Regulation of hepatobiliary transport function by nuclear receptors
Kok, Tineke

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2004

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Chapter 1

General introduction and aim of the thesis
The liver has various important physiological functions, including the formation of bile. The biliary pathway represents the major route for the excretion from the body of a wide range of compounds, including cholesterol, bilirubin, and a variety of xenobiotics. Bile formation is an active process, driven by secretion of organic molecules into the bile canicular lumen followed by passive entry of water. Bile salts comprise the major organic constituents of bile and provide the major force for bile formation. Cholestasis refers to a clinical condition in which the formation of bile is impaired. The causes underlying the development of cholestasis in patients can be extremely diverse. Bile flow impairment may occur through defects in canicular secretion from the hepatocytes or from diseases affecting the biliary tree. Transporter proteins localized at the basolateral and canicular membranes of hepatocytes play an essential role in the uptake of compounds from blood and their subsequent secretion from hepatocytes into bile. It has been shown in the past few years that a number of nuclear receptors play a prominent role in the transcriptional control of transporter genes. In addition, nuclear receptors also appear to be involved in transcriptional control of genes involved in bile salt and cholesterol metabolism1-3.

The aim of the research described in this thesis was to define the role of nuclear receptors in control of hepatobiliary transport function. Therefore, we have studied the role of the peroxisome proliferator-activated receptor \( \alpha \) (PPAR\( \alpha \)), the liver X receptor (LXR), and the farnesoid X receptor (FXR) in control of expression of hepatobiliary transporters \( \text{in vivo} \) and related changes in expression levels directly to their physiological consequences on bile formation. The \( \text{in vivo} \) effects of nuclear receptor deficiency were studied by using specific strains of gene knockout mice and consequences of nuclear receptor activation by using specific pharmacological and physiological ligands. To provide background information relevant to these studies, the following sections provide an overview of liver physiology, hepatobiliary transport, bile salt and cholesterol metabolism, and nuclear hormone receptors. An overview of the current state of knowledge of ATP binding cassette (ABC) transporters, nuclear receptors and corresponding knockout mice models is provided in review format in Chapter 8.

THE LIVER

The liver is involved in the metabolism and subsequent disposal of (potentially) harmful substances from the body, either via secretion into bile or, after their delivery to the kidneys, via secretion into urine. The liver consists of different cell types: hepatocytes (parenchymal cells), endothelial cells, Kupffer cells (macrophages), and stellate cells (Figure 1)4. The hepatocytes comprise about 70% of the cell population of the total liver. Hepatocytes are polarized cells with distinct basolateral (in contact with the blood) and apical (lining the bile canaliculus) membrane domains, separated from each other by tight junctions. The basolateral membrane contacts the space of Disse. The canicular membranes of adjacent hepatocytes form the bile canaliculi, which connect with the terminal bile ducts. The bile ducts join to form the common bile duct which ends up in the gallbladder in the majority of mammalian species, including humans and mice. Cholangiocytes (epithelial cells) line the intrahepatic bile ducts5. Endothelial cells are fenestrated and constitute a permeable barrier between the hepatocytes and the blood stream. This allows exchange of fluids between the blood and the space of Disse, but hinders the passage of cells. Kupffer cells
General introduction

play an important role in immune response and phagocytosis, while hepatic stellate cells, located in the space of Disse, usually contain vitamin A-rich lipid droplets and are key players in fibrogenesis.

Blood enters the liver via two routes. Blood coming directly from the intestine enters via the portal vein (~75%) and the remainder enters via the hepatic artery. The portal vein transfers nutrients that have been absorbed from the intestine towards the liver. Hepatocytes are responsible for the synthesis, degradation, and storage of a number of substances; they play a central role in carbohydrate and lipid metabolism and they secrete most of the proteins found in plasma. The portal area of the liver, where the bile ductules are located, is called zone 1. Blood leaves the liver via the central vein, which is located in the pericentral zone (zone 3). There are marked functional and morphological differences between hepatocytes localized in different zones and many metabolic processes are localized in the distinct zones. Metabolic processes that require oxygen are found mainly periportally while, oxygen-independent processes are located mainly in the perivenous zone. Bile flows through the canalicular network in the direction opposite to blood flow, i.e., from zone 3 to zone 1, and collects in the bile ducts (see Figure 1).

Figure 1. Schematic representation of the structure of the liver. The hepatocytes are separated from the bloodstream by a single thin sheet of endothelial cells with interspersed Kupffer cells. Small holes in the endothelial sheet allow exchange of molecules between the hepatocytes and the bloodstream. The hepatocytes also form a system of bile canaliculi into which they secrete bile. The bile ducts discharge bile into the gallbladder and finally the intestine. Adapted from.
Chapter 1

BILE FORMATION

Bile is essential for the absorption of dietary lipids and lipid-soluble vitamins from the intestine. Bile salts present in bile are required for the solubilization of dietary lipids in the intestinal lumen. During cholestasis or impairment of bile flow, bile salt deficiency occurs in the small intestinal lumen leading to lipid malabsorption. Furthermore, the biliary pathway represents the major route for elimination of a range of compounds, including bilirubin, xenobiotics, and cholesterol. In the latter way, bile is important in the maintenance of cholesterol homeostasis. Cholesterol is considered to leave the body almost exclusively after its excretion into bile, either as a free compound or after its conversion into bile salts. The main organic constituents of bile are bile salts, phospholipids, cholesterol, a variety of proteins, glutathione, amino acids, and bilirubin11-14.

Bile formation represents an osmotic process that is unique to the hepatocyte as a polarized epithelial cell. Membrane transport proteins that primarily determine bile formation are located at the basolateral (uptake) and canalicular (secretion) domains of the hepatocyte15. Bile salt secretion is the major driving force for bile formation, which is referred to as the bile salt-dependent fraction of bile flow. Other solutes generate the so-called bile salt-independent fraction of bile flow, of which glutathione (GSH) is a major contributor16. The last step of hepatobiliary transport, i.e., the secretion of substances into the canalicular lumen, is to a large extent mediated by ATP-binding cassette (ABC) transporter proteins. These transporters couple ATP hydrolysis to the transport of specific substrates, which enables uphill transport into the canalicular lumen. In recent years an impressive progress has been made in the discovery, cloning, and characterization of the basolateral and canalicular carrier proteins. After secretion into the canalicular lumen, bile is further concentrated in the gallbladder before reaching the intestine (except for species like the rat, in which the gallbladder is absent). Primary bile is modified in the bile ducts where the cholangiocytes add water, chloride and bicarbonate, which also contributes to bile formation5.

HEPATIC TRANSPORT SYSTEMS

The formation of bile requires multiple transport systems at the basolateral and canalicular membrane domains of hepatocytes. Transport specificities, localization and regulatory mechanisms for a number of these transporters are currently known. A schematic picture of these transporters, as localized in the hepatocyte, is given in Figure 2.

Hepatic uptake transporters
The specific architecture of the liver sinusoids allows the passage of protein-bound compounds through endothelial fenestrae where basolateral uptake systems of hepatocytes can extract bile salts and other organic molecules from albumin. The uptake of bile salts from blood into the hepatocyte involves two processes. The first is co-transport with sodium coupled to the electrochemical sodium gradient (maintained by Na⁺K⁺-ATPase) and the second involves sodium-independent bile salt/organic anion exchange. The uptake is predominantly mediated by members of the solute carrier superfamily (Slc).

Sodium-dependent uptake of taurine-and glycine-conjugated bile salts is mediated by the Na⁺-taurocholate co-transporting polypeptide (Ntcp; Slc10a1)17,18. Ntcp is the main transporter for the uptake of bile salts from portal blood into hepatocytes. Ntcp is expressed
General introduction

exclusively in hepatocytes localized strictly to the basolateral plasma membrane and its expression is not zonated\textsuperscript{19,20}. Recently it has become clear that \textit{Ntcp} gene transcription is controlled by a network of hepatocyte-enriched transcription factors and ligand-activated nuclear receptors\textsuperscript{21,22}. Sodium-independent hepatocellular uptake of organic anions is mediated by several members of the organic anion-transporting polypeptides (Oatps) family of membrane transporters. Oatps represent multispecific transporters which mediate hepatocellular uptake from the blood of a variety of amphiphatic organic compounds, like bile salts, bilirubin, steroids, thyroid hormones, peptides, bromosulfophthalein, mycotoxins, and numerous drugs\textsuperscript{23}. The most abundantly expressed Oatp transporter in rodent liver is Oatp1 (SL21a1). Oatp1 is able to transport conjugated and unconjugated bile salts. Oatp1 probably works as an exchanger and accepts anions and/or glutathione, which appears to be important for the driving force\textsuperscript{24}. Besides Oatp1, Oatp2 (SL21a5) and Oatp4 (SL21a10) are also present at the basolateral membrane of the hepatocyte\textsuperscript{25,26}. Oatp2 and Oatp4 show overlapping substrate specificities with Oatp1. An overview of hepatic uptake transporters can be found in several excellent reviews\textsuperscript{3,17}. After basolateral uptake, bile salts and other solutes are transferred across hepatocytes by largely undefined processes for canalicular secretion.

**Canalicular ABC transporters**

Transporter proteins located at the canalicular membrane are responsible for the biliary secretion of bile salts, phosphatidylcholine, cholesterol, and reduced glutathione (GSH) as well as for the excretion of potentially toxic compounds. These secretion processes are ATP-dependent and mediated by so-called ABC transporter proteins\textsuperscript{2}. The ABC transporters are also described in Chapter 8 and will only shortly be discussed in this section.

The structure of ABC transporters is highly conserved. Most ABC transporters have two sets of 6 membrane spanning domains (Figure 3), although some have an additional amino-terminal extension with 5 transmembrane domains. Two intracellular loops contain Walker A and B motifs, which are involved in the binding and hydrolysis of ATP. This

---

**Figure 2.** \textit{Hepatobiliary transport systems in rodent liver}. At the basolateral membrane, compounds are taken up by the Na\textsuperscript{+}-taurocholate co-transporting polypeptide (Ntcp) and by several members of the organic anion-transporting polypeptides (Oatps). Several ATP-binding cassette (ABC) transporter proteins, located at the canalicular membrane, are involved in secretion of substances into the canalicular lumen. Only proteins, whose membrane localization is unequivocally established, are included. See text for further details.
provides the energy needed for the transport against a concentration gradient\textsuperscript{27,28}. A number of ABC transporters are half-transporters, consisting of only 6 transmembrane domains and containing a single intracellular ATP-binding domain. ABC half-transporters are thought to heterodimerize into functional pumps. Based on their sequence homology, the members of the ABC superfamily have been divided into several subclasses\textsuperscript{29}. Besides the plasma membrane, ABC transporters are also localized in membranes of intracellular organelles such as the peroxisomes, endoplasmic reticulum and mitochondria\textsuperscript{27}. The focus of this section will be on transporters located at the plasma membrane of hepatocytes.

**The Abca family: Abca1**

Abca1 is probably the most studied transporter of the Abca family. The exact subcellular localization of the protein has not definitively been reported yet, although recent studies suggest that Abca1 is localized at the basolateral surface of hepatocytes\textsuperscript{30} and is cycling intracellularly between plasma membrane and endosomes\textsuperscript{31}. The role of Abca1 in hepatocytes is currently unknown, but may involve formation of pre-\(\beta\)-high-density lipoprotein (HDL) particles\textsuperscript{32,33}. Abca1 is widely expressed, and besides the liver, also abundant in peripheral macrophages and in the intestine\textsuperscript{34}. In macrophages, Abca1 is probably facilitating the efflux of cellular phospholipids and cholesterol to lipid-poor ApoA-I, thereby producing nascent or pre-\(\beta\) HDL\textsuperscript{35,36}. Intestinal Abca1 has been suggested to be involved in cholesterol efflux from enterocytes into the lumen, thereby regulating the efficiency of intestinal cholesterol absorption\textsuperscript{37,38}. Yet, more recent studies indicate a basolateral localization of the protein in the intestine\textsuperscript{39}. Mutations in the human \textit{ABCA1} gene are associated with Tangier disease, a disorder characterized by the absence of HDL in plasma and by the accumulation of cholesteryl ester in various tissues\textsuperscript{40-42}. A study described in this thesis (chapter 5) makes use of an \textit{Abca1} knockout mouse on a DBA/1 background\textsuperscript{43}. Abca1-deficient mice are also characterized by an absolute absence of plasma HDL.

**The Abcb family: Mdr1a, Mdr1b, Bsep and Mdr2**

Four important members of the Abcb family are expressed mainly or exclusively in
hepatocytes: Mdr1a, Mdr1b, Mdr2, and Bsep. Rodent multidrug resistance transporters Mdr1a and Mdr1b (Abcb1a/Abcb1b) have a single homologue in humans, i.e., MDR1 (ABCB1). Mdr1a and Mdr1b are expressed at the canalicular membrane of hepatocytes\(^44\). Besides the liver, Mdr1a/b are also expressed in the small intestine, in the kidney, and in many other tissues\(^45,46\). Mdr1a/b excretes a very broad spectrum of amphiphatic drugs out of cells, thereby conferring multidrug resistance against these substances\(^47\).

Mdr2 (Abcb4) P-glycoprotein (P-gp) is a phosphatidylcholine translocator, which is also expressed in the canalicular membrane of the hepatocyte\(^48,49\). Its expression is higher in the periportal zone \(^1\)\(^50,51\) than in zone 3. The function of Mdr2 (MDR3 in humans) has been elucidated by disruption of the \(Mdr2\) gene in the mouse\(^52\). Mdr2 translocates phospholipids from the inner leaflet of the canalicular membrane to the outer leaflet, which faces the canalicular lumen. Phospholipids are expelled from the outer leaflet of the canalicular membrane in a process that is stimulated by bile salts. Little is known about the actual mechanism by which bile salts promote secretion of phospholipids, but it appears to involve vesiculation from the outer leaflet of the membrane\(^53\). The secretion of phospholipids is of crucial importance in the protection of the cellular membranes of the biliary tree from the high concentrations of detergent bile salts\(^54\). Human \(MDR3\) gene mutations are associated with progressive familiar intrahepatic cholestasis (PFIC) type 3\(^55,56\), which is a disease featured by low biliary phospholipid concentrations, increased cholestatic serum markers and high plasma \(\gamma\)-glutamyltranspeptidase levels (see also review chapter 8). Recent studies demonstrate that in families of these patients the prevalence of gallstones and intrahepatic cholestasis of pregnancy is increased\(^57-59\).

Bile salts are pumped across the canalicular membrane via the bile salt export pump (Bsep; Abcb11). This protein is a hepatocyte-specific transporter, present in high levels in the canalicular membrane\(^60\) in a homogenous expression pattern. Mutations in the human \(BSEP\) gene are the cause of progressive familiar intrahepatic cholestasis (PFIC) type 2\(^61,62\). This disease is characterized by low biliary bile salt concentrations, elevated serum bile salt concentrations, and normal \(\gamma\)-glutamyltranspeptidase levels.

The \(Abcc\) family: \(Mrp1\), \(Mrp2\), \(Mrp3\) and \(Mrp6\)

The multidrug resistance protein transporters (Mrp; Abcc) are all organic anion pumps, but they differ in substrate specificity and tissue distribution. Mrp1, 2, 3, and 6 are all expressed in the liver and characterized by the same structural feature, i.e., they have 17 transmembrane segments\(^63\).

Mrp1 (Abcc1) is expressed in most celltypes at the basolateral membrane\(^64\). It transports a variety of drugs conjugated to GSH, to sulfate or to glucuronate, as well as anionic drugs and dyes, but also neutral amphiphatic drugs\(^65\). Mrp2 has an overall substrate specificity similar to that of Mrp1. In contrast to Mrp1, Mrp2 is localized at the apical membrane\(^65,66\). Besides the liver, Mrp2 is also expressed in the kidney and in the intestine\(^67,68\). Mrp2 contributes to bile formation by transporting GSH, which is a major driving force for the bile salt-independent bile flow\(^16\). \(MRP2\) gene mutations in humans are the cause of Dubin-Johnson syndrome, which is characterized by a conjugated hyperbilirubinaemia\(^69,70\).

Mrp3 is another organic anion transporter, basolaterally localized like Mrp1, and also present in liver, intestine, and kidney. Mrp3 differs from Mrp1 and 2 in that it appears not very well able to transport bile salts\(^71\). Mrp3 is upregulated in the liver under some cholestatic conditions and has the ability to transport bile salts\(^72\). Mrp6 is also expressed basolaterally in hepatocytes. The substrate specificity of Mrp6 is still not completely known\(^73\). For more information about the Mrp family, the reader is referred to an excellent review by
The Abcg family: Abcg5 and Abcg8

Members of the Abcg family, like Abcg5 and Abcg8, are so-called half-transporters. Abcg5 and 8 are both expressed in the liver and the intestine\textsuperscript{74,75}. ABC half-transporters are thought to dimerize into functional pumps. Based on the outcome of recent studies, using knockout\textsuperscript{76} and transgenic mice\textsuperscript{32}, it appears that Abcg5/Abcg8 in the liver is responsible for secretion of biliary cholesterol. Mutations in the $\text{ABCG5}$ or $\text{ABCG8}$ gene are the cause of sitosterolemia\textsuperscript{74,77,78}. Patients with this inherited disorder accumulate plant sterols, such as sitosterol, in their circulation. Furthermore, the biliary secretion of sitosterol, other phytosterols, and cholesterol is impaired and the absorption of plant sterols from the intestine is strongly increased.

BILE SALT METABOLISM

Bile salts, the major organic solutes in bile, are amphiphatic steroidal compounds derived from the enzymatic conversion of cholesterol in the liver\textsuperscript{79}. Bile salts are efficiently reabsorbed in the intestine and transported back to the liver. Conversion of cholesterol into bile salts is of crucial importance in the control of cholesterol homeostasis.

Bile salt biosynthesis

*De novo* synthesis of bile salts predominantly occurs in the hepatocytes and involves at least 17 different enzymes. The reactions are catalyzed by enzymes located in the endoplasmic reticulum, mitochondria, cytosol, and peroxisomes. Two major pathways exist in bile salt biosynthesis: the neutral (or classic) pathway and the acidic (or alternative) pathway (Figure 4)\textsuperscript{79,80}. The rate-limiting enzyme cholesterol 7α-hydroxylase (Cyp7a1) plays a key role in the neutral pathway, whereas sterol 27-hydroxylase (Cyp27) is the regulatory enzyme in the acidic pathway. Sterol 12α-hydroxylase (Cyp8b1)\textsuperscript{79} controls the ratio of cholate and chenodeoxycholate synthesis in both the neutral and the alternative
pathway and thereby regulates the biliary bile salt hydrophobicity index. In humans, the major newly synthesized (primary) bile salts are cholate and chenodeoxycholate. In mice, the most abundant bile salt is (muri)cholate, which makes the bile salt pool of mice much more hydrophilic than that of humans. Prior to secretion into bile, primary bile salts are conjugated with either glycine or taurine in the liver. Bile salt structure can be modified by interactions with intestinal bacteria leading to the formation of secondary bile salts.

Bile salts regulate their own synthesis in a negative feedback system by binding to the bile salt-activated nuclear farnesoid X receptor (FXR; NR1H4; see 1.7)80,82. Upon activation of this receptor, the expression of a second nuclear receptor, the short heterodimer partner (SHP; NROB2) is induced which, in turn, inhibits the activity of the tissue-specific factor liver receptor homologue-1 (LRH-1; NR5A2), which controls expression of Cyp7a182. Recent studies indicate that control of Cyp7a1 is highly complex and also involves FXR/SHP-independent mechanisms83,84.

**Bile salt transport and the enterohepatic circulation**

Bile salts are concentrated up to 1000-fold in bile in comparison to the circulating bile salt plasma concentration. This steep concentration gradient is generated through active bile salt transport by Bsep. Upon contraction of the gallbladder (caused by entry of fat-containing food in the intestine), the majority of bile salts, which are associated into mixed micelles with phospholipids and cholesterol, enter the intestinal lumen. There, they act as detergents to emulsify dietary fats and lipid-soluble vitamins. The vast majority of bile salts is efficiently reabsorbed from the small intestine through a combination of sodium-independent absorption in the proximal small intestine and active sodium-dependent absorption in the distal ileum17. Bile salts then return to the liver via the portal vein, where they are taken up by the hepatocytes and resecreted into bile. This efficient conservation and recycling of bile salts is called the enterohepatic circulation (Figure 5)12,17. In adult

![Figure 5. Schematic scheme of the enterohepatic circulation of bile salts. Bile salts can be taken up by liver cells via the Na+-taurocholate co-transporting polypeptide (Ntcp) and resecretion of bile salts into bile occurs via the bile salt export pump (Bsep). Transport in the intestine is mainly mediated by the apical sodium-dependent bile salt transporter Asbt. Ileal bile acid-binding protein (Ibabp) is a small soluble protein present in enterocytes, and is thought to be involved in facilitating uptake of bile salts and their intracellular trafficking in the small intestine. In this way, bile salts are recycling between liver, intestine and blood. A small part is lost by fecal excretion but this is compensated for by de novo synthesis of bile salts from cholesterol. See text for further details.]
humans, the total bile salt pool is approximately 2 g and circulates 6 to 10 times per 24 h through the enterohepatic pathway. About 0.5 g of bile salts is lost per day through fecal excretion. Under steady state conditions, fecal loss of bile salts is compensated for by de novo synthesis of bile salts from cholesterol\textsuperscript{12,23}. Thus, biliary bile salts are either derived from synthesis from cholesterol in the hepatocytes (~5%) or from the circulating bile salt pool in the body (~95%).

Vectorial hepatocellular secretion of bile salts from blood into bile, the first major step in the enterohepatic circulation of bile salts, has been discussed in the section ‘Hepatic transport systems’. Bile salt uptake can take place via Ntcp, and resecretion of bile salts into bile occurs via the transporter Bsep. Bile salts can also be absorbed by large cholangiocytes, present in the bile ducts after which they recirculate back to the hepatocyte. This shunting of bile salts between hepatocytes and cholangiocytes is called cholehepatic shunting\textsuperscript{17}. Efficient reabsorption of bile salts in the intestine and delivery to the portal circulation is the second major transport step of the enterohepatic circulation. Bile salt uptake in the proximal part of the intestine is thought to occur mainly via sodium-independent passive diffusion of unconjugated bile salts. However, the sodium-independent bile salt transporter Oatp3 (Slc21a7) was reported to be expressed at the proximal rat jejunum\textsuperscript{85}, but its relative importance remains to be clarified. Bile salt uptake in the distal part of the small intestine is sodium-dependent and mainly mediated by the apical sodium-dependent bile salt transporter Asbt (or Ibat) (Slc10a2)\textsuperscript{17}. Asbt belongs to the same family as Ntcp, the solute carrier superfamily (Slc). Asbt is expressed at the apical domain of ileal enterocytes\textsuperscript{86,87}. Both primary and secondary conjugated and unconjugated bile salts are substrates for Asbt. ASBT in humans seems to be the major intestinal bile salt uptake system as emphasized by the observation that ASBT mutations result in bile salt malabsorption\textsuperscript{88,89}. Ileal bile acid-binding protein (Ibabp or Ilbp) is a small soluble protein of which expression is restricted to the terminal ileum\textsuperscript{90}. Ibabp is thought to be involved in facilitating uptake of bile salts and their intracellular trafficking in the small intestine\textsuperscript{91,92}. Bile salts are able to induce Ibabp expression via the bile salt-activated nuclear farnesoid X receptor (FXR; see 1.7 )\textsuperscript{93}. The Asbt splice variant t-Asbt is localized basolaterally and may be involved in efflux of bile salts from enterocytes towards portal blood\textsuperscript{17,94}. Another potential candidate for bile salt efflux is Mrp3/MRP3, which has also been identified in both rat and human small intestine\textsuperscript{95,96}.

**CHOLESTEROL METABOLISM**

The liver is the main organ in control of cholesterol metabolism. It shows a relatively high rate of de novo cholesterol synthesis through a process initiated by the key enzyme 3-hydroxy methylglutaryl-Coenzyme A (HMG-CoA) reductase\textsuperscript{97}. As explained before, the liver is the only site for the conversion of cholesterol into bile salts and its secretion into bile, either directly or in the form of bile salts. Furthermore, the liver is involved in the uptake and hydrolysis/metabolism of plasma cholesterol (esters) in the form of HDL, low-density lipoprotein (LDL), or remnant particles, and in the esterification of excess of free cholesterol and the secretion of cholesterol into plasma associated with very low density lipoprotein (VLDL) or pre-β-HDL.

**Reverse cholesterol transport**

Reverse cholesterol transport is a key process in maintenance of whole body cholesterol
Reverse cholesterol transport involves the efflux of excess cholesterol from peripheral cells (including macrophages in the vessel wall) towards nascent HDL, its transport to the liver followed by hepatic uptake mediated by scavenger receptor class B type I (SR-BI), biliary secretion in the form of cholesterol or bile salt, and finally disposal into feces. HDL-mediated reverse cholesterol transport is generally assumed to underlie the well-known epidemiological relationship between high HDL cholesterol levels and low risk for development of atherosclerosis. Yet, recent findings indicate that reverse cholesterol transport is not determined by the plasma level of HDL. Cholesterol accumulation in macrophages is considered to represent an early event in the development of atherosclerosis. Reverse cholesterol transport is therefore considered to be an anti-atherogenic process. Efflux of cholesterol from peripheral cells is now known to be mediated in part by Abca1. This transporter is described in the section ‘hepatic transport systems’. In macrophages, Abca1 is involved in delivery of cholesterol and phospholipids to nascent HDL.

Cholesterol absorption
A relatively large but variable part of the cholesterol originating from diet and bile is taken up in the intestine. Recent data suggest that the net cholesterol absorption is the result of both uptake (passive) and active secretion back to the lumen. Transporters mainly implicated in this secretory route are Abca1, Abcg5 and Abcg8. At this moment, it remains unclear whether or not these ABC transporters are the primary transporters involved in control of intestinal cholesterol absorption.

NUCLEAR RECEPTORS
Nuclear receptors comprise a superfamily of transcription factors that are, in many cases, activated by the binding of small (lipid-soluble) molecules (or ligands). Nuclear receptor ligands control a variety of functions whose dysregulation will eventually lead to life-threatening diseases such as cancer, hyperlipidemia, cholestasis, diabetes, and atherosclerosis. Because the ligand molecules can be easily modified and synthetized, nuclear receptors have become promising pharmacological targets. It has been shown in the past few years that nuclear receptors play a prominent role in the transcriptional control of transporter genes. Besides that, they are also important in transcriptional control of genes involved in bile salt, lipid, and cholesterol metabolism.

Nuclear receptors have a typical modular structure (Figure 6A). A poorly-conserved region called activation function 1 (AF1), which is responsible for ligand-independent transcriptional activation and involved in the coordinated interaction of co-activators and co-repressors is found at the amino-terminus. Adjacent is the highly conserved DNA-binding domain (DBD), which contains two zinc-finger motifs and is responsible for recognition and binding to the DNA sequences comprising the specific response elements. A small Hinge region facilitates the three-dimensional functional organization of the multiple domains. Near the carboxy-terminal region, a moderately conserved ligand-binding domain (LBD) determines the affinity of receptors for various ligands. The last part, activation function 2 (AF2), provides ligand-dependent transactivation. It must be noted that some receptors vary significantly from the typical nuclear receptor domain organization.

Nuclear receptors bind to consensus response elements located in genes and can bind DNA as monomers, homodimers or heterodimers. Most of the heterodimeric complexes
contain the retinoid X receptor (RXR; NR2B1)\(^{107,108}\). Receptors belonging to the class II receptors include PPARs, LXR and FXR, which heterodimerize with RXR prior to binding to response elements (REs) (Figure 6B). In general, the response element is composed of two 6 basepair elements (hexamers; prototypically 5'-AGGTCA-3') separated by 0-8 nucleotides, although most DNA response elements do not contain the perfect AGGTCA hexamer. Surrounding and intervening DNA sequences may also affect binding affinity and function. Binding sites can be classified as direct repeats (DR), inverted repeats (IR), and everted repeats (ER) (Figure 6C).

**Figure 6. Nuclear receptor organization, transcriptional regulation and consensus DNA response elements.** (A) Representation of the functional domains of nuclear receptors. Nuclear receptors consist of different domains: the activation function 1 (AF1), the DNA-binding domain (DBD), the Hinge region, the ligand-binding domain (LBD), and the activation function 2 (AF2). The primary functions of the DBD and LBD domains are to recognize specific DNA sequences and ligands. See text for further details. (B) Mechanism of transcriptional regulation by nuclear receptors. Upon ligand activation, nuclear (class II) receptors heterodimerize with RXR and bind to response elements (RE) within the promoter sequences of target genes, thereby regulating their transcription. (C) Sequences of consensus DNA-binding sites for nuclear receptors. Note that there can be significant variability in the AGGTCA hexamer sequence. n = spacing between hexamers.

**PPARα (NR1C1)**
Three peroxisome proliferator-activated receptor (PPAR) genes have been identified in mammals: PPARα (NR1C1), PPARβ (NR1C2) and PPARγ (NR1C3), which are all activated by polyunsaturated fatty acids, eicosanoids, and various synthetic ligands\(^{109}\). PPARα is highly expressed in the liver and was found to respond to hypolipidemic drugs, such as fibrates\(^{110}\). Fibrates are widely used in the treatment of hyperlipidemia\(^{111}\). Fatty acids serve as natural PPAR ligands. The generation of Ppara knockout mice has established that PPARα is critical in the coordinate transcriptional activation of the fatty acid oxidation.
machinery in the liver. A physiological condition in which PPAR\(\alpha\) becomes activated is during fasting\(^{112}\). By modulating gene expression, PPAR\(\alpha\) stimulates hepatic fatty acid oxidation to supply substrates that can be metabolized by other tissues. Studies described in this thesis (chapter 2 and 3) make use of Ppar\(\alpha\) knockout mice created by Gonzalez et al.\(^{112}\). A critical feature of PPAR\(\alpha\)-deficient mice is that they cannot induce the oxidation of fatty acids appropriately under fasting conditions.

**LXR (NR1H3)**

The liver X receptor (LXR) has become recognized as an important regulator of whole-body cholesterol metabolism\(^{37}\). There are two distinct gene products, LXR\(\alpha\) (NR1H3) and LXR\(\beta\) (NR1H2), which have similar target DNA-binding elements. LXR\(\alpha\) is expressed predominantly in liver, kidney, intestine, fat tissue, spleen, and in macrophages, while LXR\(\beta\) is expressed ubiquitously\(^{37,114}\). Natural ligands for LXR are oxysterols, e.g. 22 (R)-hydroxycholesterol and 24 (S)-hydroxycholesterol\(^{115,116}\). Oxysterols are oxidized derivatives of cholesterol that serve as intermediary substrates in the rate-limiting steps of steroid hormone and bile salt synthesis\(^{117}\). LXR acts as a cholesterol sensor that responds to elevated cellular sterol concentrations, which induces expression of genes involved in cholesterol disposal. Synthetic agonists, like T0901713, are able to activate LXR.

**FXR (NR1H4)**

In 1999, it was reported that bile salts are high-affinity ligands for the farnesoid X receptor (FXR; NR1H4)\(^{91,118}\). FXR was initially named after its low-level response to farnesol. FXR is highly expressed in liver, intestine, kidney and cholangiocytes, all tissues and cells that are exposed to bile salts\(^{37}\). Bile salts, such as chenodeoxycholate, deoxycholate, cholate and their conjugates are natural ligands for FXR. The first Fxr knockout mouse was described by Sinal et al.\(^{119}\), who showed that FXR is involved in control of bile salt and lipid metabolism. The Fxr knockout mice used in studies described in this thesis (chapter 6) have not been described before and were generated by homologous recombination by Tularik Incorporated (South San Francisco, CA, USA).

**OUTLINE AND AIM OF THE THESIS**

The formation of bile is an important function of the liver. The biliary pathway represents the major route for the excretion from the body of a wide range of compounds, including cholesterol, bilirubin, and a variety of xenobiotics. Furthermore, bile is essential for the absorption of dietary lipids and lipid-soluble vitamins from the intestine. The hepatocyte is central in the process of generating bile. Bile formation is an active process, driven by secretion of organic molecules from hepatocytes into the bile canalicular lumen that is followed by passive entry of water. Bile salts comprise the major organic constituents of bile and provide the main driving force for bile formation. Hepatobiliary secretion of compounds is mediated by the coordinated action of multiple transport systems present at the basolateral (uptake) and canalicular (secretion) membrane domains of hepatocytes. The last step of hepatobiliary transport, i.e., the secretion of substances into the canalicular lumen, is to a large extent mediated by ABC transporter proteins. Many of the basolateral and canalicular carrier proteins have been cloned and characterized over the past decade and the molecular basis of several forms of inherited cholestatic liver diseases has been elucidated. Cholestasis is a clinical condition that is functionally defined as an impairment
Chapter 1

of bile flow, which may occur through defects in canalicular secretion processes. In the past few years, several nuclear receptors have been discovered that play a prominent role in the transcriptional control of transporter genes and also of genes involved in bile salt, lipid and cholesterol metabolism. Dietary compounds, certain endogenous metabolites and drugs may alter hepatic transporter activity, and thus bile formation, through activation of these nuclear receptors. Ligands of nuclear receptors can be easily modified and synthetized and, therefore, nuclear receptors have become promising pharmacological targets for treatment of diseases like artherosclerosis, hyperlipidemia, and cholestasis. The aim of this thesis was to define the role of nuclear receptors in control of hepatobiliary transport function. Most data published thus far on transcriptional regulation of transporter expression are from in vitro studies and their relevance for the in vivo situation has remained largely undefined. Therefore, we have studied the roles of three different nuclear receptors, i.e., PPARα, LXR and FXR, in control of expression of hepatobiliary transporters in vivo and related changes in expression levels directly to their physiological consequences on bile formation. The in vivo effects of nuclear receptor deficiency were studied by using specific strains of gene knockout mice and consequences of nuclear receptor activation were studied by using specific pharmacological and physiological ligands.

**Peroxisome proliferator-activated receptor α (PPARα)**

PPARα is involved in control of the fatty acid oxidation machinery in the liver, but also has a role in regulation of bile salt metabolism. Fatty acids serve as their natural ligands. Synthetic PPARα agonists, i.e., lipid-lowering fibrates, may affect bile composition as they have been shown to influence expression of rate-limiting enzymes in bile salt synthesis and expression of the canalicular phospholipid translocator Mdr2. Therefore, wild-type and PPARα-deficient mice were treated with a diet supplemented with ciprofibrate or subjected to a fasting-refeeding schedule (chapters 2 and 3) and bile formation and expression of hepatobiliary transporters were studied. Fasting is the physiological condition in which PPARα becomes activated by fatty acids that are liberated from adipose tissue. Specifically, the potential role of PPARα in the regulation of Mdr2 in mice and its human homologue MDR3 has been addressed. A peroxisome proliferator response element (PPRE), which is potentially able to bind the PPARα/RXR complex, was found ~4.9 kb upstream of the transcription initiation site in the human MDR3 gene promoter (chapter 4).

**Liver X receptor (LXR)**

LXR has been recognized as an important regulator of whole-body cholesterol metabolism. Natural ligands for LXR are oxysterols, which are oxidized derivatives of cholesterol that serve as intermediary substrates in the rate-limiting steps of steroid hormone and bile salt synthesis. The transporter Abca1, a known LXR target gene, is essential for high-density lipoprotein (HDL) formation and is generally considered rate-controlling for reverse cholesterol transport. Abca1-deficient mice are characterized by a complete absence of plasma HDL. Activation of LXR by synthetic agonists has been promoted as a potentially promising way to stimulate the so-called reverse cholesterol transport pathway. As HDL is thought to be a major source for bile-destined cholesterol, we have studied the effects of LXR activation (using the synthetic agonist T091317) on bile formation, specifically focusing on biliary cholesterol excretion in Abca1-deficient mice (chapter 5).

**Farnesoid X receptor (FXR)**

The bile salt-activated receptor FXR is involved in control of both bile salt and lipid metabolism. Activated FXR inhibits expression of the gene encoding cholesterol 7α-hydroxylase (Cyp7a1), which catalyzes the first and rate-controlling step of bile salt
synthesis, and induces the expression of the bile salt export pump \((Bsep)^{128,129}\). These findings were mainly obtained from \textit{in vitro} studies and consequences on \textit{in vivo} bile formation and bile salt metabolism were unclear. For that reason, the role of FXR on the enterohepatic circulation of bile salts \textit{in vivo} has been determined in detail by quantifying bile salt kinetics in FXR-deficient mice using a stable isotope dilution method (\textit{chapter 6}). The kinetic parameters were related to bile formation and the expression of hepatic and intestinal transporters involved in the enterohepatic circulation of bile salts. A similar series of studies were conducted in rats treated with the synthetic FXR agonist GW4046 to evaluate the effects on the enterohepatic circulation of bile salts upon chronic activation of FXR (\textit{chapter 7}).

Finally, the current state of knowledge obtained from application of hepatic ABC transporter knockout mouse models and nuclear receptor knockout mouse models has been reviewed in \textit{chapter 8}. Findings from mouse models were compared to characteristic hallmarks of inherited diseases in humans caused by mutations in homologous transporters. The nuclear receptor knockout mice were discussed as far as they affect ABC transporter expression and bile formation. The thesis concludes with a general discussion in which a picture of transport regulation by nuclear receptors based on our own data is provided.
REFERENCES


Chapter 1


Chapter 1


