The role of glycosphingolipids in insulin signaling and lipid metabolism
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Citation for published version (APA):
Bijl, N. (2009). The role of glycosphingolipids in insulin signaling and lipid metabolism

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Chapter 8

Bile acids and their role in cholesterol homeostasis

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Cellular Lipid Metabolism. ed. by Christian Ehnholm
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.
Chapter 8

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 lipophylic 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 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; Zimber
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.

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 than 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
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 7α-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 3β-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 7α-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.

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.

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 cata-
bolic 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, 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).

### 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.

**Box 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 12a-hydroxylase, CYP8B1), sinusoidal 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 (OSTa, OSTb). The development of specific FXR agonists (GW4064, fexaramine, AGN34, 6a-ethyl-chenodeoxycholic acid) and the generation of FXR Δ/Δ mice have provided powerful tools to study the pathways that are in part controlled by FXR (Hubbert et al. 2007).
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 1).

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 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 tauroursodeoxycholic 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 2).

**Box 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.
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 7α-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 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 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. 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.
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.

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 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, TNFα and IL-1β, 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 HNF4α, 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).
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 gastrointestinal tract, 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.

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 aniontransporting 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 derivate 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 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).
3.2 Intestine
The 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 LRH-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. I-BABP 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: Ostα and Ostβ (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 Ostα/β also further supported the central role for FGF15 signalling in control of bile synthesis. Ostα/β 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 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 longknown ability of oxysterols to inhibit cholesterol synthesis in animal cells.

4.1 Cholesterol Absorption in the Intestine

In an aqueous milieu the solubility of unesterified cholesterol is about 1 μM. 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 bioinfomatics approach, the NPC1L1 protein was identified as a putative cholesterol transporter in intestinal cells and target for ezetimibe.
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.

4.2 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.
4.3 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 per-
fusion 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. 8.2). 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 cholesterol secretion (TICE) was unaltered in Abcg8 knockout mice (van der Velde et al. 2007). In addition, Srb1 does not mediate TICE, in contrast the rate of TICE was 2-fold higher in Srb1 knockout mice. Also, 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-δ (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-δ, suggesting that the effects are mediated through this nuclear receptor.
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 μmol/day. Approximately 40% of this cholesterol is reabsorbed from the intestine and this should result in an output in the faeces of 4–5 μmol/day. Twice as much (10 μmol/day) is found, indicating that a significant amount (5 μmol/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.

Which lipoprotein serves as the 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 acyl-CoA; 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.

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 pre-
dominant 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; Zimber 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; Zimber 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.

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-a-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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