007); and they developed obesity and insulin resistance, even at a young age (Hibuse et al. 2005). Studies with adipocytes from these mice showed that AQP7 disruption lead to elevated adipose glycerol kinase activity and accelerated triglyceride synthesis (Hibuse et al. 2005). These studies demonstrate that glycerol can be used for triglyceride synthesis in adipocytes. Under most conditions glycerol is, however, efficiently released through the AQP7 channel to serve as a substrate for gluconeogenesis in the liver. In accord with this view, AQP7 knockout mice displayed severe fasting-induced hypoglycemia (Hibuse et al. 2005).

### ***13.5.4 Acylation-Stimulating Protein***

Acylation-stimulating protein (ASP) is often described as a lipogenic hormone (Kalant and Cianflone 2004). It is produced in adipose tissue from complement C3, primarily through the alternative pathway of complement activation (Paglialunga et al. 2008). ASP acts by binding to a G protein-coupled membrane receptor, C5L2, and has been shown to increase glucose transport and triglyceride synthesis in adipocytes (Kalant et al. 2005). C3 knockout mice (which lack the precursor and are therefore deficient in ASP) are lean, yet hyperphagic (Paglialunga et al. 2008). The increased energy intake appears to be balanced by increased fatty acid oxidation in muscle. ASP levels in blood are increased in obesity, both in humans (Kalant and Cianflone 2004) and in mice (Paglialunga et al. 2008). The proposed mechanism for ASP is that it enhances triglyceride synthesis by an effect on the DGAT enzyme.

In accord with this, studies of arteriovenous differences of triglycerides and fatty acids over adipose tissue in lean and obese individuals after a meal showed a strong correlation between fatty acid trapping in the tissue and ASP levels in blood (Kalant et al. 2000).

### **13.6 Conclusion: an Integrated View of the Lipase Systems in Adipose Tissue**

The intracellular lipase system with ATGL and HSL, as well as the LPL system, are regulated at two levels. Relatively slow changes in gene transcription set the potential activity of the systems in accord with the environment, but for rapid hour to hour responses to changes in nutritional state and other factors, both systems rely on post-transcriptional mechanisms. In this review we do not discuss the long-term adaptations; our focus is the rapid changes that occur against a background of essentially stable mRNA and protein levels.

It is often stated that intracellular lipolysis and LPL action are regulated in a reciprocal manner. This would seem expedient to ensure a smooth transition when net release of fatty acids during fasting changes to uptake after a meal. There is however, as yet, no established molecular mechanism that couples the two processes directly to each other. On the contrary, they operate on different time-scales.  $\beta$ -Adrenergic stimulation, insulin and several other hormones can change the rate of intracellular lipolysis within minutes by phosphorylation/dephosphorylation of relatively long-lived proteins in the lipase system, perilipin, HSL and perhaps other. Changes in LPL activity at its site of action, the vascular endothelium, are accomplished by changes in the amount of short-lived LPL molecules by several different mechanisms. Two intracellular mechanisms are: (a) synthesis of LPL can be specifically blocked by a protein complex that binds to and blocks translation of LPL mRNA and (b) changes in the ER milieu can lead to changes in the partitioning between export of active lipase molecules and degradation of misfolded lipase molecules. After the enzyme has been secreted from the adipocytes, angiotensin-like proteins can convert active LPL dimers into inactive LPL monomers (Sect. 13.3.4.6).

Even though these mechanisms are separate and different, they all depend on the synthesis and secretion of new LPL molecules. Therefore the changes in LPL activity take a few hours to be fully developed. This is in accord with what is observed in experiments on rats (Bergo et al. 2002; Picard et al. 1999). It is also in accord with the results of studies of arteriovenous differences over adipose tissue in man. When fasted individuals are given a meal it takes 2 h before LPL-mediated hydrolysis increases appreciably (Fielding and Frayn 1998). Hence, the mechanisms by which intracellular lipolysis and LPL activity changes occur by different mechanisms and act on different time-scales.

An additional level of control that acts on LPL but not directly on the ATGL-HSL system is exerted at the vascular endothelium. When fatty acids and other

products of lipolysis accumulate, they shut off LPL action by eliminating the effect of apoCII, which is a necessary activator for LPL.

This is not to say that there is no co-ordination between intracellular lipolysis and the LPL reaction at the vascular endothelium, but such co-ordination is not brought about by common mechanisms for regulation, or by direct crosstalk between the lipases. The lipases probably feed their products, fatty acids and monoglycerides into common pools and the ultimate result, net deposition or mobilization, is determined by the balance between rates of lipolysis and rates of esterification.

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