Towards treatment of cholestatic liver disease in children via interference with transcriptional regulation of hepatic transport systems

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TOWARDS TREATMENT OF CHOLESTATIC LIVER DISEASE IN CHILDREN VIA INTERFERENCE WITH TRANSCRIPTIONAL REGULATION OF HEPATIC TRANSPORT SYSTEMS

JAAP MULDER

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Contents

CHAPTER 1

Introduction

Introduction

The liver performs multiple crucial functions [1]. In addition to its role in detoxification of endogenous and exogenous compounds, the liver generates bile that is essential for excretion of waste products and for fat absorption, acts as a central integrator of whole body energy metabolism and is the site of extensive protein synthesis (e.g., albumin and clotting factors) and modification of hormones and vitamins (e.g., thyroid hormone and vitamin D) [2].

Anatomy and histology

Positioned in the right upper quadrant of the abdomen (**Figure 1A**), the liver receives both arterial blood from the hepatic artery and venous blood that comes from the gastro-intestinal tract via the portal vein (**Figure 1B**). The latter constitutes

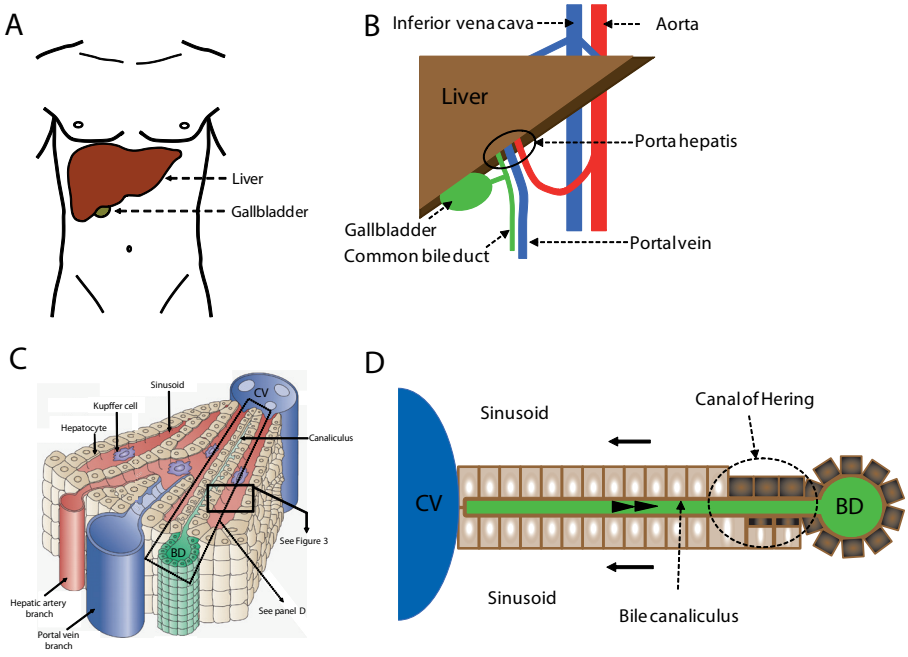

Figure 1. ***Liver anatomy and histology.*** (A) Position in abdomen. (B) Schematic representation of liver blood supply and drainage. (C) Microscopic anatomy of liver lobule (Adapted by permission from MacMillan Publishers Ltd: Adams DH, Eksteen B. Nat Rev Immunol 2006, **www.nature.com/nri/**) 81. (D) Schematic representation of bile canaliculus. Canaliculi are formed by tightly joined hepatocytes and drain via Canals of Hering into bile ductulus, which are lined (in part) by cholangiocytes. Bile flow direction is indicated by arrowheads. Sinusoidal blood flow direction is indicated by arrows. BD, bile ductulus; CV, central vein.

approximately two-thirds of total liver blood supply. Blood leaves the liver via the hepatic veins, which drain into the inferior vena cava just below the diaphragm [3].

The hepatic artery and the portal vein run together through the lesser omentum and start to branch close to the porta hepatis (**Figure 1B**). These initial branches supply the left and right liver lobes, but this branching continues until 10th-12th order branches merge into sinusoids lining the hepatocytes (**Figure 1C**). In the sinusoids, a fenestrated endothelial layer allows for direct contact between blood and hepatocytes, facilitating bi-directional transport of blood constituents and hepatocyte-derived compounds across the basolateral membrane of the hepatocytes. Sinusoids drain into the central veins, which continue to merge and eventually form the hepatic veins [3-5].

The biliary tree runs in close proximity, but "antiparallel" to the arterial and portal systems. Its smallest branches, the bile canaliculi, are formed by tightly joined hepatocytes (**Figure 1D**). These junctions not only prevent exchange of constituents between the intercellular canaliculi and the sinusoids, but also separate the hepatocellular plasma membrane into basolateral (facing the sinusoid) and apical (or canalicular) domains. As a consequence hepatocytes are highly polarized cells. Bile canaliculi merge forming a mesh network, which drain via the canals of Hering into the bile ductules (**Figure 1D**). Starting at the canals of Hering, the biliary tree is lined with specialized epithelium, i.e., cholangiocytes. Bile ductules converge into progressively larger bile ducts, eventually leaving the liver as the hepatic duct. In humans and mice, newly formed bile is temporarily stored in the gallbladder. Bile is secreted into the duodenum upon gallbladder contraction via the common bile duct. Gallbladder contraction induced by a fatty meal is a neuro-endocrinally controlled process, including release of cholecystokinin and actions of the autonomous nervous system [1, 4, 6].

Throughout the past, histologists have given different definitions of the functional units of the liver. Examples of three of these units are shown in **Figure 2**. The classic lobule, which was first described by Kiernan in 1833, is a polygon* with a central vein and the surrounding centripetal sinusoids fed by the tributaries from portal triads located at peripheral sides. The classic lobule thus includes all hepatocytes drained by a single central vein. This unit can, however, only be identified microscopically in a few species (e.g., pig and polar bear) due to septa of connective tissue at its boundaries. Since such septa are not present in normal human liver and the classic lobule does not provide a histological basis for understanding deranged hepatic organization and function, additional definitions of the functional units of the liver have been proposed, including the portal lobule and the hepatic acinus (**Figure 2**). The former includes all hepatocytes drained by a single bile ductule and the latter constitutes a three-dimensional mass of hepatocytes arranged around the terminal branches of the hepatic artery and portal vein, which is irregular in shape

* This polygon is often represented schematically as a hexagon.

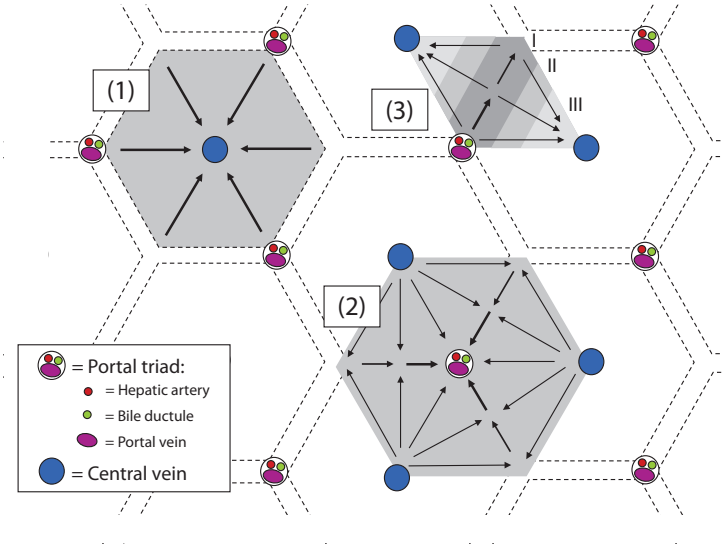

Figure 2. ***The schematic representation of functional units of the liver:*** (1) classic lobule, (2) portal lobule and (3) hepatic acinus. Grey scale in the latter indicates to relative blood oxygen-content (dark grey = high oxygen = acinar zone I; light grey = low oxygen = zone III). See text for further details. Arrows indicate direction of (1) sinusoidal blood flow, (2) bile drainage and (3) arterial blood flow.

and drains into at least two central veins [1, 6]. There is, however, still no consensus on which definition is the most useful. The choice of definition used affects the terminology used to refer to specific regions/zones within the liver, where hepatocytes fulfil different functions. The acinar zone I (or periportal region) is supplied with relatively oxygen-rich blood. The acinar zone III (or pericentral region) receives relatively oxygen-poor blood with zone II (or mid-zonal region) receiving blood with intermediate oxygen-contents. Highly oxygen-dependent processes are mostly concentrated in zone I (e.g., oxidative energy metabolism, gluconeogenesis and bile salt secretion), while less oxygen-dependent processes are mainly occurring in zone III (e.g., glycolysis, liponeogenesis, drug-detoxification) [1].

Cells of the liver

The majority of the total cell population in the liver (i.e., 60-65%) is made up of hepatocytes, which are also called parenchymal liver cells [7]. Several other cell types are present throughout the liver in close proximity to hepatocytes. These so-called non-parenchymal cells consist mainly of endothelial cells (15-20%), Kupffer cells (8-12%), pit cells (1-2%), hepatic stellate cells (3-8%) and cholangiocytes (3-4%) [7]. **Figure 3** shows a schematic representation of a liver sinusoid.

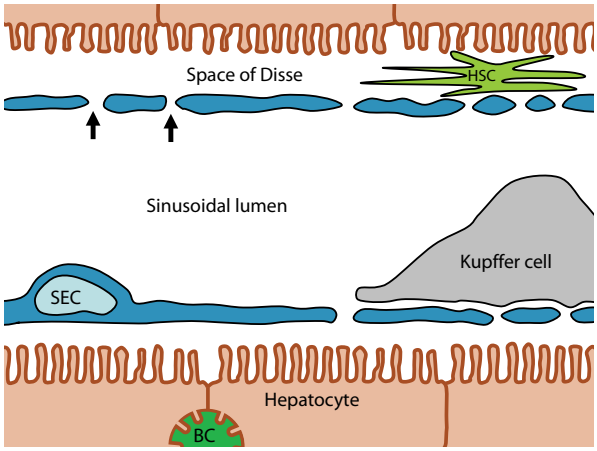

Figure 3. ***Schematic representation of the liver sinusoid.*** SEC, sinusoidal endothelial cell; HSC, hepatic stellate cell; BC, bile canaliculus. Arrows indicate endothelial fenestrations. (Pit cells are not shown)

Hepatocytes carry out the bulk of typical liver functions, including bile formation, protein synthesis, lipid and glucose metabolism and detoxification. Both the basolateral and canalicular plasma membranes of the polarized hepatocytes contain microvilli. This greatly increases the available surface area for transmembrane transport processes. To be able to perform their metabolic tasks, hepatocytes contain many mitochondria and extensive endoplasmic reticulum (both smooth and rough). Hepatocytes located in different acinar zones have different morphologic characteristics matching their activity profile [1, 4, 6].

The sinusoidal endothelial cells separate the basolateral membrane of hepatocytes from the sinusoidal lumen and thus create the so-called space of Disse. Due to the fenestrations, the absence of a basement membrane and the lack of tight intercellular attachments, solutes can move freely in and out of the space of Disse which further facilitates bi-directional transport between hepatocytes and blood. Besides creating this sieve-like physical barrier, the sinusoidal endothelial cells are also biologically active, e.g., contributing to hemodynamic regulation and inflammatory responses through secretion of vaso-active substances and cytokines, and endocytotic activities [6].

Kupffer cells line the endothelial cells inside the sinusoidal lumen. These liver resident macrophages are bone-marrow derived cells that play an important immunological role in the liver [8]. They clear particles, both infectious and non-infectious, from the circulation in a highly-effective manner [6]. More recently, Kupffer cells have also been shown to be involved in the regulation of injury repair [9]. Activated Kupffer cells secrete a whole range of cytokines and other (inflammatory) mediators and thus regulate many hepatic responses in a paracrine fashion [8]. Besides Kupffer

cells, another type of immune cell is often present in the sinusoids, i.e., the liver-associated lymfocytes or Pit cells. These cells have been designated as resident liver natural-killer cells and are important in the protection against metastatic cancer cells, viruses, intracellular bacteria and parasites [6, 10].

Within the space of Disse, one finds hepatic stellate cells. These cells, also referred to as Ito cells, perform multiple tasks. They store vitamin A, are involved in the regulation of microvascular tone, produce extra-cellular matrix proteins and mediate the regenerative response of the liver [6, 11]. Interestingly, hepatic stellate cells appear to be better known for their role in liver pathology than in liver physiology. Considering their production of matrix proteins and their role in regeneration, it is not surprising that hepatic stellate cells are important players in the development of liver fibrosis in response to a wide-spectrum of liver insults. Much of the research into hepatic stellate cell biology has thus focused on this particular pathophysiological process [11]

Cholangiocytes constitute another very important component of the non-parenchymal liver cell population. First appearing at the level of the canals of Hering, these cells line the bile ductules and larger-sized branches of the biliary tree as well as the gallbladder [4]. Cholangiocytes are actively involved in the formation of bile and the regulation of its composition through active transport of various bile constituents [12]. It has been presumed that in humans 40% of actual bile flow is generated by the biliary epithelium [13].

Bile formation/flow and enterohepatic circulation

One of the primary functions of the liver is the synthesis and secretion of bile salts. Bile salts are derived from cholesterol. Via two major pathways, i.e. the neutral/classical and the acidic pathways, a series of enzymatic reactions leads to the generation of two primary bile acids, i.e., cholic acid (CA) and chenodeoxycholic acid (CDCA) [14]. These bile acids are efficiently conjugated with the amino acids taurine or glycine. This conjugation renders CA and CDCA more hydrophilic and acidic. Due to this increased acidity conjugated bile acids are present as anionic salts at physiological pH and are thus referred to as bile salts [15].

Bile salts are secreted against an uphill concentration gradient from the hepatocytes into the canaliculi. This energy-dependent process is carried out by the bile salt export pump (BSEP †), a member of the ATP-binding cassette (ABC)-transporter family also known as ABCB11 ‡ (**Figure 4**). Bile salt secretion is the primary driving force for the generation of bile flow [16]. It leads to the passive transport of water and

† According to general convention, names of human proteins and genes are presented in uppercase with the latter in italics, while those of other species are presented in lowercase. The presentation of human proteins/genes will be used unless there is a difference between human and non-human names. In this case, the non-human name will be given once.

‡ Official nomenclature of these transporters is based on phylogenetic classification. Transporter names are grouped into families, e.g. ABC or solute carrier (SLC). For reasons of clarity, however, the traditional protein/gene names of transporters (e.g. BSEP, NTCP) will be used in this thesis.

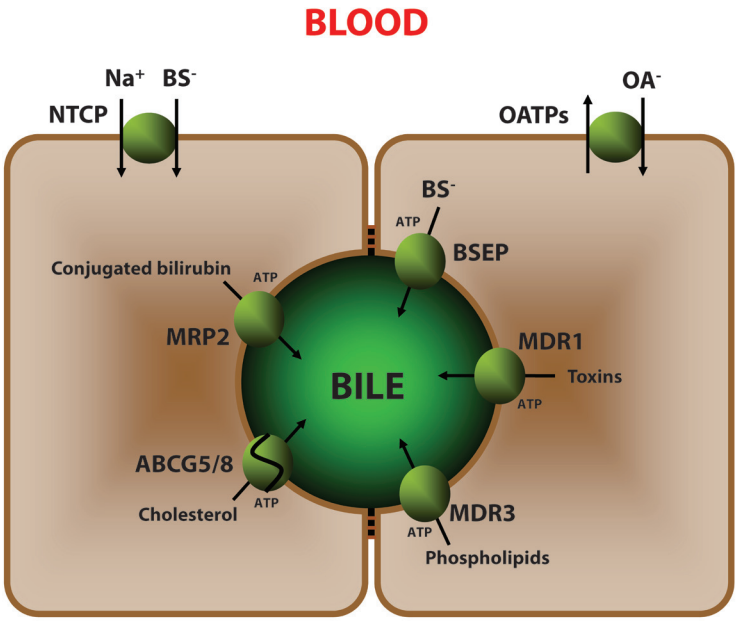

Figure 4. ***Hepatocellular transporters involved in bile formation and basolateral/sinusoidal bile salt uptake.*** Two adjacent hepatocytes are tightly joined, which creates the canalicular lumen. BS, bile salt; OA, organic anion; ATP, adenosine-triphosphate. *Of note*, microvilli present on basolateral and canalicular membranes are not shown.

electrolytes across the canalicular membrane. In addition to this bile salt-dependent fraction of bile flow, active transport of bicarbonate and glutathione into the canalicular lumen further induces bile flow (bile salt-independent fraction) [16]. Several other transporters are present in the canalicular membrane that contribute to the formation of bile (**Figure 4**). These transport, amongst other compounds, phospholipids (multidrug resistance protein (MDR)-3/Mdr2 or ABCB4), cholesterol (ABCG5/8), conjugated bilirubin (multidrug resistance-associated protein (MRP)-2 or ABCC2) and toxins (MDR1/Mdr1b or ABCB1) [17]. Although thorough modification of its composition will occur during passage through the biliary system and storage in the gallbladder, bile will eventually reach the intestinal tract, where bile salts can fulfil their functions in lipid digestion and absorption (see below) and biliary waste products will be excreted via the feces.

Due to their hydrophilicity, bile salts permeate the apical membrane of enterocytes poorly. Thus, bile salts can exert their role in lipid digestion and absorption throughout the small intestine. In the distal ileum, however, bile salts are very efficiently taken up by the apical sodium-dependent bile salt transporter (ASBT or SLC10A2) located on the apical membrane of the enterocytes. The high efficiency

of this process is illustrated by the fact that under normal conditions approximately 95% of intestinal bile salts are reabsorbed [14].

After trans-enterocyte transport, bile salts are secreted via the organic solute transporter (OST)-α/β heterodimer, at the basolateral side and transported back to the liver via the portal circulation. In the liver, bile salts are taken up again at the basolateral side of the hepatocytes by sodium-taurocholate co-transporting polypeptide (NTCP or SLC10A1) or organic anion transporting peptides (OATPs or SLC21A), completing the enterohepatic circulation. The hepatic extraction of bile salts from portal blood is also a highly efficient process (70-90%) [18].

In addition to the enterohepatic circulation, bile salts also recirculate within the liver and biliary tree via the "cholehepatic shunt", i.e., after canalicular secretion, bile salts are taken up by cholangiocytes and return to the liver to be resecreted into bile [19]. Both the quantitative and qualitative importance of this shunt remains to be determined. The recent identification of OSTα/β as a bile salt transporting heterodimer and its expression in cholangiocytes further support this concept [20].

Bile salts are not inert compounds. During their intestinal transit, bile salts can also undergo several modifications. Dehydroxylation of primary bile salts by the intestinal flora yields deoxycholate and lithocholate [21] and, to lesser extent, ursodeoxycholate (UDCA [§]) [22]. These secondary and tertiary bile salts become an integral part of the total bile salt pool.

Intestinal actions of bile salts

Bile salts are amphipathic molecules that act as detergents and facilitate intestinal lipid digestion and absorption by emulsification of dietary lipids and subsequent formation of mixed micelles [23]. Together with biliary and dietary phospholipids, bile salts emulsify large water-insoluble fat droplets into smaller droplets and thus render the lipid contents of these droplets more accessible for intraluminal lipases. Mixed micelles further facilitate the lipid absorption process [2]. Along with dietary fats and their digestion products, many other fat-soluble compounds are also absorbed by the enterocytes via emulsified lipid droplets and mixed micelles. Examples of such compounds are the fat-soluble vitamins A, D, E and K. Intestinal bile salts are, thus, essential for adequate uptake of both macronutrients and micronutrients. A lack or reduction of intestinal bile salts will have profound repercussions not only on growth, but also on processes as bone mineralization (vitamin D) and blood coagulation (vitamin K).

Regulation of bile salt homeostasis and protection against bile salt overload

Although the detergent-characteristics of bile salts are crucial for their intestinal actions, these also pose a problem. Bile salts can cause cellular damage through

§ This abbreviation officially refers to the bile acid from, but is also used to refer to the bile salt ursodeoxycholate.

disruption of cell membranes and induce hepatocellular apoptosis and necrosis [24]. Hence, physicochemical barriers need to be present to protect membranes against high local bile salt concentrations and intracellular bile salt concentrations need to be tightly regulated.

Physicochemical protection against the "membranolytic" effects [25] of bile salts is provided by at least two different mechanisms. Gallbladder epithelial cells secrete mucus to protect the apical membrane against highly concentrated bile [1]. Hepatocytes also secrete phospholipids via the canalicular transporter MDR3 into canalicular bile (**Figure 4**). These form mixed micelles with bile salts and thus, protect the apical membranes facing the canalicular and ductular lumina against high local concentrations of bile salts. The importance of this process is illustrated MDR3-deficiency, which is the underlying defect of progressive familial intrahepatic cholestasis (PFIC) type 3. This hereditary cholestatic liver disease is characterized biochemical markers of bile duct damage and ensuing progressive liver damage due to low biliary phospholipids concentrations [26].

High intracellular concentrations of free bile salts are prevented by binding to cytosolic proteins, most notably 3-hydroxy steroid hydrogenase [27], and by transcriptional regulation bile salt homeostasis. The latter is mediated to a large extent by members of the nuclear receptor (NR) superfamily (see below) with the farnesoid X receptor (FXR, NR1H4) playing the most important role. FXR is directly activated by bile salts [28-30] and it is highly expressed in organs and cells involved in bile salt transport [31, 32]. FXR heterodimerizes with its obligate partner, the retinoid X receptor (RXR)-α, (NR2B1). The actions of bile salt-activated FXR in hepatocytes and enterocytes are summarized in **Figure 5**. Upon activation by bile salts, FXR directly induces bile salt export (via BSEP and OSTα/β) and indirectly suppresses bile salt import (via NTCP and ASBT) and synthesis (via CYP7A1) [33]. Although FXR-mediated control of bile salt homeostasis was initially thought to be an intracellular process, more recent studies have shown that FXR-activation also leads to endocrine and autocrine signaling via induction and release of fibroblast growth factor (FGF)-19 (rodent orthologue Fgf15) [34-36] (**Figure 5**).

Besides FXR, at least two other NRs can be activated by bile acids, i.e., the pregnane X receptor (PXR, NR1I2) and the vitamin D receptor (VDR, NR1I1) [37, 38]. These receptors, however, are activated by the secondary bile acid lithocholic acid and may be more important in protection against overload of toxic bile acids than in regulation of basal bile salt synthesis and transport.

The remarkable degree of control of bile salts over their own homeostasis is illustrated by the very effective treatment of several bile salt synthesis defects by bile salt supplementation [39]. This group of inborn errors of metabolism is characterized by the production of cytotoxic bile acid intermediates that accumulate in hepatocytes and lead to apoptosis and/or necrosis [21] as well as the lack of intestinal bile salts. Treatment consists of oral supplementation with the primary bile acid CA [39, 40].

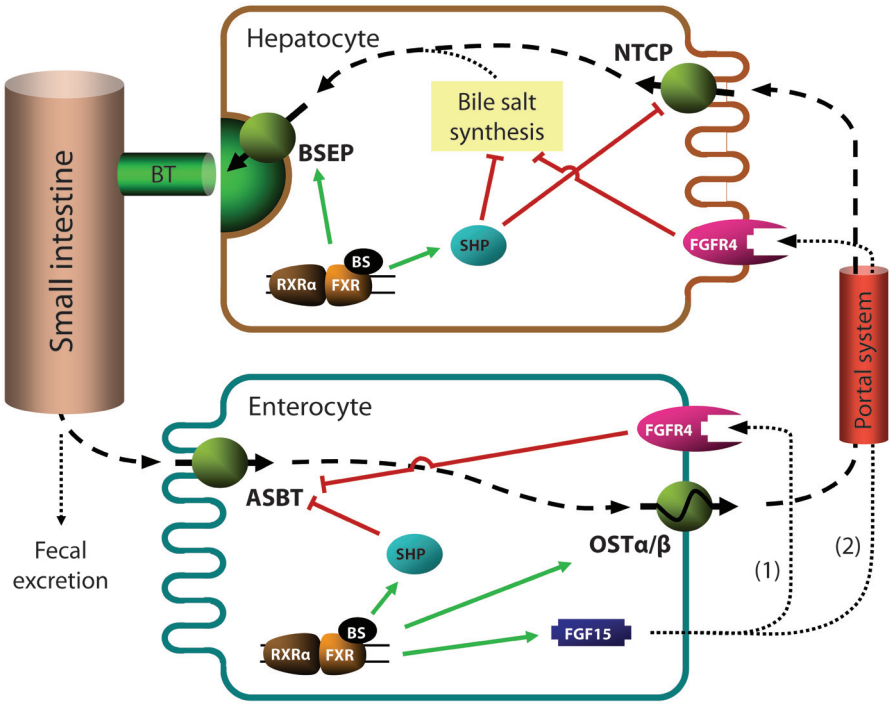

Figure 5. ***FXR-mediated transcriptional regulation of the enterohepatic circulation and bile salt synthesis.*** Bile salt (BS)-activated FXR in conjunction with RXR(α) directly induces expression of BSEP, OSTα/β, FGF15 and SHP (green arrows). The latter two lead to indirect suppression of ASBT expression and bile salt synthesis (red lines). Secreted FGF15 acts in autocrine (1) and endocrine (2) manners via the FGF-receptor 4 (FGFR4). The components of enterohepatic circulation are shown by the dashed lines. BT, biliary tree (see text for other abbreviations).

CA-supplementation does not only lead to adequate levels of intestinal bile salts facilitating lipid digestion and absorption, but it simultaneously leads to the suppression of endogenous synthesis of cytotoxic bile acid intermediates. The effectiveness of this treatment is demonstrated by the dramatic improvement of the prognosis of afflicted children. In the case of 3β-hydroxy-C27-steroid oxidoreductase deficiency or Δ4-3-oxosteroid 5β-reductase deficiency, the prognosis of patients changes from liver transplantation-bound to very good [39].

Defining cholestasis and cholestatic liver disease

The term "cholestasis" is derived from the Greek words χολη (= "bile") and στασις (= "stoppage") and has been translated as "stoppage or suppression of bile flow, having intrahepatic or extrahepatic causes" [41]. Although this literal translation appears

to be a straightforward definition, it still harbors a sense of ambiguity [42]. "Stoppage" suggests an obstruction of already generated bile flow, while "suppression" may be interpreted as a reduction in the generation. Moreover, the all-embracing addition of "having intrahepatic or extrahepatic causes" does not provide any precision.

Not only linguists, but pathologists, clinicians and physiologists have all provided their own definitions of "cholestasis". This has led to at least eleven definitions throughout the years (Table 1) [42]. Although this may seem redundant, one has to bear in mind that these definitions were provided by authors with different perspectives and interests in an applicable definition during different eras with corresponding states of knowledge. Hence, one might expect that today additional definitions could be proposed, e.g., based on the current availability of new diagnostic procedures.

To further add to the confusion, "cholestasis" is sometimes also used to indicate liver disease, i.e., clinical symptoms, signs and (permanent) liver damage due to cholestasis. These conditions, however, should be referred to as "cholestatic liver disease" to make a distinction between the pathophysiological observation and a true disease state. Fortunately, cholestasis will not always lead to (permanent) liver injury (e.g., intermittent cholestasis due to cholelithiasis). However, it is difficult to define the exact point of transition from cholestasis to cholestatic liver disease.

As can be deduced from the definition of cholestasis by Dorland's [41], its causes have often been grouped into "extrahepatic" and "intrahepatic". Although this anatomical classification is straightforward and applicable (e.g., with regard to surgical access), it does not account for important pathophysiological differences. Hence, other classifications have been proposed, including the pathophysiological distinc-

Table 1. ***Definitions of cholestasis (adapted from McIntyre (42))***

Dictionaries		
1	a	Stoppage or suppression of the flow of bile, having intrahepatic or extrahepatic causes
	b	An arrest in the flow of bile
Pathological		
2		Macroscopic (green liver and hepatomegaly)
3		Light microscopy (canalicular bile plugs and bile pigment in Kupffer cells and hepatocytes)
4		Ultrastructural (dilated canaliculi with fewer and blunted microvilli, alterations in endoplasmic reticulum and Golgi apparatus)
Clinical		
5		Jaundice, dark urine, pale stools, pruritus
Biochemical		
6		Elevated conjugated bilirubin, alkaline phosphatase and cholesterol
Physiological		
7		A primary hepatocellular alteration of secretion of micelles containing bile salts
8		Decrease in bile flow (measured)
9		Diminution of the volume of that fraction of bile that is dependent on bile acids
10		A reduction of bile salt output into bile and into the intestine
11		Failure of normal amounts of bile to reach the intestine

tion of "obstructive" vs. "hepatocellular" cholestasis [5]. The former category includes conditions in which the flow of "normal" (canalicular) bile is obstructed with secondary accumulation of bile products and subsequent damage to biliary epithelia and hepatocytes, while the latter category includes conditions in which initial bile formation is impaired. This pathophysiological classification separates the various causes largely along the same line as the anatomical classification except for those conditions where bile flow is obstructed at the level of the small intrahepatic bile ducts.

The above emphasizes that one ought to clearly state what one means when using the term "cholestasis". Despite the fact that Javitt and Arias expressed their concerns about the promiscuous use of the term "cholestasis" more than 40 years ago [43], it continues to cause debate [44, 45]. Addition of the pathophysiological cause is probably the most informative.

Cholestatic liver disease in infancy and childhood

Cholestasis and cholestatic liver disease can have many different causes. The most common causes in the neonatal and pediatric population are listed in Table 2. The particular relevance of this class of diseases for these populations is illustrated by its relative contribution as cause of end-stage liver disease necessitating liver transplantation in infants and children (**Figure 6**) [46]. Amongst the different cholestatic conditions, biliary atresia is worldwide the most common diagnosis leading to liver transplantation [47-51].

There are several reasons why infants and children seem to be affected more frequently by cholestatic liver disease than adults. Firstly, many types of cholestatic liver disease are due to congenital or genetic disorders, which generally present early in life. Secondly, the most frequent form of acquired cholestatic liver disease in children, i.e. biliary atresia, exclusively affects infants. This is thought to be due to an age-specific susceptibility to a viral insult on the biliary tree [52]. Thirdly, the normal developmental pattern of bile formation also explains why children and, especially premature, infants are more susceptible than adults to disturbances in this process and thus prone to develop cholestasis [53]. Factors responsible for this difference are thought to include relatively larger contribution of bile-salt independent fraction to bile flow, which may be more sensitive to various insults than the bile salt-dependent fraction [54], due lower (immature) transporter expression in infants and children [5, 55] and the smaller bile salt pool size and synthesis rate [56].

Another type of cholestatic liver disease that primarily affects infants and children is parenteral nutrition-associated cholestasis (PNAC). Although its exact cause remains to be elucidated, PNAC illustrates how a combination of factors can eventually lead to cholestatic liver disease. Parenteral nutrition is used in infants that cannot be fed enterally since they are either born prematurely or with gastro-intestinal anomalies requiring resection of (parts of) the intestinal tract. As a consequence of

Table 2. ***Causes of cholestasis in infancy and childhood (5, 61, 78-80)***

Structural	
	Extrahepatic biliary atresia
	Choledochal cyst
	Bile duct hypoplasia
	Bile duct paucity (Alagille's syndrome / non-syndromic)
	Primary sclerosing cholangitis
	Congenital hepatic fibrosis
Infectious / immunological	
	Viral (e.g., cytomegalovirus, herpes, toxoplasmosis, enterovirus, parvovirus)
	Bacterial (sepsis)
	Tuberculosis
	Autoimmune hepatitis
Metabolic / genetic	
	α1-Antitrypsin deficiency
	Cystic fibrosis
	Cholelithiasis
	Galactosaemia
	Tyrosinaemia
	PFIC1-3 / BRIC
	North American Indian familial cholestasis
	Bile acid synthesis defects
	Peroxisomal disorders
	Hypothyrodism
	Wilson's disease
Toxic	
	Parenteral nutrition
	Drugs
Others	
	Langerhans' cell histiocytosis
	Perinatal asphyxia
	Budd-Chiari, veno-occlusive disease
	Inspissated bile syndrome (AB0-incompatibility)
	Idiopathic neonatal hepatitis

PFIC, progressive familial intrahepatic cholestasis; BRIC, benign recurrent intrahepatic cholestasis

dependency on parenteral nutrition, patients generally require indwelling catheters making them more susceptible to sepsis. Along with the immaturity of both bile formation and immunity, these factors render infants particularly premature infants more susceptible to develop PNAC [57-59].

Curative treatment of cholestatic liver disease

Since cholestatic liver disease comprises a heterogeneous group of conditions, many different, disease-specific treatments exist with, unfortunately, widely differing efficacies. Structural anomalies, such as choledochal cysts, are treated

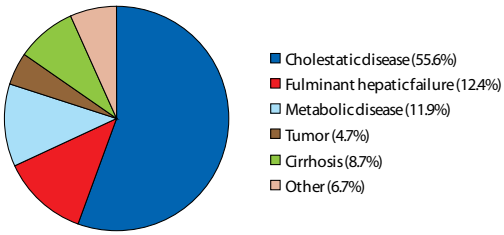

Figure 6. ***Primary liver disease diagnoses amongst children undergoing liver transplantation according to the 1995-2002 SPLIT-registration.*** (Derived from McDiarmid *et al.* (46).)

with the surgical intervention (cyst resection, cholecystectomy and Roux-en-Y hepaticojejunostomy) [60, 61]. The prognosis after cyst resection is generally good, although life-time follow-up is warranted due to potential malignant degeneration [60]. Cholestasis caused by infection often requires antimicrobial treatment, while cholestasis caused by the administration of drugs or toxins will generally subside upon withdrawal of these agents. As already mentioned patients with bile salt synthesis defects may benefit from oral bile salt replacement.

Unfortunately, many patients cannot be treated effectively. This may be due to the condition of the patient that precludes withdrawal of the toxin (e.g., parenteral nutrition dependent infants who cannot be fed enterally at short term) or due to the causative defect that will make proper bile formation impossible (e.g., PFIC types 1-3) or will continue to prevent adequate bile drainage (e.g., bile duct paucity). The prognosis of these patients depends largely on the underlying pathology.

Biliary atresia is an intriguing example of cholestatic liver disease as it can initially be treated surgically by hepatoporto-enterostomy, which is aimed at restoring bile-drainage. The efficacy of this so-called Kasai-procedure [62] is dependent on multiple factors, including age at surgery (best if age <30 days) and surgical expertise [63]. In 40-60% of patients bile flow was restored as evidenced by disappearance of jaundice, but in the remaining patients, the failure to restore bile drainage leads to further, rapid progression of the liver injury generally necessitating liver transplantation within the first year of life [63, 64]. Unfortunately, patients in which bile drainage was re-established also often redevelop cholestasis and, although the majority of them survive more than 10 years with their native liver, most will eventually require liver transplantation [65, 66].

With the advent of orthotopic liver transplantation, the prognosis of patients suffering from end-stage liver failure improved dramatically. Liver transplantation was first performed in 1963 by dr Thomas Starzl in Denver, Colorado in the United States [67], but did not become a reliable clinical alternative until effective immunosuppressive agents became available [68]. Along with improved surgical tech-

niques, better peri-/post-transplant (immunosuppression) regimes, patient selection and graft allocation have further increased post-transplantation survival rates [69]. Amongst all age groups, post-transplantation patient survival rates are the highest in children of the age 1-18 years, followed by infants (0-1 year). Current overall patient survival rates at 1- and 5-year post-transplantation hover around 80% in these groups [68].

Symptomatic treatments

Besides the curative treatments mentioned in the previous paragraph that are highly dependent on the type of cholestatic liver disease, several symptomatic treatments exist that can be applied more universally in patients with cholestatic liver disease. Two of the major effects of cholestasis are the accumulation of compounds normally excreted into bile in liver and elsewhere, and the lack of adequate levels of intestinal bile salts.

The former leads to symptoms such as jaundice and pruritus. Especially, the latter can be extremely debilitating and have profound effects on the quality of life. Although the exact pathogenesis of cholestatic pruritus remains unclear, several symptomatic treatments have been shown to be effective, including UDCA, intestinal bile salt sequestrants (e.g., cholestyramine, colesevelam), the PXR-agonist rifampicin, opioid antagonists (e.g., naloxone and naltrexone) and the selective serotonin-reuptake inhibitor, sertraline [70]. Pruritus due to extrahepatic cholestasis can sometimes be treated by endoscopic or surgical intervention, e.g., partial external biliary diversion. However, intractable intrahepatic pruritus may even warrant liver transplantation in the absence of end-stage liver failure [70].

As already mentioned, the lack of intestinal bile salts will have repercussions for the digestion and absorption of dietary fats as well as the absorption of fat-soluble micronutrients. At the same time, the metabolic rate is also known to be increased in patients with cholestatic liver disease. This combination may not only lead to general failure-to-thrive, but also to more specific nutritional deficiencies such as rickets or acute hemorrhagic emergencies due to vitamin D and K deficiencies, respectively. To achieve adequate caloric intake, the intake is increased to 120-150% of estimated daily requirements with medium-chain fatty acids constituting the majority of the dietary fats, since these can be taken up directly by enterocytes. Fat-soluble nutrients also need to be supplemented in relatively high doses [71, 72].

Ursodeoxycholic acid

In current treatment regimes of cholestatic liver disease, a special place is reserved for ursodeoxycholic acid (UDCA). UDCA is the most abundant bile acid in black bear's bile (ursus = "bear", in Latin), which has been used in traditional Chinese medicine to treat liver disease for more than thousand years [73]. Regarded as tertiary bile salts, UDCA-conjugates are also present in the endogenous human

bile salt pool, albeit as a minor fraction (1-3%) [73]. In comparison to other bile salts, UDCA-conjugates are more hydrophilic and non-cytotoxic [22]. The rationale behind the clinical use of UDCA is bile salt "displacement" and its choleretic effect [21]. Orally administered UDCA is absorbed by the intestine, effectively conjugated in the liver and secreted into bile. After administration in usual doses, UDCA-conjugates will constitute 30-60% of the bile salt pool and thus render it less cytotoxic by displacing the regular, more toxic bile salts [22]. Simultaneously, UDCA-conjugates will enhance biliary secretion and thus enhance the elimination of other potentially toxic compounds from the hepatocytes [73]. Moreover, UDCA is known to have several other beneficial effects, including the inhibition of hepatocellular apoptosis under cholestatic conditions and the induction cholangiocellular bicarbonate secretion [73]. Although UDCA administration has been shown to generally improve biochemical abnormalities in pediatric cholestatic liver disease, its effect on clinical outcome has been shown to be variable and to be dependent on the type of disease [57, 58, 74-76]. Thus, its true efficacy with regard to clinical outcome remains to be further investigated.

With regard to NR-regulation of bile salt homeostasis, it is important to note that UDCA is, at most, only a weak FXR-agonist [77]. Some authors actually consider UDCA to be an FXR-antagonist [21].

Need for additional treatment strategies

The overall prognosis of patients suffering from cholestatic liver disease, especially the hepatocellular types, has improved greatly over the past decades. This improvement can, however, largely be attributed to successful liver transplantation programs. Besides oral bile salt replacement in some bile salt synthesis defects, few medical treatments exist that dramatically affect the prognosis of this group of patients. In the light of scarcity of organ donors and the fact that liver transplantation remains a complex procedure with significant morbidity and mortality, the quest for new treatment strategies continues. Although these would ideally be directly curative, therapies aimed at modulation of the disease progression may be more realistic.

Nuclear receptors and their ligands in new treatments

After the extensive physiological studies of bile formation the 1970s-1980s and the identification and characterization of the actual transporters in the 1980s-1990s, the past decade has brought us much improved understanding of the regulation of these transporters. NRs were found to play an important role in these processes. Besides FXR, several other NRs were shown to be involved in the regulation of hepatobiliary transporters including the liver X receptor (LXRα/β, NR1H3/2), the small heterodimer partner (SHP, NR0B2), the liver receptor homolog (LRH)-1 (NR5A2) and the hepatocyte nuclear factor (HNF)-4α (NR2A1) [14]. As a class of ligand-activated transcription factors, NRs provide the opportunity to make the logical next step after identification of regulatory mechanisms, i.e., intervention in the transcriptional regulation of the transport systems.

Scope of this thesis

Cholestatic liver disease comprises a heterogeneous group of conditions frequently affecting infants and children. It has a wide spectrum of causes ranging from congenital or acquired obstruction of the biliary tree to impaired bile formation due to genetic deficiencies, infectious and toxic insults. Unfortunately, few effective therapies exist besides liver transplantation and current treatments are mainly symptomatic. Therefore, there exists a need to expand our therapeutic arsenal to improve the quality of life and prognosis of young patients with cholestatic liver disease. This thesis describes a set of attempts to gain further insight in the possibilities of using pharmacological ligands of nuclear receptors (NR) to intervene in the pathogenesis of cholestasis

Our focus has primarily been on cholestasis induced by inflammation, which belongs in the category of hepatocellular cholestasis. In **Chapter 2**, we reviewed clinical and pathophysiological aspects of inflammation-induced cholestasis (IIC) and the roles that NRs play as both mediators in its pathogenesis and as potentially therapeutic modifiers of IIC. NR biology is briefly discussed with special attention to the hitherto often underappreciated role of co-regulators in NR-regulated gene transcription. Inflammatory signaling has been shown to affect NR-function and, since the latter is important in basal hepatobiliary transporter expression, this appears to be one of the mediating steps in the development of IIC. Several NRs have been shown to have anti-inflammatory effects, especially in macrophages.

Considering the important role of Kupffer cells, as liver resident macrophages, in the pathogenesis of IIC and the recent findings of the anti-inflammatory effects of PPARγ and LXR in macrophages, we determined the effects of synthetic ligands of these two NRs in a mouse model of IIC (injection of lipopolysaccharide). In **Chapter 3**, the effects of the PPARγ-ligand rosiglitazone on LPS-induced suppression of hepatobiliary transporters are described, while in **Chapter 4**, the effects of the LXR-ligand T0901317 on these transporters are presented. Both compounds were shown to be able to attenuate the effects of LPS, but, unexpectedly, they appeared to primarily act on hepatocytes rather than on Kupffer cells. Rosiglitazone and T0901317 were also shown to differently affect specific inflammatory signaling pathways. Although T0901317 was found to be effective in suppressing the inflammatory response, this came at the expense of massive steatosis due to hepatocellular LXR-activation. To determine whether Kupffer cell-targeted LXR-activation might be a feasible alternative avenue to the inflammatory response while avoiding the hepatocellular side-effects, *in vitro* follow-up studies with T0901317 were performed with primary Kupffer cells. The results of these studies are presented in **Chapter 5**.

Previously, investigations into the pathophysiology of IIC had mainly focused on the effects of inflammation on actual bile flow generation by secretion of bile salts and the regulation of the transporters responsible for these processes. Bile, however, also contains other components, e.g., water, phospholipids, cholesterol and endo-/

xenobiotics. The effects of inflammatory signaling on the secretion of these components had received relatively little attention. In **Chapter 6**, we analyzed the effects of inflammation on hepatobiliary cholesterol secretion.

Finally, in **Chapter 7**, our overall results are discussed and put in a clinical and experimental perspective. Potential directions of future investigations are given.

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CHAPTER 2

Nuclear receptors: mediators and modifiers of inflammation-induced cholestasis

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ABSTRACT

Inflammation-induced cholestasis (IIC) is a frequently occurring phenomenon. A central role in its pathogenesis is played by nuclear receptors (NRs). These ligand-activated transcription factors not only regulate basal expression of hepatobiliary transport systems, but also mediate adaptive responses to inflammation and possess anti-inflammatory characteristics. The latter two functions may be exploited in the search for new treatments for IIC as well as for cholestasis in general. Current knowledge of the pathogenesis of IIC and the dual role NRs in this process are reviewed. Special interest is given to the use of NRs as potential targets for intervention.

Introduction

Inflammation-induced cholestasis (IIC) is a frequently occurring, well-recognized clinical entity. Molecular mechanisms underlying IIC have been partially unravelled over the past decades, facilitated by the increasing knowledge of mechanisms of bile formation and regulation of the transport systems involved, identification of nuclear factors controlling transporter gene expression and a broader understanding of molecular aspects of the inflammatory response. It is now evident that several nuclear receptors (NRs), i.e., ligand-activated transcription factors, play key roles in the regulation of bile formation and the pathogenesis of IIC. These receptors are not only important under physiological conditions but their involvement expands to pathophysiological situations, both as mediators, i.e., in a disease-promoting role, and as important modulators of adaptive responses. Recently, NRs have also been proposed as targets for intervention in IIC. This review focuses on the various roles of NRs in processes that lead to cholestasis during inflammation and on the ways in which NRs can be exploited for design of treatment options.

Clinical aspects of inflammation-induced cholestasis (IIC)

The link between inflammation and cholestasis has been recognized for centuries, with jaundice as the primary symptom of impaired bile formation [1-4]. The underlying mechanisms of this association, however, have gone unexplained for a long time [5].

Cholestasis associated with sepsis is generally regarded as the prototypical example of IIC, but bile formation is also affected in other conditions associated with an inflammatory state and cholestasis may thus be considered as a consequence of the so-called acute phase response (APR). The APR consists of a set of rapid, well-coordinated responses initiated by infection or tissue damage leading to the production of various soluble mediators (e.g., proteases, clotting factors, cytokines, etc.) aimed at restoration of homeostasis [6, 7]. The APR also includes a broad suppression of many core intermediary metabolic functions within the liver – notably albumin synthesis and the metabolism of carbohydrates, fats, and bile acids are affected, the latter of which directly contributes to cholestasis. Conditions besides sepsis that are associated with cholestasis include extrahepatic infections such as bacterial pneumonia and urinary tract infections [1-3, 8-10], but this group can likely be expanded with conditions involving a systemic inflammatory response syndrome followingburn injury, severe trauma and major surgery [11]. The importance of circulating pro-inflammatory mediators in the pathogenesis of IIC was illustrated by the side-effects of therapeutical administration of these mediators to humans. Treatment of cancer patients with recombinant cytokines (TNFα or IL-2) in phase I/II clinical trials was shown to lead to hyperbilirubinemia and cholestasis [12, 13]. Cholestasis seen in certain non-metastatic paraneoplastic syndromes, such as Stauffer's syndrome, appears to be caused by secreted cytokines too [14, 15]. Considering the plethora of conditions

associated with IIC, it is not surprising that jaundice is frequently observed in intensive care units for children, most notably neonates, and adults. The importance of sepsis as an underlying cause of clinical cholestasis has often been overlooked [16].

The presence and severity of cholestasis appears to be associated with poor prognosis of septicaemia [17]. This obviously does not imply that cholestasis itself is the causative factor of poor outcome: cholestasis is more likely an indicator of the severity of sepsis. Therefore, current treatment modalities are mainly aimed at treating sepsis with antibiotics and further supportive care and not at restoration of hepatic secretory function. Yet, it is easily appreciated that cholestasis per se will have immediate repercussions for the metabolism and elimination of drugs and toxins. Moreover, intestinal function will be impaired with reduced bile flow, with subsequent complications of malabsorption as well as bacterial overgrowth and translocation, further worsening the cholestatic state. The long-term effects of sepsis-associated cholestasis are largely unknown.

Experimental models of IIC

The pathogenesis of IIC has been studied using a variety of *in vivo*, ex vivo and *in vitro* models. These models generally involve the induction of a hepatic APR. A frequently used *in vivo* model involves administration of endotoxin, i.e., lipopolysaccharide (LPS), to rodents [18]. LPS, a component of the outer membrane of Gram-negative bacteria, is a ligand for two different pattern-recognition receptors, i.e., Toll-like receptor (TLR*)-4 [19] and CD14 [20]. LPS signaling is dependent on a complex arrangement that includes binding to soluble proteins (LPS-binding protein) and both TLR4 and CD14. Mice deficient in either Tlr4-signaling (mutant strain C3H/HeJ or null-mice C57BL/1-ScCr) or Cd14 are resistant to LPS [19, 21]. These receptors are present at the surface of several cell types within the liver, including Kupffer cells (KC), and LPS binding elicits an immune response in these cells [22]. KCs are the resident liver macrophages and central mediators of the inflammatory cascade leading to IIC. Mice of the C3H/HeJ strain are often used as LPS-resistant control mice [23-26].

IIC has also been studied using different activators of innate immunity, such as zymosan [27] or lipoteichoic acid [28], or individual pro-inflammatory cytokines [29-31]. Other models include administration of chemical agents to rodents, e.g., turpentine [32-34] or surgical procedures to induce polymicrobial sepsis (e.g., cecal-ligation and puncture (CLP) [35-39]).

Isolated perfused rodent livers [40-43] allow for well-controlled experiments with regard to perfusate composition, use of tracers, etc. Precision-cut slices from

* According to general convention, names of human proteins and genes are presented in uppercase with the latter in italics, while those for other species are presented in lowercase. Throughout this review, uppercase is used when human and other species are discussed simultaneously and when general topics/mechanisms are discussed. When proteins and encoding genes are discussed simultaneously, only the protein nomenclature is used for reasons of clarity.

both human and rodent liver have also been used to study the effects of LPS on cytokine expression and transporter expression [44-46].

In addition to using intact animals/organs, IIC has been examined at the (sub)cellular level using primary hepatocytes or hepatoma cell-lines [47, 48]. Treatment with (individual) cytokines or medium obtained from activated Kupffer cells or macrophages mimics the *in vivo* response at the hepatocellular level down-stream of KC activation [49].

Nuclear receptor (NR) biology

NRs are ligand-activated transcription factors that play important roles in many aspects of metazoan life, including embryonic development, cell differentiation and maintenance of cellular homeostasis [50]. NRs are expressed differentially amongst tissue and throughout day-night cycles [51, 52]. NRs are assumed to have arisen from constitutively active transcription factors [53] having acquired the ability to be activated by hormones (e.g., glucocorticoid receptor (GR), estrogen receptor (ER)) or to sense local environmental and nutritional cues (e.g., liver X receptor (LXR), farnesoid X receptor (FXR)). This allows not only for concerted gene responses throughout the organism, initiating hormone-appropriate responses, such as the stress-response after glucocorticoid release, but also for cell-specific responses to altered local environmental conditions, as exemplified by induction of cholesterol efflux transporters during cellular sterol overload by LXR stimulation.

Classes and structures of NRs

The various NRs share several structural similarities: a DNA-binding domain (DBD), a ligand-binding domain (LBD) and activation function domains (AF)-1 and AF-2 (**Figure 1A**). A DBD contains two well-conserved zinc-finger domains, which determine the affinity for specific DNA sequences known as response elements (cis-acting elements) [50]. The LBD determines the ligand-specificity of NRs and will thus differ most significantly between NR family members [54]. Ligand binding will lead to conformational changes in the NR molecule, resulting in altered transcriptional activities through re-organization of the transcription complex at the promoter, generally involving removal of co-repressors and recruitment of co-activators. The AF-1 (situated at the N-terminus) and AF-2 (actually contained within the LBD at the C-terminus) mediate interactions with co-regulators.

The NR superfamily has been subdivided according to different classifications. Traditionally, NRs have been divided into three functional groups [50]: 1) classic NRs such as GR, ER, etc. These NRs generally form homodimers and are activated with high affinity by steroid hormones in an endocrine fashion. 2) heterodimers with retinoid X receptor (RXR) and partners such as LXR, FXR, retinoic acid receptor (RAR), peroxisome proliferator activated receptors (PPARs), that are activated at lower affinity by metabolites or nutrients such as fatty acids and oxysterols, and 3)

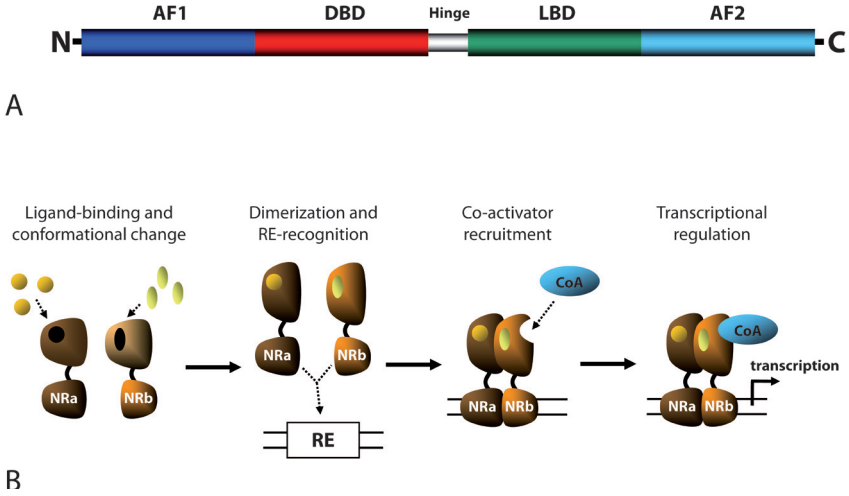

Figure 1. A.) Schematic of NR structure (N = N-terminus, AF = activation function domain, DBD = DNA-binding domain, LBD = ligand-binding domain, C = C-terminus); B.) Schematic of general mechanism of transactivation by ligand-activated NRs (NRa/b, generic NR heterodimer partners, RE = response element, CoA = co-activator). Of note, ligand-binding and conformational change do not necessarily occur prior to DNA-binding, as they may also induce release of co-repressors from RE-bound NRs).

orphan NRs, referring to transcription factors expected to be NRs based on gene/protein structure, for which no specific ligands have been identified yet, or appear to lack a functional LBD based on structural analysis. Once members of this class of NRs have been assigned (specific) ligands, they become adopted, as occurred recently with the identification of heme as ligand of REV-ERBα and REV-ERBβ [55, 56].

More recently, NRs nomenclature has been revised in a way analogous to that for cytochrome p450 (CYP) enzyme systems using a coded numbering system. In this system, NRs are classified into 6 distinct groups based on molecular phylogeny [57]. This system allows for classification of NRs from different species as well as NRs identified by genetic analyses without clarified functional and/or biological characteristics [53].

Inherent to their function as transcriptional regulators, NRs selectively recognize and bind to short DNA sequences located in gene-regulatory elements, either in close proximity to the transcriptional start sites (promoters) or in more distant elements (enhancers). These "cis-acting" response elements (REs) share several characteristics. REs that mediate transcriptional activity of RXR-heterodimers generally consist of two hexamers that are separated by one to eight nucleotides and are direct, everted or inverted repeats. However, some of the NRs that form heterodimers with RXR may also regulate transcriptional activity by binding as a monomer (e.g., FXR [58, 59]).

Mechanisms of genomic actions

A key characteristic of NRs that sets them apart from other transcription factors is altered activity upon ligand binding. Although in reality much more intricate due to the involvement of many proteins [53], the general mechanism of NR-regulated gene expression is depicted in **Figure 1B**. It involves a conformational change of the NR upon binding of its ligand. Some NRs will then relocate to the nucleus, bind to their corresponding REs and recruit co-activator molecules. Other NRs are already bound to their response elements while being unliganded and associated with co-repressor proteins. Upon ligand binding, these NRs will release co-repressors and start to recruit co-activators. The recruitment of co-activators is a process that involves a multitude of proteins and has specific spatial and temporal characteristics. The conformational changes that occur upon ligand-binding are thought to invoke a closure of the ligand-binding pocket by helix 12 rendering the surface of the NR more available for binding of co-activators, while co-repressors become less able to bind and are released. Since NR-binding to promoters is a cyclic process, i.e., a continuous binding and removal of NRs from the response elements, increased stability of the co-activator complexes will shift the balance from inhibition to stimulation of transcription. One of the important molecular actions of these multi-functional co-activators is chromatin relaxation through histone acetyltransferase activity or mediating the recruitment of other proteins with such function. This will render target genes more accessible for the transcriptional machinery. Co-repressors such as the nuclear receptor co-repressor (NCoR) or silencing mediator of retinoid and thyroid hormone receptor (SMRT) either have histone deacetylating properties or stimulate the recruitment of other co-repressors with such enzymatic activity, the so-called histone deacetylases (HDACs), and thus reverse chromatin relaxation and inhibit gene transcription. In addition to (de)acetylating modification of histones, co-regulators can also modify histones via (de)methylation and (de)phosphorylation mechanisms [60]. There are also non-histone-mediated actions by which co-regulators affect gene transcription, including ATP-dependent remodelling of chromatin and the recruitment of both basal transcription factors and co-regulators [60, 61]. Co-regulators are regarded as the actual determinants of NR-mediated transcriptional regulation and their tissue-specific expression patterns are responsible for the specific effects of NRs and their ligands in different tissues [62].

Non-genomic actions of NRs

Besides the "classical" NR mode of action, some of the NRs exert effects on gene transcription without DNA-binding, i.e., "non-genomically" by protein-protein interactions [53]. The small heterodimer partner (SHP) is one example of a NR that acts non-genomically as it lacks a DBD. SHP is often seen as a transcriptional repressor that acts by binding and interfering with the action of some NRs and transcriptional activators. SHP, however, is not the only NR that has non-genomic actions. There is substantial evidence that many NRs that possess a DBD regulate gene expres-

sion through other domains in the protein and without DNA-binding. This group includes GR, ER, PPARγ and LXR, which are known to regulate gene expression via traditional REs, but have also been shown to suppress inflammatory signaling via non-genomic interactions.

Clinical relevance of NR ligands

The aspect of ligand-induced modification of NR activity and their generally well-matched sets of transcriptional targets has led to the concept that NRs represent attractive targets for pharmacological intervention in a wide range of pathophysiological processes. An estimated 20% of all prescriptions in the United States exert their effects via NRs [63]. Interestingly, some of these were already used clinically without knowledge of their primary target or molecular mode of action, such as hypolipidemic fibrates (e.g., clofibrate) and antidiabetic thiazolidinediones (e.g., rosiglitazone) which were later shown to be PPARα and PPARγ ligands, respectively [64-66]. For several drugs, their identification as ligands of NRs, more specifically of the pregnane X receptor (PXR) and constitutive androstane receptor (CAR), provided an explanation for their known interference with metabolism of other drugs. Examples of the latter group include phenobarbital, a CAR ligand [67-69], and the PXR ligands rifampicin and nifedipine [70]. These compounds regulate drug metabolism through activation of CAR and PXR and subsequent changes in the expression of CYP and other genes involved in drug metabolism.

NRs of specific relevance to IIC

A subset of NRs is of particular interest in relation to IIC. This subset includes RXRα for its central role as obligate heterodimerization partner for other class II NRs, RARα for its role in control of basal hepatocellular gene expression, FXR as bile acid sensor, PXR and CAR as xenobiotic sensors involved in detoxification pathways, LXR and PPARγ for their recently identified anti-inflammatory activities and the orphan receptors liver receptor homologue (LRH)-1, hepatocyte nuclear factor (HNF)-4α and SHP, involved in the transcriptional regulation of various genes involved in bile formation and hepatobiliary transport. Some of the characteristics of these NRs are summarized in Table 1. In the paragraphs reviewing anti-inflammatory properties of NRs involved in IIC, GR will also be discussed as the prototypical example of NRs with anti-inflammatory properties. Some other NRs, e.g., ER or progesterone receptor (PR), are described to further illustrate general principles regarding NR biology.

Mechanisms underlying IIC

It is generally accepted that IIC results from impairment of normal hepatobiliary transport functions through the effects of pro-inflammatory mediators [4, 18]. These mediators can either reach the liver via the systemic circulation or be produced

Table 1. NRs involved in bile-formation and IIC, examples of their natural and synthetic ligands and examples of their direct, IIC-related, target genes.

NR	Official name	Ligands		Examples of direct target genes
		Natural	Synthetic	
RXRα	NR2B1	9cis retinoic acid	LG268, LG1069	(Obligate heterodimer partner)
RARα	NR1B1	All-trans retinoic acid	TTNPB	NTCP, MRP2, CYP7A1
FXR	NR1H4	Chenodeoxycholic acid, cholic acid	GW4064	BSEP, SHP, OSTα/β
LXRα/β	NR1H3/ NR1H2	Oxysterols	T0901317, GW3965	ABCG5/8, (rodent) Cyp7a1
PPARγ	NR1C3	15d-PGJ2, fatty acids	Thiazolidinediones	
PXR	NR1I2	5β-pregnane-3,20-dione	PCN, rifampicin, nifedipine	MRP2, MDR1, CYP-families
CAR	NR1I3	Androstenol	TCPOBOP	MRP2, CYP-families
Orphans				
HNF4α	NR2A1	-	-	ABCG5/8, CYP8b1
LRH-1	NR5A2	-	-	CYP7A1, (human) ABCG5/8
SHP	NR0B2	-	-	(No DBD)

15d-PGJ2 = 15-deoxy-Δ-12,14-prostaglandin J2; TCPOBOP = 1,4-bis-2-(3,5-dichloropyridyloxy)-benzene; PCN = pregnenolone-16α-carbonitrile. (Adapted from Karpen [54].)

locally in response to a variety of stimulants, including endotoxin [11, 18]. Pro-inflammatory mediators are able to affect hepatobiliary transport functions via multiple signal transduction pathways, targeting events at the membrane, cytosol and the nucleus.

Physiology of bile formation

Bile formation represents an osmotic process, driven by active secretion of cholephils by hepatocytes into the minute bile canaliculi [71]. These canaliculi are separated from the circulation by tight junctions connecting adjacent hepatocytes. The active secretion of osmotically active solutes, most notably bile salts, leads to the passive transport of water and electrolytes into the canaliculi, thus generating bile flow [71]. Total hepatic bile flow is considered to be the sum of bile salt-dependent flow (BSDF) and bile salt-independent flow (BSIF). The latter is mainly driven by the secretion of substances such as glutathione [72, 73] and bicarbonate [74]. The canaliculi join to form bile ductules that are lined with cholangiocytes and eventually converge into the major bile ducts that drain into the duodenum. Although bile ducts were earlier primarily regarded as a drainage system, it is now clear that the bile duct epithelium plays an active role in the generation of bile flow and regulation of bile composition [75]. Approximately 10-13% and 40% of total bile flow in rats and humans, respectively, is driven by secretin-stimulated secretion of chloride and bicarbonate by cholangiocytes [76, 77]. Not surprisingly, substantial compositional differences exist between canalicular and ductular bile. In humans and mice, but not in rats, bile is stored and

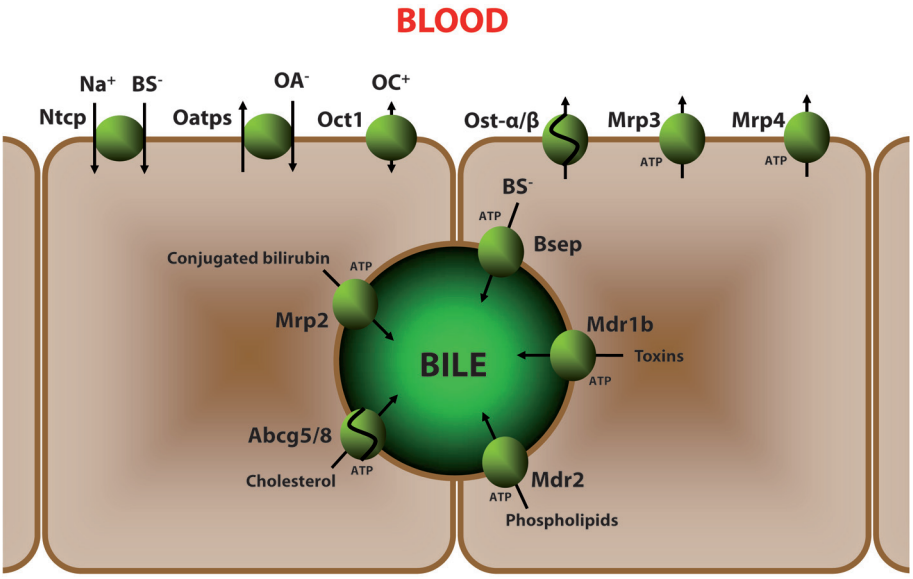

Figure 2. ***Schematic of hepatobiliary transporters involved in bile formation and adaptive responses to cholestasis (see text for details).*** Adjacent hepatocytes and a bile canaliculus are shown. (BS = bile salt, OA = organic anion, OC = organic cation)

further modified by concentration in the gallbladder.

Over the past decades, knowledge of the molecular basis of bile formation has greatly expanded by the identification of several essential transporters that contribute to the process [78]. The most important hepatocellular transporters involved in bile formation as well as in protection of hepatocytes against bile salt overload are shown in **Figure 2**. At the basolateral side of hepatocytes, the Na^+-taurocholate co-transporting polypeptide (NTCP), organic anion transporting proteins (OATPs) and organic cation transporter (OCT)1 are responsible for sodium-dependent and sodium-independent uptake of bile salts, organic anions and cations. At this side of the cell, members of the multidrug resistance-associated proteins (MRPs), i.e., MRP3 and MRP4, are present too. These transporters exert hepatocyte-protecting effects during (extrahepatic) cholestasis most likely by facilitating the basolateral efflux of retained and potentially harmful substances including bile salts [79, 80]. Recently, an additional bile salt transporter complex has been identified, i.e., the organic solute transporter (OST)-α/β heterodimer [81, 82], which appeared to be the elusive bile salt efflux transporter at the basolateral side of enterocytes [83, 84]. OSTα/β heterodimers are expressed in the liver, both in cholangiocytes and hepatocytes [85], differentially between species. In cholangiocytes, it may very well play a role in "cholehepatic

shunting"of bile salts, while in hepatocytes it appears to function as an overflow efflux transporter, as its expression is markedly induced under cholestatic conditions in an FXR-dependent manner [86, 87].

At the canalicular membrane of hepatocytes, several transporters are localized that are responsible for the biliary secretion of various biliary components. Many of these transporters belong to the ATP-binding cassette (ABC) transporter family and actively transport their substrates against concentration gradients into the canalicular lumen. The bile salt export pump (BSEP or ABCB11) mediates biliary secretion of monovalent bile salts. MRP2 (ABCC2) is responsible for efflux of divalent anions including conjugated bilirubin and sulfated bile salts. Multidrug resistance (MDR)-3 P-glycoprotein (ABCB4, rodent orthologue Mdr2/Abcb4) is involved in phospholipid secretion into the canalicular lumen by functioning as a floppase [88]. ABCG5 and -8 are two halftransporters that facilitate sterol export and MDR1 (ABCB1, rodent orthologue Mdr1b/Abcb1) is involved in the excretion of many organic cations (endobiotics, xenobiotics). The importance of the various individual transporters in the process of bile formation is demonstrated in various human syndromes as well as various animal knock-out / mutant models [88].

Several other (non-ABC) transporter proteins are also present on the canalicular membrane including FIC1 and Niemann-Pick-1-like-1 protein (NPC1L1). The importance of FIC1 (ATP8B1) is evident since mutations in the ATP8B1 gene have been recognized as the genetic defects underlying progressive familial intrahepatic cholestasis type 1 (PFIC1, hence its name FIC1), also known as Byler's disease. Different, milder mutations in ATP8B1 lead to benign recurrent intrahepatic cholestasis type 1 (BRIC1). Although it is known to be a member of sub-family of ATP-transporters that act as aminophospholipid flippases, its exact modes of action under physiological conditions and in the development of PFIC1 and BRIC1 remain to be established [88]. NPC1L1 has been identified as the transporter responsible for cholesterol uptake in enterocytes and as the target of the cholesterol-lowering drug ezetimibe [89]. NPC1L1 is highly expressed in human (but not mouse) liver [89], where it might mediate re-uptake of cholesterol from the canalicular lumen [90]. The physiological relevance of this process is, however, unknown.

Impaired bile formation during inflammation

LPS-treatment of perfused rat livers reduces bile flow as well as bilirubin and dye transport [29, 40, 41, 43, 91-93]. Although LPS-treatment was initially thought to primarily affect BSIF [41, 92, 94], BSDF was also shown to be affected [29, 43, 93, 95], indicating that both components of bile flow are impaired upon inflammation. Similar results were obtained in another sepsis model, i.e., CLP [36].

LPS interferes with normal bile flow generation via several mechanisms, which all ultimately lead to reduced activity of the transporters and enzymes involved in the process. With regard to BSIF, LPS reduces Na^+-K^+-ATPase activity [30, 92] and also

impairs glutathione secretion [94]. The latter has been linked to reduced mRNA and protein expression of Mrp2, but this transporter has also been shown to undergo intracellular re-localization upon LPS-treatment [42, 96-98].

It is generally accepted that canalicular transport is rate-controlling in overall hepatobiliary transport [93, 99]. Hence, impaired biliary secretion is likely to lead to accumulation of potentially toxic substances in hepatocytes. Simultaneous effects on basolateral transporters, whether there be a reduction of influx (NTCP, OATPs) or an increase in efflux (MRP3, MRP4, OSTα/β), will therefore strongly influence the degree of bile salt retention and potential cellular damage. Due to their important contribution to bile formation, effects of inflammatory signaling on the cholangiocytes also contribute significantly to the pathogenesis of IIC. Spirli et al. [100] analyzed the effects of inflammatory mediators on biliary fluid secretion using isolated bile duct units. These authors found that a mixture of cytokines, but not individual cytokines, reduced cAMP-dependent fluid secretion in isolated bile duct units [100]. At the same time, they observed an impaired biliary epithelial barrier function, which most probably contributes to the emergence of cholestasis [100]. These data suggest that effects on biliary epithelium indeed have a role in IIC, but further studies are necessary to gain insight into the quantitative and qualitative importance of the effects of inflammation on the biliary epithelium.

Important for bile flow generation, and bile salt metabolism in general, is the synthesis of bile salts by the hepatocytes. This process is tightly regulated and involves the actions of several NRs, e.g., FXR, SHP, LRH-1, HNF4α and LXR [101]. Due to the highly efficient reabsorption of bile salts by the epithelia of biliary system and in the distal ileum, leading to cycling of bile salts via the cholehepatic shunt and in the enterohepatic circulation, respectively, the loss of bile salts per cycle is small. Therefore, the quantitative contribution of newly synthesized bile salts to the bile formation process is limited. Inflammation-induced suppression of the expression and activity of Cyp7a1 and Cyp27 [102, 103], which catalyze the first step of respectively the classic/neutral and the acidic pathway of bile salt synthesis from cholesterol [101], will only become relevant after prolonged sepsis/inflammation. On the other hand, effects of inflammation on intestinal and gallbladder motility, leading to impaired enterohepatic cycling, may contribute to impaired bile formation.

Inflammatory cascade and Kupffer cells

IIC can be elicited by various inflammatory mediators, which are either reaching the liver from the circulation or are produced locally (**Figure 3**). The Kupffer cells (KC), resident liver macrophages, play a central role in local production [104]. KCs form the primary line of defense against intestine-derived toxins that enter the liver via the portal circulation, as is illustrated by the nearly complete clearance of endotoxin from portal blood by KCs [105]. Activation of KC by LPS occurs via several different signal transduction pathways [22]. The importance of KCs in the pathogenesis of IIC

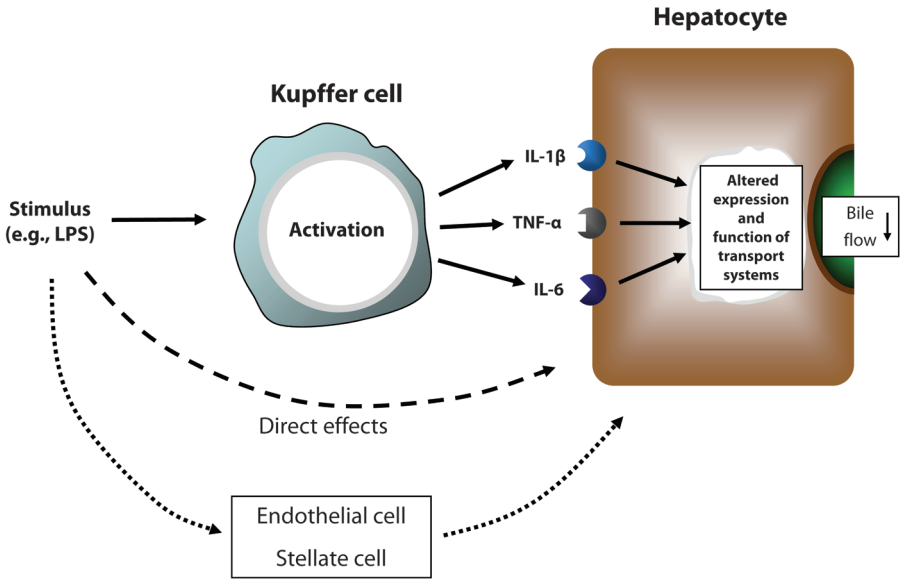

Figure 3. ***Linking inflammatory signals to hepatocellular effects.*** Inflammatory stimuli can interfere with normal hepatocellular function either via activation of Kupffer cells and subsequent release of pro-inflammatory mediators (top), through direct effects on hepatocytes (middle) or through effects on other non-parenchymal cells (bottom).

has been demonstrated in various *in vivo* studies in which suppression of transporter expression by LPS-administration was found to be reduced when KC had been inactivated by gadolinium chloride [106, 107] or selectively removed using liposomal clodronate [108].

Mediators affecting bile formation in hepatocytes include KC-secreted pro-inflammatory cytokines such as IL-1β, TNFα and IL-6. The importance of these cytokines was supported by experimental findings showing that individually administered cytokines elicited similar responses *in vivo* as LPS did [30, 31, 34, 109]. In addition, cytokine-inactivation through the administration of anti-TNFα and anti-IL-1β antibodies blunted the cholestatic response to endotoxin-treatment both *in vitro* and *in vivo* [29, 49, 110], despite the fact that there appeared to be some degree of redundancy in the cytokine-signaling [111].

Although the involvement of KCs in the pathogenesis of IIC is evident, it has also become clear that KC-independent mechanisms contribute. Suppression of Ntcp expression after LPS administration persisted in KC-depleted livers, albeit that the degree of suppression was lower than in control livers [108]. It is plausible that either direct effects of LPS on hepatocytes or LPS activation of other non-parenchymal liver cells

can be involved in the pathogenesis of IIC, e.g., endothelial cells and stellate cells. Endothelial cells, for instance, are known to express TLR4 and to respond to inflammatory stimuli [112, 113]. The importance of endothelial cells is further illustrated by the findings of Laschke et al. [114] using a combination of LPS and D-galactosamine (LPS/Gal) in mice. Pre-treatment of mice with antibodies against the adhesion molecule P-selectin, which is present on endothelial cells upon LPS-treatment [115], reduced the recruitment of leukocytes and concomitantly diminished the effects of LPS/Gal on bile flow, transporter gene expression profile and liver morphology, while levels of inflammatory cytokines were not affected [114]. These results indicate that processes such as leukocyte recruitment are also of critical importance in the pathophysiology of sepsis-associated cholestasis. The mechanistic link is between reduced leukocyte recruitment and inhibition of cytokine activity remains to be determined.

Finally, activated KCs not only secrete cytokines but also inflammatory mediators such as nitric oxide (NO) and prostaglandins [116]. Although NO had been shown to stimulate bile flow [117], LPS-induced NO-production did not have a significant effect on LPS-induced suppression of bile flow [94]. Prostaglandins have also been shown to reduce bile flow and bile salt secretion [118, 119].

Inflammatory signaling in hepatocytes

Pro-inflammatory cytokines and other inflammatory mediators affect hepatocellular function via various, sometimes parallel, pathways [7, 120]. These include, but are definitely not limited to, NF-κB and mitogen-activated protein kinase routes. Examples of how these pathways can contribute to the development of IIC include NF-κB-mediated induction of Mdr1b expression [121], c-jun N-terminal kinase (JNK)-mediated nuclear export of RXRα *in vitro* [47, 122] and extracellular regulated kinase-mediated inhibition of interferon-regulated factor-3 nuclear import [48]. Of note, the specific roles of these pathways have often been examined *in vitro* using hepatocytes or hepatoma cells treated with (individual) cytokines. Although this is an elegant approach to elucidate underlying mechanisms, one has to bear in mind that in the *in vivo* situation multiple, parallel pathways may be active with a certain degree of redundancy, while LPS affects hepatocytes directly to some extent as well [123, 124].

Hepatobiliary transporters

Inflammatory signaling has been shown to differentially affect hepatocellular transport function at both transcriptional and post-transcriptional level. The effects of LPS on the expression of several physiologically relevant transporters in rats and mice are summarized in Table 2. This list, however, is not complete and ought to be seen as an indicator of general effects. The localization of the various transporters is shown in **Figure 2**. Most extensively studied transporters in this regard are Ntcp, Bsep and Mrp2. These three transporters are generally suppressed, both at mRNA and protein level. To our knowledge, no information is available on the effects of LPS on the expression of the canalicular transporters Fic1 and Npc1l1 or on that of the

Table 2. ***In vivo effects of LPS on hepatobiliary transporters in rodent (mouse/rat) models.***

Transporter	Official name	mRNA	References	Protein	References
Basolateral					
Ntcp	Slc10a1	↓	30, 34, 108, 110, 111, 126, 133, 141, 152, 195, 281-285	↓	30, 108, 281, 282, 284, 285
				↔	195
Oatp1	Slco1a1	↓	34, 109-111, 126, 284, 285	↓	284-286
		↔	286		
Oatp2	Slco1a4	↓	109, 110, 126, 152, 284, 285	↓	284, 285
		↔	111		
Oatp4	Slco1b2	↓	24, 111, 126, 284, 285, 287	↓	285
Oct1	Slc22a1	↓	126		
Mrp3	Abcc3	↓	34, 109, 152		
		↔	111, 288		
		↑	126	↓	288
Mrp4	Abcc4	↔	111, 285, 288		
Canalicular					
Bsep	Abcb11	↓	33, 34, 109, 111, 125, 126, 133, 152, 283-285, 289	↓	43, 283
				↔	285
Mrp2	Abcc2	↓	34, 42, 43, 96, 106, 109, 110, 126, 133, 152, 283, 284, 288, 290	↓	43, 96, 152, 283, 288, 290
		↔	111		
Mdr1b	Abcb1b	↓	33, 125		
		↔	111, 152, 284	↔	284
		↑	96, 126		
Mdr2	Abcb4	↓	33, 125, 152		
		↔	96		
Abcg5/8		↓	133, 161		

basolateral Ostα/β transporting complex.

Most of the transporters are suppressed under inflammatory conditions with rodent Mdr1b (Abcb1b) being the most consistent exception. Although some demonstrated suppressive effects of LPS on Mdr1b expression [33, 125], other groups have observed an induction of Mdr1b *in vivo* [96, 126] (Mulder et al. (unpublished data)). Mdr1b, as a member of the multidrug resistance protein family serves as an inducible efflux transporter for organic cations, xenobiotics and toxins [78] and is directly regulated by NF-κB signaling [121, 127]. This role of inducible efflux transporter appears to be in contrast to that of its closely related family member Mdr1a, which is more consistently expressed [96].

Water channels, the so-called aquaporins (AQP), have so far received relatively little attention in IIC research, despite the expression of at least 7 family members in the hepatobiliary system [128]. A recent report by Lehmann et al. showed that LPS-treatment of rats led to down-regulation of Aqp8 protein expression, while Aqp9 expression was not affected [129]. The suppression of Aqp8 expression was TNFα-dependent and post-transcriptionally mediated through both lysosomal and proteasomal degradation [129]. This led to a reduced osmotic water permeability of the canalicular membrane, which was suggested to contribute to the pathogenesis of inflammation-induced cholestasis [129].

NR expression and function during inflammation

The observed reduction in hormone sensitivity during inflammatory conditions, for instance growth hormone (GH) resistance during chronic inflammation [130], illustrates that there is a direct link between inflammation and altered NR function. This link has been addressed experimentally in many studies. Although some NRs are differentially regulated, e.g., induction of Nur77 in LPS-stimulated macrophages [131], inflammation suppresses expression and function of most NRs studied at multiple levels (**Figure 4**). This general response is considered to be a crucial step in the negative hepatic APR [132-134].

RXRα

In view of its importance as obligate heterodimer partner of class II NRs, the effects of inflammatory signaling on RXRα expression and function have been studied extensively. Beigneux et al. [132] showed that both mRNA and protein expression of Rxrα in livers of Syrian hamsters were strongly reduced upon treatment with either LPS or cytokines, which led to reduced binding activity of Rxr-homodimers. This effect was shown to be mediated, at least partially, by reduced Rxrα transcription [132]. Similar effects of LPS on hepatic Rxrα gene expression were obtained in mice [135] and rats [136] as well as in non-hepatic tissues, i.e., adipose tissue [137] and heart [138].

At least two other mechanisms have been identified by which inflammatory signaling alters RXRα transcriptional activity. Firstly, as part of the protein complex originally known as Footprint B binding protein, Rxrα was shown to be a key regulator of the rat Ntcp gene expression [139, 140]. Follow-up studies revealed that the nuclear abundance of this protein is rapidly reduced upon LPS-treatment of mice with a simultaneous, transient appearance in the cytosolic compartment, suggesting active nuclear export followed by degradation [133]. At the same time, Jnk activity, important in the regulation of Ntcp promoter activity by Rxrα during inflammation [47], was increased [133]. This led to a reduced mRNA expression of NR-target genes, despite preserved expression of most heterodimer partners [133]. Subsequent *in vitro* studies provided a potential mechanism for the nuclear export of RXRα [122]. JNK-dependent phosphorylation of a serine residue at position 260 of RXRα was shown to induce nuclear export and subsequent proteasomal degradation [122]. Reduced nuclear

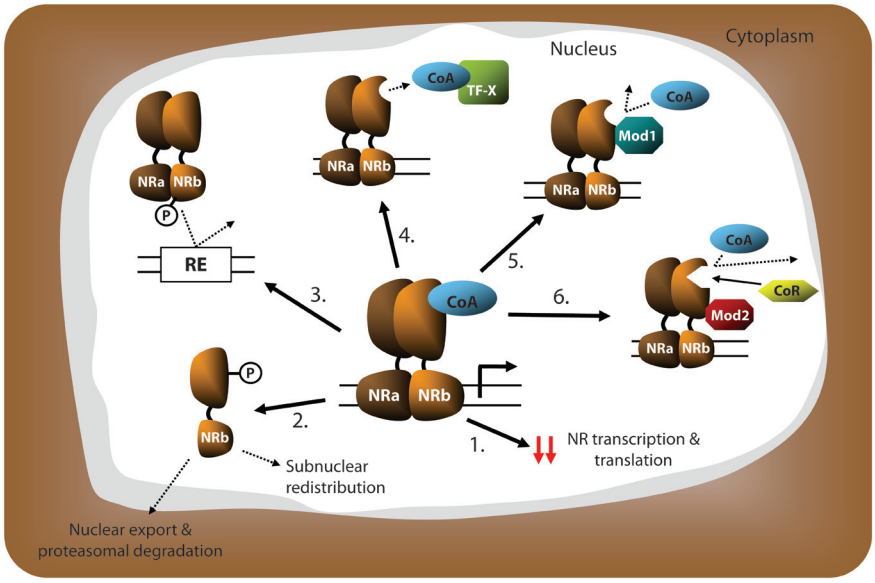

Figure 4. ***General effects of inflammatory signaling on NR expression and function:*** 1.) suppression of NR transcription or translation; 2.) signal dependent relocalization and degradation; 3.) reduced DNA binding by post-transcriptional modification of NR (e.g., phosphorylation (P)); 4.) competition for co-activator (CoA) by inflammation-induced transcription factor (TF-X); 5) inhibition of CoA recruitment by NR-modification (Mod1) leading to steric hindrance and/or conformational change; 6) increased affinity for co-repressor (CoR) binding by NR-modification (Mod2). (Other abbreviations: NRa/b, generic NR heterodimer partners, RE = response element, CoA = co-activator.)

protein levels of Rxrα have also been reported by others [141]. Secondly, Gu et al. [142] presented another mechanism involved in altered RXR function during inflammation. Activated NF-κB suppressed DNA-binding of the PXR:RXR heterodimer to the promoter of the CYP3A4 gene and this effect was in part mediated by direct interaction between the p65 subunit of NF-κB and RXR [142]. Although this has not been shown to occur with other heterodimers, it may represent a more generally occurring mechanism and thus not only pertain to the PXR:RXR heterodimer.

These results combined suggest that inflammatory signaling suppresses RXRα expression and function in multiple ways. Considering its important role in the function of class II NRs, the regulation of RXRα alone could already be a central mediator of the negative hepatic APR.

RARα

In contrast to RXRα, limited information is available on the effects of inflammatory signaling specifically on RARα expression. TNFα treatment of mice led to a transient reduction in nuclear protein levels of Rarα in liver, which corresponded

well with transiently reduced mRNA expression [31]. Interestingly, treatment with IL-1β also led to reduced nuclear protein levels of Rarα, but at a later timepoint, while Rarα mRNA expression was not altered [31]. This indicates that the reduction in nuclear Rarα by inflammatory cytokines is not only regulated at the transcriptional level.

Results obtained with individual cytokines were different from those with LPS-treatment. Ghose et al. [133] showed that Rarα mRNA expression and nuclear protein levels remained unaffected in mice after LPS-treatment, while nuclear Rxrα protein levels were rapidly reduced. The cause of these differential effects of individual cytokines and LPS remains unclear, but may be related to the dose of inflammatory mediators used, as Rarα nuclear protein levels were strongly reduced after very high dose of LPS in mice [141].

FXR

Since its identification as an intracellular bile salt sensor [143, 144], FXR has been shown to play a central role in control of expression of transporter genes such as BSEP as well as in the adaptive response to a bile salt challenge [145]. Studies with Fxr-null mice revealed an impaired hepatocellular protection against bile salt overload [145]. The expression of FXR at both mRNA and protein level is reduced upon LPS- or cytokine treatment of rodents or cytokine-treatment of hepatoma cells [31, 136, 146]. In the CLP-model, expression and binding activity of Fxr:Rxr were also reduced [39]. These results not only illustrate that FXR is similarly affected by inflammatory signaling as other NRs and thus may contribute to the pathogenesis of IIC, but also indicate that impaired FXR function will most likely prevent a proper adaptive response during IIC as well.

CAR and PXR

CAR and PXR play critical roles in xenobiotic metabolism and detoxification [147]. These NRs are distinct NR family members and show important differences in their LBD, with PXR being more promiscuous [147]. Nevertheless, they are often grouped together due to several shared characteristics, including expression profile, with highest expression in liver and intestine, shared target genes as well as a subset of ligands. CAR and PXR have been studied extensively in relation to drug metabolism, which is altered by inflammation [148-150]. Car and Pxr mRNA expression levels were found to be suppressed in LPS-treated rodents [107, 133, 136, 151, 152]. Suppressed Pxr-function was shown by Kim et al. [153], who demonstrated that induction of dehydroepiandrosterone-sulfotransferase (Sult2a1) expression by pregnenolone 16α-carbonitrile was suppressed after LPS administration in mice. This effect was most likely mediated by cytokines (TNF, IL-1, but not IL-6) as these induced similar effects in hepatoma cells [153]. Interestingly, Sult2a1 expression was suppressed at much lower LPS doses than expression of Pxr and Car [153]. It is unclear whether the transactivation capacity of PXR and CAR per se is affected, since both NRs were shown to retain their

transactivational activity, albeit when overexpressed, in IL-6-treated human hepatocytes [154].

Mechanisms underlying suppression of CAR expression were analyzed by Assenat et al. [155], who showed that inflammatory signaling interfered with GR-regulated CAR-expression via NF-κB signaling. Activation of the latter led to decreased histone acetylation of the proximal CAR promoter [155]. Of note, NF-κB signaling has also been shown to affect PXR function [142, 156].

Apart from their roles in xenobiotic/drug metabolism, CAR and PXR are also involved in the protection against cholestatic liver injury in general [157] and, more specifically, against various "cholestatic compounds" such as hepatotoxic bile salts, notably lithocholate [158], bilirubin [159] and cholesterol metabolites [160]. It can therefore be anticipated that inflammation will not only affect drug metabolism, but will also render the liver less capable to deal with the ensuing cholestatic insult.

LXR

LPS-treatment of both hamsters and mice led to reduced Lxr DNA-binding in the liver [132, 133]. This occurred simultaneously with reduced Lxrα mRNA expression in hamster [132], while hepatic Lxrα mRNA expression was actually slightly increased in LPS-treated mice [133]. Reduced DNA-binding in mouse liver, associated with suppressed expression of the Lxr target gene Abcg5, was ascribed to reduced nuclear Rxrα levels [133]. Treatment of mice with either TNFα or IL-1 was found to slightly reduce liver Lxrα mRNA level [135]. In vitro experiments with Hep3B cells confirmed suppression of LXRα mRNA expression, reduced transactivating activity and suppressed expression of its target gene sterol regulatory element binding protein-1c [135]. In contrast to LPS-treated livers tissue and cytokine-treated hepatoma cells, LPS treatment of macrophages did not affect Lxr DNA-binding [161], indicating cell-specific effects. Expression of the Lxrβ isoform was not significantly reduced upon LPS-treatment in mouse liver [132].

PPARγ

Hepatic Pparγ mRNA expression was suppressed by LPS treatment of Syrian hamsters [132]. Similar results were obtained in mice, where LPS-induced suppression of Pparγ mRNA and protein expression was shown to be dependent on TNFα release [162]. In vitro, TNFα and IL-1β were also shown to suppress PPARγ expression in Hep3B cells [135].

The mechanism underlying the reduced gene expression of PPARγ by inflammatory signaling may involve NF-κB as was shown for LPS-induced suppression of Pparγ in macrophages [163].

HNF4α

HNF4α is a transcriptional regulator of many genes involved in hepatic lipid and bile salt metabolism [164]. HNF4α appears to be constitutively active, supposedly due to a permanent association of lipids with its LBD [165]. However, HNF4α is still considered an orphan NR, since no specific ligand has been identified. Considering the broad impact of HNF4α on hepatocellular gene expression profiles [166], it has been postulated that effects of inflammatory signaling on HNF4α function might provide a central mechanism for initiation of the hepatic APR [134].

Inflammatory signaling affects HNF4α function via multiple, often simultaneously acting, mechanisms. These mechanisms include reduction of mRNA expression, acceleration of proteasomal degradation, reduction of DNA-binding through post-transcriptional modifications and inhibition of co-activator recruitment [134, 141, 167-174]. Since these data suggest that inflammatory signaling indiscriminately leads to reduced HNF4α activity, it is important to emphasize that HNF4α activity can also be regulated in an opposite manner by other inflammation-related cues. Kuo and colleagues showed that in a different *in vitro* model of sepsis/shock, i.e., combined treatment of hepatocytes with cytokines and hydrogen peroxide, alteration of the specific phosphorylation pattern of HNF4α was critical for interaction with a specific co-activator and thus for enhancement of cytokine-induced iNOS expression by oxidative stress [175-177]. This indicates that post-transcriptional modification of HNF4α in the context of inflammatory processes has promoter-specific effects.

Finally, HNF4α was also shown to undergo tyrosine-phosphorylation signal-dependent intranuclear redistribution [178]. Whether this also occurs in the setting of inflammation is unclear. It may, however, provide a new level of complexity to the regulation of NR function.

LRH-1

Gerbod-Giannone et al. [179] identified another mechanism by which NR function can be inhibited during inflammation. Inflammation-induced production of α1-antitrypsin leads to increased production of the α1-antitrypsin-derived peptide C-36. This peptide was shown to specifically reduce DNA-binding of Lrh-1 and to inhibit Lrh-1 regulated gene transcription (including Cyp7a1, α-fetoprotein). C-36 physically interacted with Lrh-1, but did not bind to its DBD suggesting that C-36 induced conformational changes in Lrh-1 [179]. This interaction between bio-active peptides and a NR suggests that there may be more of these unanticipated interactions.

SHP

The expression of the atypical NR SHP is regulated by several other NR superfamily members, including FXR, ER and HNF4α, but also via an AP-1 binding site [145,

[180-182]. The latter is most likely regulated by inflammatory signaling. LPS-treatment of mice leads to strongly reduced hepatic Shp mRNA expression [133, 141]. However, the exact mechanism underlying this effect and whether reduced mRNA levels are translated into reduced protein expression and activity, remains to be elucidated. Considering the general repressive effect of SHP on other NRs, it is interesting to speculate on the functional consequences of reduced SHP expression. The net outcome will depend on whether reduced repression by SHP or reduced expression of the other NRs prevails.

Effects of inflammation on co-factor expression and function

The importance of co-regulators in NR action on gene transcription implies that interactions between inflammatory signaling and these co-regulators will directly affect NR-regulated gene transcription. Effects of inflammation on co-factor expression and function can be categorized into three different general categories, i. reduction of co-regulator expression/abundances, ii. competition between transcription factors for critical co-factors and iii. post-transcriptional modification of co-factors (**Figure 5**).

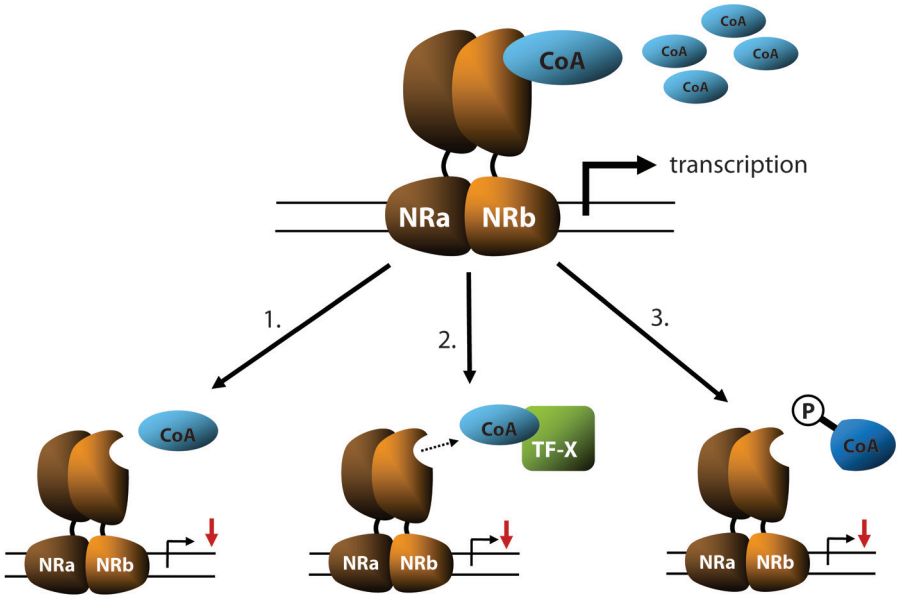

Figure 5. ***General effects of inflammatory signaling on co-activators (CoA):*** 1.) reduction of CoA abundance; 2.) competition for CoA-recruitment by inflammation-induced transcription factors (TF-X); 3.) post-transcriptional modification of CoA (e.g., phosphorylation (P)) leading to reduced NR-binding. (Other abbreviations: NRa/b, generic NR heterodimer partners.)

Kim et al. showed that inflammation leads to a reduced expression of a subset of co-factors *in vitro* and *in vivo* [135]. Cytokine treatment (IL-1 and TNF, but not IL-6) of hepatoma cells and cytokine- or LPS-treatment of mice led to a specific reduction in mRNA expression of the co-activators PPARγ co-activator (PGC)-1α, PGC-1β and steroid receptor co-activator (SRC)-1, while the expression of other co-activators, including CRE-binding protein (CBP), as well as co-repressors NCoR and SMRT was not affected [135]. This selective effect in combination with reduced NR expression led to reduced NR-mediated transcription using reporter constructs [135]. This supports the concept that a specific reduction in co-activating complexes shifts the balance between transactivating and transrepressing complexes towards the latter, ultimately suppressing gene transcription. Reduced expression of specific subsets of co-activators upon inflammatory stimuli was not only seen in liver, but also in other organs and tissues, including heart [138], adipose tissue [137] and uterine smooth muscle cells [183].

Leite et al. [183] provided evidence that there is a critical window of co-activator abundance, as the activity of the progesterone receptor (PR) is reduced during inflammation despite the fact that protein levels are not altered. This suggests that the concomitantly reduced levels of co-activators SRC-1 and SRC-2 are responsible for reduced PR function [183]. This is further supported by the fact that exogenous SRC-1 or SRC-2 can at least partially reverse the effects of TNFα on PR function [183].

The concept of critical co-activator levels within the nucleus for proper NR function also provides the basis for another mechanism by which inflammation may interfere with NR function, namely competition between transcription factors. Despite large functional and structural differences, the individual NRs often recruit the same co-factors as other types of transcription factors [60]. Therefore, initiation of inflammatory signaling within cells and the subsequent activation of transcription factors such as NF-κB and AP-1 will lead to the recruitment of co-activators. If co-activators are not redundantly present within cells, competition for co-activators can lead to insufficient co-activator-NR interaction and thus to reduced NR function. This principle of competition has been shown to occur between GR and NF-κB, as these transcription factors were shown to both be dependent on co-activators SRC-1 and CBP for maximal activity [184]. Again, exogenous supplementation of either co-activator was able to reverse this competition [184]. Similar findings were reported for other NRs, including RXR [185].

A third mechanism by which inflammation can affect co-factor function is by post-transcriptional modification. It is known that, similarly to NRs, co-factors can be subjected to (de)phosphorylation, (de)acetylation and (de)methylation and their activity can further be controlled by proteolytic processes and shuttling between nucleus and cytoplasm in response to various signals [186]. For example, TNFα-induced, inhibitor of κB kinase-mediated phosphorylation of SRC-3 leads to preferential nuclear localization of this co-activator and enhanced NF-κB mediated gene

transcription [187]. Although this is an example of increased co-activator activity, it is plausible that inflammatory signaling may lead to reduced activity of co-activators in other settings. The multiple sites in co-regulators for post-transcriptional/translational modification provide a means to rapidly regulate co-regulator function with a need to alter gene expression [188].

Thus, inflammatory stimuli can have multiple effects on co-factors that may directly translate into disturbed NR function. The overall effect, however, is most likely context-dependent, i.e., specific for type of stimulus and tissue involved, and not easily predicted. Moreover, a recent report by Lu et al. [189] adds another level of complexity of NR-co-factor function. These authors showed that the co-activator Src-3 is specifically involved in suppressing the innate immune response. Src-3 knock-out mice were shown to be highly sensitive to LPS-treatment. Surprisingly, Src-3 was shown to act as a suppressor of mRNA translation. This unexpected interaction between inflammation and co-factor function warrants caution in predicting how co-factors will affect inflammatory processes.

Pharmacological/experimental interventions in models of IIC

Better insight into the pathogenesis of specifically IIC and the negative APR in general has provided the opportunity to explore different, intriguing approaches to intervene in these processes. These approaches have been aimed at different levels of the cascade leading to IIC (**Figure 3**).

Administration of high-density lipoprotein particles [190, 191], recombinant LPS-binding protein [192] or TLR4 antagonists, e.g., M62812 [113] have been used to attenuate the initial step of KC activation. Liposomal siRNA against TNFα was used to specifically suppress production of this cytokine in KC, which rapidly take up liposomes [193]. Inhibition of signaling downstream of KCs has been achieved by using cytokine-inactivating antibodies, e.g. anti-TNFα, anti-IL-1β [29, 49, 110], or inhibitors of hepatocellular signaling, e.g., JNK-inhibitors as SP600125 [122].

Different roles of NRs in IIC: mediators and modifiers

Thus far, this review has mainly dealt with effects of inflammatory signaling on NR function that contribute to the pathogenesis of IIC. Inflammatory signaling has multiple potential effects on NR function through reduction of NR mRNA and protein expression, subcellular localization, post-transcriptional modifications with subsequent reduced DNA-binding and/or co-regulator recruitment and altered co-regulator expression or activity (Figures 4 and 5). In this regard, NRs can be seen as mediators in the development of IIC. On the other hand, NRs can also play modifying roles, which can be divided into two modes of action. NRs can exert adaptive responses aimed at restoration of normal hepatocellular homeostasis and NRs have been shown to have anti-inflammatory properties.

Modifier function 1: adaptive responses

During cholestasis, in particular of extrahepatic origin, hepatocellular accumulation of bile salts and other potentially toxic compounds will lead to an adaptive cellular response [194]. This means that, in theory, accumulation of bile salts could lead to an adapative response during IIC too. High intracellular bile salt concentrations will activate FXR and thus induce their export from the liver while reducing import and production. However, this adaptive response requires "sound" NR signaling. It is, however, unlikely that such an appropriate adaptive response can be fully engaged, since inflammation affects NR signaling in many different ways. Although the quantitative contribution of adaptive responses remains to be determined, it probably is limited. The latter idea is best illustrated by the reported findings of Zollner et al.: bile-duct ligation and bile salt treatment of mice leads to increased Shp mRNA expression, while LPS-treatment strongly suppresses Shp mRNA expression [141, 195]. The latter suggests that in the context of LPS, direct suppressive effects of inflammatory signaling on SHP expression overrule the anticipated stimulating effect of bile salt accumulation.

In this regard, it is important to mention the concept of pharmacologic enhancement of normal adaptive responses [196] as a means to intervene in the pathogenesis of IIC. Although inflammation generally impairs NR function, boosting the remaining activity using pharmacological agents may provide an avenue to at least attenuate the effects of inflammation on hepatobiliary transporter expression.

Modifier function 2: anti-inflammatory actions

Several NRs have been shown to possess anti-inflammatory properties. Although most of the studies exploring these NR properties have dealt with other models of inflammatory diseases, most notably atherosclerosis, it implies that use of NR ligands to suppress inflammatory signaling may be a useful means to intervene in the pathogenesis of IIC as well. This approach has been explored already by several groups [23, 197, 198]. The following sections will further focus on the mechanisms of anti-inflammatory effects of NRs (**Figure 6**).

GR

GR has been studied extensively for its anti-inflammatory properties [199]. Glucocorticoids remain the mainstay of therapeutic options for a wide variety of conditions involving a detrimental inflammatory response, including sepsis, asthma, and autoimmune diseases [200]. GR, more in particular the GRα isoform, appears to have multiple effects on inflammatory signaling, both through activation of gene expression (transactivation) and through inhibition of gene expression (transrepression) [201]. Examples of transactivation include the induction of the anti-inflammatory cytokine IL-10, the IL-1 receptor antagonist and the inhibitor of NF-κB. The transrepressive mechanisms appear to be more diverse and include, but are not limited to,

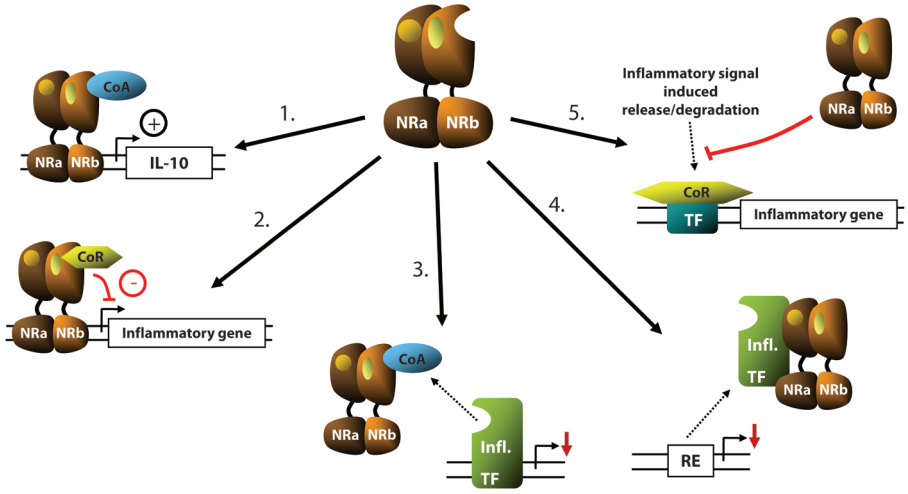

Figure 6. ***Mechanisms behind anti-inflammatory actions of NRs:*** 1.) induction of anti-inflammatory mediators through transactivation; 2.) transrepression of (pro-)inflammatory genes; 3.) competition for co-activators (CoA) with inflammation-induced transcription factors (Infl. TF); 4.) direct interaction with transcription factors induced by inflammatory signaling; 5.) interference with release of co-repressors (CoR) from transcription factors associated with inflammatory genes. (Other abbreviations: NRa/b, generic NR heterodimer partners, RE = response element, CoA = co-activator.)

inhibition of gene expression, competition for co-factors, the physical interaction between GR and other transcription factors, and effects on chromatin-remodelling [201]. Glucocorticoids have been found to be effective in various experimental settings of inflammation-induced cholestasis, both *in vitro* and *in vivo* [39, 42, 126, 202]. However, despite these effects in experimental settings, this has not led to the widespread clinical use of glucocorticoids for IIC.

PPARγ

Although it had initially been recognized as an important regulator of lipid and glucose metabolism as well as cellular differentiation, PPARγ was among the first class II NRs to be attributed with anti-inflammatory properties [203, 204]. Much of this early work dealt with the development of atherosclerosis, in which peripheral macrophages play an important role. It is, therefore, not surprising that the anti-inflammatory effects of PPARγ and its ligands have been studied particularly well in this cell-type [205]. Since the discovery of their anti-inflammatory potential, PPARγ ligands have been applied experimentally in various models of inflammation-related disorders, e.g., rheumatoid arthritis [206], inflammatory bowel disease [207] and lung disease [208].

Considering the role of KCs, as resident liver macrophages. in the pathogenesis of endotoxemic shock, Uchimura and colleagues [209] investigated the potential effect of the PPARγ agonist pioglitazone and of the RXR agonist Ro47-5944, on LPS-stimulated rat Kupffer cells and found that both were able to suppress the production of TNFα and NO and the transactivation activity of NF-κB. This effect, however, was not mediated by reduced DNA-binding of the transcription factors AP-1/NF-κB [209]. Since no Pparγ/Rxr response element was found in either the Tnfα or iNos promoter, these authors suggested activated Pparγ/Rxr to interfere non-genomically with the transcriptional activity of the pro-inflammatory transcription factors, perhaps through competition for important co-activators. This concept had been already been addressed by Li et al. [210], who showed that in macrophages Pparγ trans-repressed iNos gene expression through interaction with co-activator Cbp.

A different mechanism for PPARγ induced transrepression was elucidated by Pascual et al. [211] who showed that the transrepression of NF-κB signaling by ligand-activated Pparγ involved prevention of the clearance of NCoR-Hdac3 from the basally repressed promoter of the iNos gene. This clearance is normally initiated upon a pro-inflammatory stimulus, but ligand-dependent SUMOylation of Pparγ prevented this process. In retrospect, this mode of action appears to be in agreement with the anti-inflammatory effects of Pparγ/Rxr in rat Kupffer cells [209], which might also involve prevention of co-repressor clearance.

The effect of PPARγ agonist treatment in the setting of IIC has been addressed by Ghose et al. [197], who showed that pre-treatment of mice with rosiglitazone partially preserved gene expression of critical hepatobiliary transporters involved in bile formation. This, however, did not seem to be mediated by KCs, as cytokine expression and production were not significantly altered by rosiglitazone-pretreatment, but most likely involves the preservation of nuclear Rxrα levels in the hepatocytes [197]. Miyake et al. [23] used rosiglitazone in a mouse model of hepatic inflammation induced by a bile-acid containing pro-atherogenic diet. Rosiglitazone was able to suppress the effect of diet-induced suppression of Cyp7a1, which was indirectly shown to be dependent on macrophage/KC inflammatory signaling [23].

Lastly, PPARγ ligands also exert anti-inflammatory actions through gene transactivation. Similarly to the induction of IL-10 and the IL-1 receptor antagonist by glucocorticoids, PPARγ ligands induce the soluble IL-1 receptor antagonist in the THP-1 macrophage cell-line [212].

LXR

LXR is critical for maintenance of cellular cholesterol homeostasis [213, 214]. In peripheral macrophages, Lxr activation leads to an increased expression of transporters involved in reverse cholesterol transport, including Abca1 [215] and Abcg1 [216, 217] and functionally to a reduction of atherosclerosis in relevant mouse models [218-220]. LXR activation in hepatocytes induces expression of the canalicular half-transport-

ers Abcg5 and Abcg8 and stimulates biliary cholesterol excretion [221, 222]. Joseph et al. [223] reported that Lxr activation inhibited the macrophage inflammatory gene response both *in vitro* and *in vivo*. This effect could be exerted by either isoform of Lxr and appeared to involve inhibition of NF-κB signaling [223]. Follow-up studies by Castrillo et al. [224] demonstrated that ligand-activated Lxr inhibited inflammatory signaling, leading to macrophage matrix metalloproteinase (Mmp)-9 expression at least in part through interference with NF-κB signaling downstream of its binding to DNA. No evidence was found for interference with AP-1 signaling or for direct Lxr:Rxr binding to the 5'-flanking region [224]. Since then, multiple groups have reported on this anti-inflammatory effect of LXR(-agonists) in macrophages [225-228] and other cell-types, including lymphocytes [229], keratinocytes [230], Kupffer cells [198], hepatocytes [231], microglia and astrocytes [232] and polymorphonuclear neutrophils [233]. Interestingly, Ogawa et al. [226] found LXR-agonists to inhibit inflammation-induced expression of osteopontin through interference with AP-1 signaling. It remains unclear what causes the discrepancy between this finding and the earlier observed lack of effect on AP-1 signaling [224, 225]. There may also be a difference between the anti-inflammatory actions in mice and humans as Fontaine et al. [228] showed that in human macrophages LXR(-agonists) increased the expression of TLR4 and the response to LPS-challenge via an LXR-RE in the TLR4 promoter, which is not conserved in mice.

Inspired by studies on anti-inflammatory actions of PPARγ, Ghisletti et al. [234] sought to determine whether similar mechanisms might be involved in the anti-inflammatory actions of LXRs. Interestingly, similar mechanisms involving SUMOylation (albeit by SUMO2/3 rather than SUMO1) of ligand-bound Lxr were found, which prevented the clearance of NCoR from the iNos (or other inflammatory gene) promoter [234]. Reduced clearance of NCoR by ligand activated Lxr has also been shown to be the mechanism underlying the inhibition of hepatocellular C-reactive protein expression [231].

Considering the anti-inflammatory effects of LXRs and their expression in KCs, we recently examined the potential of LXR-agonists to interrupt the inflammatory cascade leading to LPS-induced suppression of hepatobiliary transporter expression (Mulder et al. (unpublished data)). Although treatment of mice with T0901317 led to partial preservation of transporter gene expression and preserved Mrp2 protein expression, this appeared to be KC-independent, since cytokine expression was not altered. Wang et al. employed a different LXR-agonist in a more severe model of hepatic inflammation (LPS/Gal-treatment of rats) and found the LXR agonist to be protective and associated with a reduced KC-response [198].

Similarly to GR, LXR can also modify inflammatory response "genomically" through direct transactivation, e.g., through induction of arginase II, which can compete with iNOS for substrate [235]. Furthermore, it was recently shown that LXRα can directly repress gene expression through promoter specific recruitment of the

co-repressor RIP140 [236]. It is tempting to speculate that LXR may repress inflammatory genes via such a mechanism too.

RXRα and RARα

The anti-inflammatory effects of retinoids have been used clinically for several decades, especially in the treatment of various dermatological conditions (e.g., psoriasis, acne) [237]. Initial studies addressed the effects of both types of receptor on regulation of transforming growth factor (TGF)-β1 [238]. Both receptors were shown to inhibit AP-1 signaling through physical interaction [238].

Pertaining to IIC, Rxrα agonists were shown to inhibit TNFα release and NO production by primary Kupffer cell [239]. These results were confirmed by Uchimura et al. [209], who went on to show that this effect was associated with reduced NF-κB transcriptional activity. Rarα-selective ligands, on the other hand, were not effective *in vitro* [239]. The latter was surprising as all-trans retinoic acid was shown to blunt LPS and Propionibacterium acnes induced liver injury *in vivo* [240], indicating that RARα agonists can initiate anti-inflammatory mechanisms at least *in vivo*.

Ligand-bound Rxrα was able to inhibit NF-κB transcriptional activity [209] and appeared to do so similarly to Pparγ:Rxrα heterodimer, suggesting that similar mechanisms as described for PPARγ and LXR might be active (i.e., inhibition of co-repressor clearance). Na et al. showed that RXR ligands reduced LPS-induced cytokine expression in mouse macrophages via at least two mechanisms, i.e., either through physical interactions with NF-κB subunits p50 and p65, but also through co-activator competition [185]. Anti-inflammatory effects of retinoids have also been shown in other cell-types, e.g., microglia and astrocytes [232]. Finally, the observation of increased liver cytokine gene expression in hepatocyte-specific Rxrα-deficient mice after alcohol-treatment suggests that Rxrα may very well have anti-inflammatory effects under basal conditions [241].

Anti-inflammatory effects of other NRs

Besides GR, PPARγ and LXR, other NRs involved in bile formation, lipid homeostasis and bile salt metabolism have also been attributed anti-inflammatory properties.

Although FXR has been shown to modify disease processes involving inflammation (including atherosclerosis [242] and cholesterol gallstone disease [243]), limited information is available on direct effects of FXR on inflammatory signaling in liver cells. Hepatic cytokine gene expression after LPS treatment was not different between in wild-type and Fxr-null mice [141]. However, one has to bear in mind that, in this study, FXR agonists were not used to analyze the effect of pharmacological activation of FXR on LPS-induced cytokine expression [141]. In bile-duct ligated mice, FXR-activation by GW4064 led to a decreased expression of the pro-fibrogenic cytokine TGFβ [244], although no underlying mechanism was provided. It remains to be

determined whether this reduced expression is a concurrent effect of disease process modification rather than a direct effect on TGFβ expression. Interestingly, it was recently shown in models of diabetic nephropathy, which is associated with increased fibrogenesis/inflammation, that FXR agonists such as cholate and GW4064 reduced IL-6 and TGFβ mRNA expression [245]. This suggests that this effect of FXR is a more general phenomenon, because it is not limited to liver cells and because different disease processes that lead to fibrogenesis in different cell types respond similarly. Li et al. [246] showed that FXR ligands were able to inhibit inflammatory response in rat/human vascular smooth muscle cells in a FXR- and SHP-dependent manner, with concurrent reduction in NF-κB activation.

In contrast with a potential anti-inflammatory role, Qin et al. [247] showed that FXR mediates the bile salt-induced expression of intracellular adhesion molecule-1, which is involved in the recruitment of neutrophils to the site of inflammation. By this induction, FXR will actually enhance the inflammatory process. Furthermore, FXR activation led to a SHP-mediated derepression of MMP-9 in endothelial cells [248]. MMP-9, involved in tissue remodelling including atherosclerosis, was shown to be negatively regulated by LXR [224], suggesting opposite effects of these two nuclear receptors. It clearly remains to be further investigated how FXR modulates inflammatory processes overall and whether some the effects described above are concurrent effects of FXR agonists on disease progression or distinct anti-inflammatory effects.

PXR is another NR that is able to interfere with NF-κB signaling [156]. Different PXR ligands were able to suppress basal or induced expression of NF-κB target genes and this suppression was dependent on PXR expression. Furthermore, primary hepatocytes derived from Pxr-null mice showed increased expression of NF-κB target genes, indicating a general anti-inflammatory effect. A mechanistic explanation, however, was not provided for these observations. Similar observations of the anti-inflammatory characteristics of Pxr were done in a mouse model of inflammatory-bowel disease [249].

LRH-1 was recently also shown to inhibit the hepatic inflammatory response via at least two different mechanisms [250, 251]. First, LRH-1 was shown to specifically interfere with C/EBPβ transcriptional activity induced by different cytokines, while it had no effect on simultaneous induction of NF-κB and AP-1 activity [250]. Subsequent studies revealed that LRH-1 also induces expression of secreted IL-1RA by hepatocytes under inflammatory conditions [251]. The latter indicates that LRH-1 not only directly interferes with inflammatory signaling (C/EBPβ), but also indirectly through induction of an anti-inflammatory mediator (IL-1 receptor antagonist). These mechanisms appear to act synergistically. Of interest, Mueller et al. reported that Lrh-1 mediates the induction of extra-adrenal glucocorticoid synthesis, i.e. by the intestine [252], which has been linked to reduced inflammatory responses in the setting of inflammatory bowel disease [253]. It would be relevant to examine whether such mechanisms might also be at work in the pathogenesis of IIC.

Although generally regarded as a transcriptional suppressor, only limited information is available on a potential role of SHP in modulation of inflammatory signaling. Some of the mechanisms by which SHP exerts its repressive effects on gene transcription, e.g., competition for binding with co-activators through physical interaction with NRs and other transcription factors [254, 255] are very similar to those by which other NRs interfere with inflammatory signaling. It seems therefore plausible that SHP interferes with inflammatory cascades. This has already been shown for TGFβ1 signaling [256]. TGFβ1, a cytokine involved liver regeneration and fibrosis, activates, amongst other pathways, SMAD-signaling [257]. SHP was shown to inhibit TGFβ1-induced gene responses through physical interaction with SMAD3, preventing binding of the co-activator p300 [256]. As mentioned above, Li [246] showed that FXR-mediated induction of SHP expression was able to inhibit the inflammatory response in rat and human vascular smooth muscle cells by interfering with NF-κB activation. The exact molecular mechanism of action, however, remains to be revealed.

General considerations regarding the anti-inflammatory effects of NRs

Although some NRs seem to act clearly in an anti-inflammatory fashion (e.g., GR), the effects of others, i.e., FXR and LXR, appear to be rather unpredictable and are gene-specific. The inflammatory response is an extremely complex process which balances effects aimed at protection of the organism with potential detrimental effects. This balance, in turn, involves multiple delicately organized processes in different cell-types in response to multiple stimuli which change over time with various feedback systems. It might therefore be an oversimplification to expect that one particular NR will either be pro-inflammatory, neutral or anti-inflammatory as this may be cell type-, species- and context-dependent. In its overall complexity, it appears that inflammatory signaling involves several different, often redundant pathways. Considering the different modes of anti-inflammatory actions of the NRs, one could propose that targeting multiple NRs simultaneously may lead to synergistic anti-inflammatory effects. The latter has indeed been shown for combinations of GR- and PPARγ ligands [258] as well as LXR- and PPARγ ligands [259]. This concept is especially interesting with regard to clinical application of NR ligands in inflammatory therapeutic regimes as combining ligands may reduce side-effects associated with the use of single ligands [260].

Design of optimal NR ligands for intervention in IIC

In the previous sections, the involvement of NRs in the pathophysiology of IIC as well as their potential role as modifiers of this condition have been discussed. The latter concept makes use of the key characteristic of NRs that separates them from other transcription factors, namely their ligand-binding properties. Although natural ligands have been identified for several of the NRs discussed above and synthetic

ligands have become available, there remains a clear necessity for improvement. Besides optimizing the general pharmacokinetic characteristics, i.e., absorption, distribution, elimination and metabolism of NR ligands, and perhaps the application of advanced drug targeting strategies, the concept of selective NR modulators (SNuRMs) may provide an alternative approach in the quest for ideal NR ligands.

NRs function as molecular links between genomic information (genes/promoters), environmental/hormonal cues and the effector machinery for active gene transcription. However, NRs should not be regarded as rigid mediators that serve as "on/off switches", whose interactions with ligands resemble key-lock interactions. The overall outcome of ligand-binding to a NR is determined at multiple levels. First of all, although not all NRs are as promiscuous with regard to their ligand-binding as for instance CAR and PXR, NRs can generally be bound by different, more or less related ligands. Differential ligand binding will lead to different conformational changes, which will affect co-factor recruitment. Secondly, it has also been shown that very subtle differences in cis-acting response-elements can have an influence of co-factor recruitment by NRs as well as other transcription factors. The latter was illustrated by Leung et al. who showed that a single-nucleotide difference in a κB-site affected co-factor recruitment to NF-κB dimers. This illustrates the extent to which NR/transcription factor actions are fine-tuned and that DNA can be considered as an allosteric modifier of transcription factor function [261] Thirdly, co-factors can be regarded as the effector molecules of NR function. Since this large group of molecules has its own (spatial and temporal) expression profile, binding of a single ligand to a specific NR may have transcriptional effects that are context-dependent (organ, tissue, cell-type, promoter). These aspects all contribute to the complexity of DNA-NR-co-factor interaction. This feature of NR biology makes it near to impossible to accurately predict the outcome of NR-binding by a single ligand, yet, it simultaneously provides an opportunity to search for compounds that acts as SNuRMs. Kremoser et al. reviewed a series of approaches to search for effective SNuRMs [262].

A good example of a clinically used SNuRMs are the selective modulators of the estrogen receptor (SERMs), tamoxifen and raloxifene [62]. Tamoxifen is widely used as an adjuvant treatment in breast cancer care, while raloxifene is used as anti-osteoporosis agent. These drugs act as antagonists of the estrogen receptor in some tissues, but as agonists of the same receptor in other tissues. The underlying mechanism of this tissue specificity primarily involves differences in co-regulator expression and activity [62]. SERMs are actually mixed agonists/antagonists and their binding induces conformational changes, which lead to changes co-activator and co-regulator recruitment. The exact pattern of recruitment depends on co-regulator expression and post-transcriptional modification. The development/identification of such selective modulators for other NRs would potentially be a great stride forward, especially for those NRs whose general modulation/activation appears to be of benefit in certain settings, but whose further development is hampered by undesired activation of other subsets of genes.

Tissue- and gene-specific activation of LXR is probably one of the greatest challenges in the NR field. Although the anti-inflammatory [223] and plaque-reducing effects [218-220] are obvious from mouse studies, current LXR agonists have also been shown to induce de novo lipogenesis in hepatocytes with increased plasma triglycerides and hepatic steatotosis as detrimental results. The metabolic consequences of these particular agonists currently preclude their clinical use. In the search for other LXR ligands with great therapeutic potential, one can take different approaches. First of all, ligands which are preferentially taken up by macrophages or specifically targeted to KCs, rather than by hepatocytes, should in theory limit the detrimental hepatocellular LXR-activation. Organ selectivity has been described for one of the current LXR agonists, i.e., GW3965 [263]. LXRβ selective ligands would also be appealing alternatives, since LXRβ expression is lower than that of LXRα in hepatocytes, while expression levels of both are more comparable in macrophages. Although LXRβ-selectivity may a priori not be easily achieved as the LBDs of the two LXR-isoforms are highly similar, several groups have reported on LXRβ selective LXR ligands [264, 265]. In addition to cell-, organ- and isoform-specific LXR ligands, development of selective LXR modulators that are specifically active in non-hepatocyte cells or inactive on promoter of lipogenic genes would be of great therapeutic potential.

Other approaches

As described previously, co-regulators are very important in regulation of gene transcription by NRs. Initially considered to be mere executors in the regulation of gene expression by NRs, this clearly underestimates the role of co-regulators. This can be illustrated by the transcriptional regulation of phosphoenolpyruvate carboxykinase (PEPCK), an enzyme centrally involved in gluconeogenesis. The gluconeogenic response is dependent on proper HNF4α function, but binding of this NR is not altered during fasting, when PEPCK transcription is induced. This induction was regulated by increased expression of co-activator PGC1α [266, 267], illustrating both that co-regulators are more than mere bystanders in the process of gene transcription and that methods to alter co-regulator abundance/modification may provide another approach to modify NR-function. Although even more far-fetched, the concept of co-activator rescue, e.g., restoring NF-κB transactivation capacity by supplying an exogenous co-activator [268] is also intriguing. However, due to the complexity of the transcriptional complex, it is difficult to predict what the implications of alterations of single co-regulators may be *in vivo*. So far, this concept has not been pursued in the context of IIC.

Summary and perspective

IIC is a frequently occurring phenomenon and with substantial morbidity and mortality. NRs play a dual role in the pathogenesis of IIC. On one hand, NRs contribute to disease development, as they are targets of inflammatory signal-

ing themselves. Whether it be through direct effects on their expression levels or functionality or through indirect effects on their co-regulators, altered NR activity affects expression levels of important hepatobiliary transport systems. Hence, NRs can be considered to be important mediators of IIC. On the other hand, NRs also play important roles in adaptive responses, for instance during bile salt accumulation within hepatocytes through FXR activation. Several NRs have also been shown to possess anti-inflammatory effects. Therefore, NRs can also act as modifiers of IIC. The latter concept makes it interesting to determine whether NRs can be exploited as pharmacological targets to intervene in IIC, either through boosting adaptive responses or induce anti-inflammatory responses. Identification of new NR ligands or SNuRMs with characteristics as described in the previous section would be ideal.

Although sepsis-associated cholestasis is the most widely known example of IIC, cholestasis can also occur in the setting of many other inflammation-related conditions associated with a generalized APR, e.g., severe burn injury or trauma. Moreover, inflammatory signaling also appears to be involved in other conditions characterized by cholestasis. An example of the latter is biliary atresia (BA), the most common neonatal cholestatic disorder, which is characterized by progressive inflammation, fibrosis and, subsequently, obliteration of the bile ducts ultimately leading to biliary cirrhosis [269]. This multifactorial pathophysiological process appears to include a persistent (auto)immune response after a perinatal, infectious insult or aberrant bile duct formation [269]. Several cytokines, e.g., TNFα, IFNγ and osteopontin [270-272], have been implicated as potentially important mediators and are in part secreted by recruited macrophages/monocytes [273]. This indicates that anti-inflammatory agents aimed at macrophages may have a role in treatment of BA. Perhaps PPARγ or LXR agonists may be suitable agents to explore. Interestingly, glucocorticoids are often used in the treatment of BA, but only after the primary surgical intervention, i.e., Kasai portoenterostomy, with the underlying thoughts that glucocorticoids will enhance bile formation and reduce inflammation, and thus increase the chances of establishing permanent post-Kasai bile drainage [274].

Total parenteral nutrition associated cholestasis (TPNAC) is another, rather frequently occurring condition that shows the close link between inflammation and cholestasis. Although the exact pathogenesis of TPNAC remains unclear, many factors have be implicated to play a causative role, including increased portal LPS-levels and individual components of TPN formulas [275]. With regard to the latter, it was recently shown that the phytosterol, stigmasterol, can act as an FXR antagonist [276], which is expected to impair hepatic adaptive responses to bile salt retention. Implicitly, this also suggests that FXR agonists are potentially of benefit under such conditions by presumably enhancing adaptive responses. Furthermore, FXR also appears to have anti-inflammatory characteristics within the intestine [277]. This suggests that NR ligands are of potential use in TPNAC either as anti-inflammatory agents or as adaptation-boosting agents.

These two examples (BA and TPNAC) further illustrate the link between inflammation and cholestasis, which probably ought to be regarded as intertwined phenomena. Not only does inflammation lead to cholestasis (as in IIC), cholestasis per se leads to hepatic inflammation as well, as reduced intestinal delivery of bile may lead to bacterial overgrowth, translocation and an inflammatory response [11]. Finally, hepatocellular damage, regardless of its cause, will activate inflammatory signaling and thus further impair liver function in part via inducing cholestasis. Studies in bile duct-ligated mice, a well known model of acute, extrahepatic cholestasis, have shown that there is an inflammatory response that further worsens liver injury [278, 279]. The underlying mechanisms, however, remain unclear as bile-duct ligation also leads to reduced expression of hepatic transporters independently of cytokine expression [280] or degree of inflammatory response [25].

In conclusion, NRs play dual roles in the setting of IIC. Increased understanding of the pathogenesis of IIC has shown us how centrally involved NRs and their co-regulators are in the regulation of hepatobiliary transport systems. Simultaneously, this has provided us with novel therapeutic strategies aimed at maintaining or even boosting NR function during inflammation, enabling proper/necessary adaptive responses. Furthermore, future application of new anti-inflammatory agents (such as selective PPARγ or LXR modulators) would expand the therapeutic arsenal importantly making us less dependent on traditional compounds as glucocorticoids.

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CHAPTER 3

Rosiglitazone attenuates suppression of RXRα-dependent gene expression in inflamed liver

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Abstract

Background/Aims: A recently-determined target of lipopolysaccharide (LPS) and cytokine signaling in liver is the central Type II nuclear receptor (NR) heterodimer partner, retinoid X receptor α (RXRα). We sought to determine if rosiglitazone (Rosi) a peroxisome proliferator activated receptor γ (PPARγ) agonist with anti-inflammatory properties, can attenuate LPS and cytokine-induced molecular suppression of RXRα-regulated genes.

Methods: In vivo, mice were gavage-fed Rosi for 3 days, prior to intraperitoneal injection of LPS, followed by harvest of liver and serum. In vitro, HepG2 cells were treated with IL-1β, ± short-term Rosi pretreatment. RNA was analyzed by quantitative RT-PCR, while nuclear and cytoplasmic proteins were analyzed by immunoblotting and gel shifts.

Results: Rosi attenuated LPS-mediated suppression of RNA levels of several Type II NR-regulated genes, including bile acid transporters and the major drug metabolizing enzyme, Cyp3a11, without affecting cytokine expression, suggesting a novel, direct anti-inflammatory effect in hepatocytes. Rosi suppressed the inflammation-induced nuclear export of RXRα, in both LPS-injected mice and IL-1β-treated HepG2 cells, leading to maintenance of nuclear RXRα levels and heterodimer binding activity.

Conclusions: Rosi directly attenuates the suppressive effects of inflammation-induced cell signaling on nuclear RXRα levels in liver.

Introduction

Inflammation induces the negative hepatic APR, which is characterized by disruption of critical physiological processes in the liver [1, 2]. LPS-induced APR involves Kupffer cell (KC)-mediated release of the pro-inflammatory cytokines, interleukin (IL)-1b, IL-6, and tumor necrosis factor (TNF)-a, which results in the activation of cell-signaling pathways leading to the suppression of hepatic genes [3, 4]. APR leads to the pathogenesis and progression of a variety of liver diseases, including cholestasis, which results from altered expression of the bile acid transporters, including sodium/taurocholate cotransporter (Ntcp/Slc10a1), bile acid salt exporter pump (Bsep, Abcb11), and the multi-drug resistance-associated proteins (Mrp, Abcc) 2 and 3 [2, 5]. The effects of LPS on hepatic genes are attenuated in rodent models upon inactivation or depletion of KCs, or by administration of anti-cytokine antibodies [6-9]. This suggests that counteracting either the production or intracellular action of inflammatory mediators secreted by KCs may attenuate the pathogenesis of inflammation in liver diseases.

The expression of many of the genes which are repressed during negative hepatic APR are regulated by Type II nuclear receptors (NRs), which require heterodimerization with retinoid X receptor (RXR) to activate gene transcription [10, 11]. RXRα is the most highly expressed RXR isoform in the liver and plays a central role in regulating major physiological processes in the liver, including endobiotic/xenobiotic metabolism and homeostasis [12, 13]. We have recently demonstrated that reduction of nuclear RXRα protein levels by LPS administration *in vivo* and IL-1β *in vitro*, appear to be a major contributor to the repression of hepatic genes during the negative hepatic APR [14-16]. Thus, maintaining RXRα levels in the nucleus may be a new and potent means to attenuate LPS-mediated suppression of hepatic genes.

In addition to playing major roles in lipid metabolism, members of the PPAR (Nr1c1-3) subfamily of Type II NRs (α, β/δ, γ), may possess potent anti-inflammatory properties [17-19]. PPARγ ligands can inhibit the expression of inflammatory genes such as IL-1β, TNFα, IL-6, iNOS, MMP-9 and scavenger receptor A in macrophages and monocytes [17-20]. There is increasing evidence to support a protective role of PPARγ in various pathophysiological conditions including cancer, atherosclerosis, diabetes and hepatogastroenterological diseases [19, 21]. PPARγ ligands can inhibit LPS-induced NO and TNFα production in cultured KCs and the inhibition was potentiated by co-treatment with RXR agonists [22]. However, it is not known whether PPARγ agonists have any role in reducing the effects of inflammation on NR genes in hepatocytes, although recent studies in humans with non-alcoholic steatohepatitis (NASH) support PPARγ ligands as potential anti-inflammatory agents [23, 24].

In this study we sought to determine whether the PPARγ ligand, Rosiglitazone (Rosi) can attenuate the deleterious effects of inflammation on the expression of genes regulating endobiotic/xenobiotic transport and metabolism in liver. Rosi attenuated the effects of LPS on the expression of critical RXRα-regulated hepatic

genes (Ntcp, Bsep, Cyp3a11 and Lfabp), while inhibiting LPS-mediated RXRα nuclear export, resulting in increased nuclear binding activity of RXRα heterodimers *in vivo*. Surprisingly, Rosi did not affect LPS-mediated induction of cytokine expression, but appears to have a direct anti-inflammatory effect in hepatocytes. In vitro studies indicate that Rosi can act intracellularly in liver-derived HepG2 cells to prevent IL-1β-mediated nuclear export and degradation of RXRα. This suggests that PPARγ agonists can be utilized as novel therapeutic agents to modulate hepatic inflammatory responses in acute and chronic liver diseases.

Materials and methods

Mice

Male C57BL/6 mice from Charles River Laboratories (Wilmington, MA) were maintained in a temperature-and humidity-controlled environment and provided with water and rodent chow ad lib. Mice were gavage-fed 50 mg/kg/d of Rosiglitazone (Alexis Biochemicals, San Diego, CA) or corn-oil once daily for 3 days. On day 3, the animals were intraperitoneally (IP) injected with 2 mg/kg body weight of LPS (Salmonella typhimurium; Sigma Chemical Co., St. Louis, MO) or saline and livers were harvested after 1, 4, 8 and 16 hours [16]. All animal protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Experiments were performed in triplicate and repeated three to four times.

Real time quantitative PCR analysis

Total RNA was isolated from liver tissues using the RNeasy kit from Qiagen, and cDNA was synthesized using the ProSTAR™ First-Strand RT-PCR Kit (Stratagene, La Jolla, CA). Real time quantitative PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA). Quantitative expression values were extrapolated from standard curves and were normalized to cyclophilin. The sequences of the primers and probes are listed in Table 1. All data were analyzed by Kruskal Wallis H-test followed by Mann-Whitney test. P-values less than 0.05 were used as the criteria of significance.

Plasma cytokine analysis

Plasma levels of IL-1β, IL-6 and TNFα were determined simultaneously using xMAP technology (Luminex Corporation, Austin, TX) with a commercially available kit (Linco Research, St.Charles, MO).

Cell fractionation and immunoblotting

Cell extracts were prepared as previously described [15, 16, 25]. The following antibodies were used in immunoblot analysis: JNK, phospho-JNK and phospho-c-Jun antibodies (Ser 63) (Cell Signaling, Beverly, MA), IκBα and anti-RXRα (D-20) (Santa

Table 1. Primer and probe DNA sequences used for Taqman real-time PCR for RNA quantitation

Gene	Acc. nr.	Forward primer	Reverse primer	Probe	Ref.
Ntcp	AB003303	ATGACCACCTGCTC-CAGCTT	GCCTTTG-TAGGGCACCTTGT	CCTTGGGCATGAT-GCCTCTCCTC	37
Bsep	NM_021022	CTGCCAAGGAT-GCTAATGCA	CGATGGCTACCCTTT-GCTTCT	TGCCACAGCAATTT-GACACCCTAGTTGG	37
Mrp2	NM_013806	GCTGGGAGAAATG-GAGAATGTC	GACTGCTGAGGGAC-GTAGGCTA	TGGGCATATCAC-CATCAAGGGCTCC	38
Mrp3	BC048825	TCCCACTTTTCG-GAGACAGTAAC	ACTGAGGACCTT-GAAGTCTTGGA	CACCAGTGTCATTC-GGGCCTATGGC	39
Cyp3a11	X60452	GGATGAGATCGAT-GAGGCTCTG	CAGGTATTCCATCTC-CATCACAGT	CCAA-CAAGGCACCTCCCAC-GTATGA	
IL-1b	NM_008361	CAACCAACAAGT-GATATTCTCCATG	GATCCACACTCTC-CAGCTGCA	CTGTGTAATGAAAGACG-GCACACCCACC	40
TNFa	NM_013693	CATCTTCTCAAAATTC-GAGTGACAA	TGGGAGTAGA-CAAGGTACAACCC	CACGTCGTAGCAAAC-CACCAAGTGGA	40
sIL-1Ra	M57525	CTCCTTCTCATCCTTCT-GTTTCATT	GCATCTTG-CAGGGTCTTTTCC	AGAGGCAGCCT-GCCGCCCTT	-

Cruz Biotechnology, Santa Cruz, CA). Signals were developed using Tropix luminescence following the manufacturer's protocol (Applied Biosystems, Foster City, CA).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to Timchenko et al. with some modifications[26]. 10 mg of nuclear extracts were incubated on ice for 30 min with ^{32}P end-labeled oligonucleotide as described previously[15]. After binding, the samples were electrophoresed through a non-denaturing 6% polyacrylamide gel, dried and exposed to x-ray film.

Cell culture

The human hepatoma cell line, HepG2, was maintained in MEM containing Earle's salts and supplemented with 10 % certified fetal bovine serum (FBS), penicillin-streptomycin and L-glutamine. The cells were plated at 2.5 x 10^5 cells/ml and maintained in serum-containing media for 48 hours and then serum starved for 20 hours prior to treatment with 10 mM Rosi or DMSO. After 30 minutes of Rosi treatment, cells were treated with either 10ng/ml IL-1β or vehicle control (0.0001% BSA in PBS) for 30 minutes.

Immunofluorescent analysis

Mice were pre-treated with Rosi or vehicle, followed by saline or LPS injection, and livers were harvested after 1 hour. Livers were fixed in 10% buffered neutral formalin overnight at 4 °C and then stored in 70% ethanol. Fluorescent detection was performed by using anti-RXRα (D-20) antibody and fluorescein isothiocyanate

(FITC)-labeled secondary antibody and nuclei was stained with 4'-6-diamidino-2-phenylindole (DAPI). Visualization was performed with a Deltavision Spectris Deconvolution Microscope System (Applied Precision, Inc.).

HepG2 cells were grown on cover slips, treated with Rosi or DMSO for 30 minutes, followed by IL-1β or vehicle treatment for another 30 minutes. Cells were washed with cold phosphate buffered saline, and immunostaining was performed as described previously[14]. The cells were stained with anti-RXRα antibody and Alexa Fluor 555 goat anti-rabbit secondary antibody (Invitrogen, Eugene, Oregon).

Results

Rosiglitazone pre-treatment attenuates LPS-mediated suppression of RXRα-regulated hepatic genes

Administration of LPS leads to the down-regulation of hepatic genes involved in bile acid metabolism and transport[27, 28]. To determine whether the PPARγ agonist, Rosi can attenuate the effect of LPS on hepatic gene expression, four groups of mice were tested—vehicle feeding followed by saline injection (Veh/Sal), vehicle feeding followed by LPS (Veh/LPS), Rosi feeding followed by saline injection (Rosi/

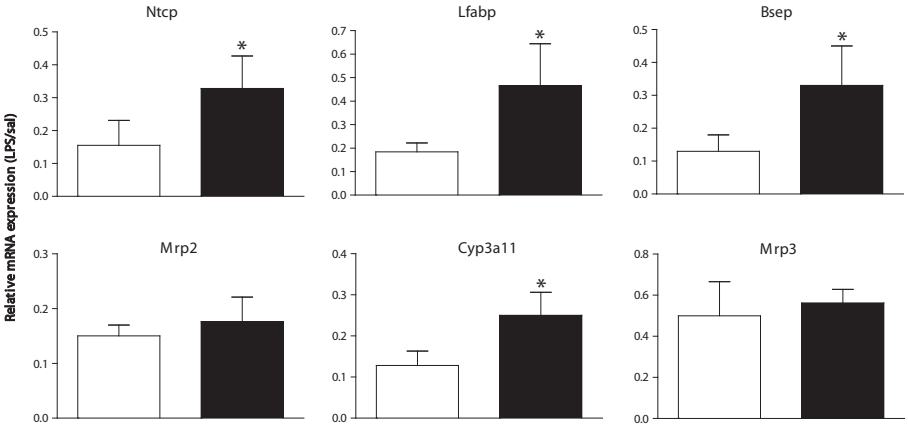

Figure 1. ***Rosiglitazone attenuates suppression of hepatic genes by LPS.*** C57BL/6 male mice were gavage-fed 50 mg/kg/d of Rosi or corn-oil for 3 days. On day 3, the animals were intraperitoneally (IP) injected with 2 mg/kg body weight of Salmonella LPS or saline and livers were harvested after 16 hours (n=6 per group). RNA was isolated from the livers and analyzed by TaqMan real-time PCR. All data were presented as mean (+SD) and standardized for cyclophilin RNA levels. The expression of the genes after LPS treatment is shown here. In case of vehicle or Rosi pre-treatment, expression in saline-injected animals was set to 1, and fold change after LPS treatment was compared to vehicle or Rosi controls respectively. The asterisks indicate significant difference ($p < 0.05$).

Sal), and Rosi feeding followed by LPS injection (Rosi/LPS). RNA was isolated from livers harvested at 16 hours after injection and analyzed by real-time PCR (**Figure 1**). The RNA levels of Veh/LPS and Rosi/LPS samples were determined relative to their controls, Veh/Sal and Rosi/Sal, respectively. RNA levels of the major bile acid transporters, Ntcp and Bsep, from Rosi/LPS treated mice increased 2-3fold compared to Veh/LPS treated control mice. RNA levels of the major bile acid and drug metabolizing enzyme, cytochrome P450 3a11 (Cyp3a11) increased ~2-fold, with Rosi pre-treatment as did RNA levels of the liver fatty acid binding protein (Lfabp). Rosi did not affect the LPS-mediated suppression of two NR-regulated transporter genes, Mrp2 and Mrp3, suggesting that Rosi exhibited gene-specific responses.

A direct hepatocyte target gene of PPARγ, fatty acid translocase (FAT)/CD36 [29] was induced by Rosi (data not shown) indicating that 50 mg/kg/d of Rosi treatment activates PPARγ in hepatocytes. Thus, Rosi is capable of activating PPARγ in liver and attenuating LPS-mediated down-regulation of key genes involved in bile acid homeostasis.

Rosi attenuates effects of LPS on RXRα subcellular localization in mouse liver

Recent results indicate that LPS reduces RXRα target gene expression by inducing its nuclear export [16]. We wanted to determine whether Rosi attenuated LPS-mediated suppression of RXRα-regulated hepatic gene expression by reducing its nuclear export and maintaining nuclear RXRα levels. As reported by us [16], in Veh/LPS-treated mice, nuclear RXRα levels were significantly reduced with a corresponding increase in cytosolic RXRα levels, compared to Veh/Sal controls (**Figure 2A**). However, LPS-mediated reduction in nuclear levels of RXRα was attenuated by Rosi pre-treatment (10% in Veh/LPS → 20% in Rosi/LPS). LPS-mediated induction in cytosolic RXRα levels was also attenuated by Rosi (80% in Veh/LPS → 1% in Rosi/LPS), indicating that Rosi pre-treatment attenuates LPS-induced nuclear export of RXRα. Rosi treatment alone did not affect the nuclear or cytosolic levels of RXRα (**Figure 2A**). Immunofluorescent analysis of formalin-fixed liver tissues show that RXRα was localized in the nucleus in Veh/Sal treated sample, and was detected in the cytosol after LPS treatment (**Figure 2B**). Rosi treatment alone had no effect on nuclear RXRα, however, Rosi pre-treatment blocked LPS-induced nuclear export of RXRα, as evidenced by the lack of cytoplasmic RXRα in Rosi/LPS-treated panel (**Figure 2B**).

In order to determine if attenuation of LPS-mediated nuclear export of RXRα by Rosi affects DNA binding activity of RXRα and its partners, EMSA was performed. Nuclear extracts were incubated with oligonucleotides containing canonical DNA elements scanning Type II NR binding sites--direct repeats of the hexad AGGTCA, separated by 2 and 4 nucleotides (DR2 & DR4), or an inverted repeat separated by 1 nucleotide (IR1)—sequences in promoter regions that regulate many genes involved in metabolism and transport in hepatocytes [10, 16]. In response to LPS treatment,

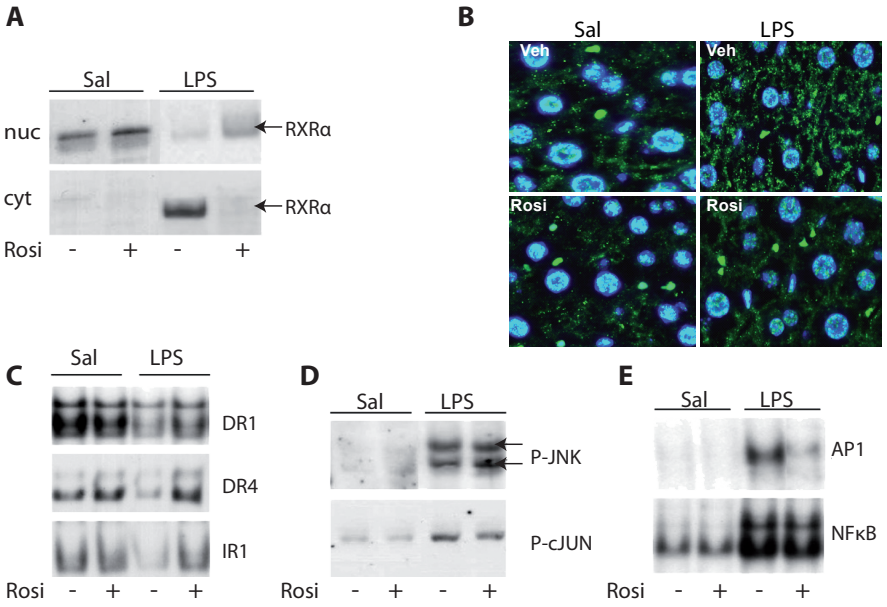

Figure 2. ***Rosiglitazone attenuates effects of LPS on RXRα localization and binding activity in vivo.*** C57BL/6 male mice were gavage-fed 50 mg/kg/d of Rosi or corn-oil for 3 days prior to saline or LPS (2 µg/g bw) injection on day 3. Livers were isolated at the 1, 4 and 16h and nuclear and cytosolic extracts were prepared. Extracts from 4-5 animals were analyzed individually and combined to account for inter-animal variability. (A) Nuclear (Nuc) and cytosolic (Cyt) extracts from 1h samples were analyzed by immunoblotting with antibodies to RXRα to determine the effects of Rosi on subcellular localization of RXRα in the presence of saline or LPS. (B) Immunofluorescence analysis of formalin-fixed liver tissues. RXRα was stained with FITC-labeled secondary antibody, nuclei were stained with DAPI, and the merged images are shown. (C) Electrophoretic mobility shift assay analysis of 16h samples where radiolabeled DR2, DR4 and IR1 elements were incubated with hepatic nuclear extracts. The samples were electrophoresed through a 6% non-denaturing polyacrylamide gel, dried and analyzed by autoradiography. (D) Phosphorylation of JNK (P-JNK) and c-JUN (P-cJUN) was determined by immunoblotting cell lysates from 1h samples with phospho-JNK and phospho-c-JUN antibodies respectively. (E) In order to determine the effects of Rosi on LPS-mediated AP-1 or NF-κB activation, binding activity of nuclear extracts (prepared from 4h samples) to consensus AP-1 or NF-κB elements was measured by EMSA.

binding to these RXRα-containing conserved sequences was reduced (**Figure 2C**), while, Rosi pre-treatment increased binding activity to all three target sequences back to baseline levels (DR2: 50% à 85%, DR4: 40% à 90% and IR1: 60% à 100% in Rosi/LPS compared to Veh/LPS).

Effect of Rosi on cell-signaling pathways *in vivo*

We next examined the role of LPS-activated cell-signaling on nuclear RXRα export in mouse liver. The mitogen-activated protein kinase, c-Jun N-terminal kinase, JNK was recently shown to be involved in nuclear export of RXRα during activation of inflammatory pathways [14, 16]. Rosi treatment did not alter the LPS-mediated increase in phosphorylation of JNK, or its substrate, c-Jun in nuclear extracts, thus indicating that Rosi has no effect on activation of JNK by LPS (**Figure 2D**).

PPARγ ligands inhibit NF-κb and AP-1 signaling pathways in LPS-treated peritoneal macrophages, although the mechanisms are unclear [18, 20]. Whether or not such mechanisms are active in whole liver is unknown. We examined the effects of Rosi on LPS-induced activation of AP-1 and NF-κb by EMSA (**Figure 2E**). Interest-

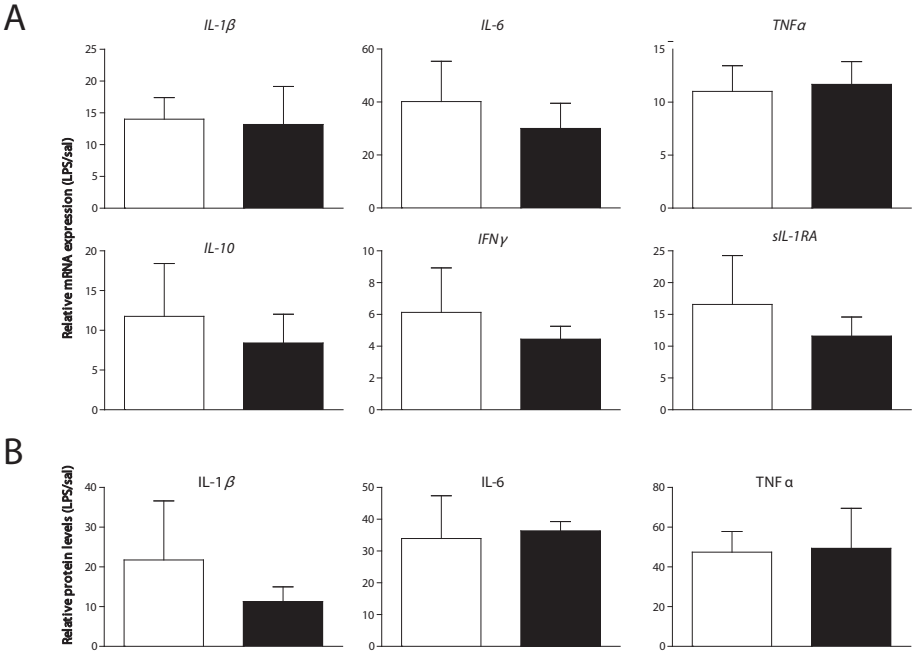

Figure 3. ***Rosiglitazone has no effect on the induction of cytokines by LPS.*** C57BL/6 male mice were gavage-fed 50 mg/kg/d of Rosi or corn-oil for 3 days. On day 3, the animals were IP injected with 2 mg/kg body weight of Salmonella LPS or saline and livers were harvested after 4 hours (n=5 per group). (A) RNA was isolated from the livers and analyzed by TaqMan real-time PCR. All data were presented as mean (+SD) and standardized for cyclophilin RNA levels. The expression of the genes after LPS treatment is shown here. In case of vehicle pre-treatment, expression in saline-injected animals was set to 1, and fold change after LPS treatment was compared to vehicle controls. See supplemental information for primers and probes. (B) Plasma levels of the cytokines were determined by xMAP technology. In case of vehicle or Rosi pre-treatment, expression in saline-injected animals was set to 1, and fold change after LPS treatment was compared to vehicle or Rosi controls respectively.

ingly, Rosi inhibits AP-1 activation, but had no effect on NF-κB activation in the presence of LPS, distinguishing the effects of Rosi in isolated macrophages from whole liver.

Rosi does not affect induction of hepatic cytokines by LPS

Rosi inhibits the expression of pro-inflammatory cytokines in cultured macrophages and in isolated KCs [18, 20, 22]. We hypothesized that the anti-inflammatory effects of Rosi *in vivo* were due to either impaired KC activity, or direct hepatocellular targeting. Compared to Veh/LPS, there was no reduction in IL-1β, TNFα, IL-6 and IFNγ RNA levels in Rosi/LPS mice. The induction of the anti-inflammatory cytokine, IL-10 and the secreted form of the IL-1 receptor antagonist (sIL-1Ra) by LPS was also not affected by Rosi (**Figure 3A**). Plasma protein levels of the cytokines IL-1β, TNFα and IL-6 were significantly increased after LPS, but were not affected by Rosi (**Figure 3B**). Thus, the effect of Rosi on LPS-mediated hepatic gene expression is not mediated by alteration of cytokine RNA and protein levels, but likely may be due to inhibition of inflammation-mediated signaling within hepatocytes.

Rosi attenuates cytokine-mediated nuclear export of RXRα in cell culture

LPS administration to mice or cytokine treatment of HepG2 cells leads to rapid nuclear export of RXRα [444, 361]. Since Rosi did not affect induction of cytokines by LPS (**Figure 3**), we explored potential direct hepatocellular mechanisms by utilizing IL-1β-treated HepG2 cells, which models the negative effects of inflammation on RXRα-regulated gene expression [15]. IL-1β treatment of HepG2 cells resulted in rapid JNK-mediated nuclear export of RXRα, while short-term (30 min) pre-treatment with Rosi inhibited this export (**Figure 4A**). Thus, Rosi inhibits IL-1β-mediated nuclear export of RXRα in HepG2 cells, in agreement with the *in vivo* results. This was confirmed by immunofluorescent analysis of HepG2 cells (**Figure 4B**), where RXRα was detected in the cytosol after IL-1β treatment (Veh/IL-1β versus Veh/Sal). RXRα remains in the nucleus in Rosi/Sal-treated cells, indicating that Rosi by itself does not affect RXRα protein levels in the nucleus. RXRα was not detected in the cytosol in IL-1β-treated cells, which has been pre-treated with Rosi (Rosi/IL-1β), indicating that Rosi pre-treatment blocked nuclear export of RXRα by IL-1β.

IL-1β-induced JNK activation contributed to reduced nuclear levels and activity of RXRα with the consequent down-regulation of target gene expression [14, 15]. Rosi did not attenuate IL-1β-induced phosphorylation of JNK, or its substrate, c-jun, indicating that Rosi affects IL-1β mediated RXRα nuclear export without altering activation of the JNK pathway (**Figure 4C**).

The fate of RXRα after nuclear export is unknown, but likely involves proteasome-mediated degradation in the cytosol 30. In HepG2 cells, IL-1β treatment led to reduced RXRα levels in whole cell extracts, which was significantly reversed by pre-incubation with Rosi (**Figure 4D**). These inhibitory effects on proteasome-

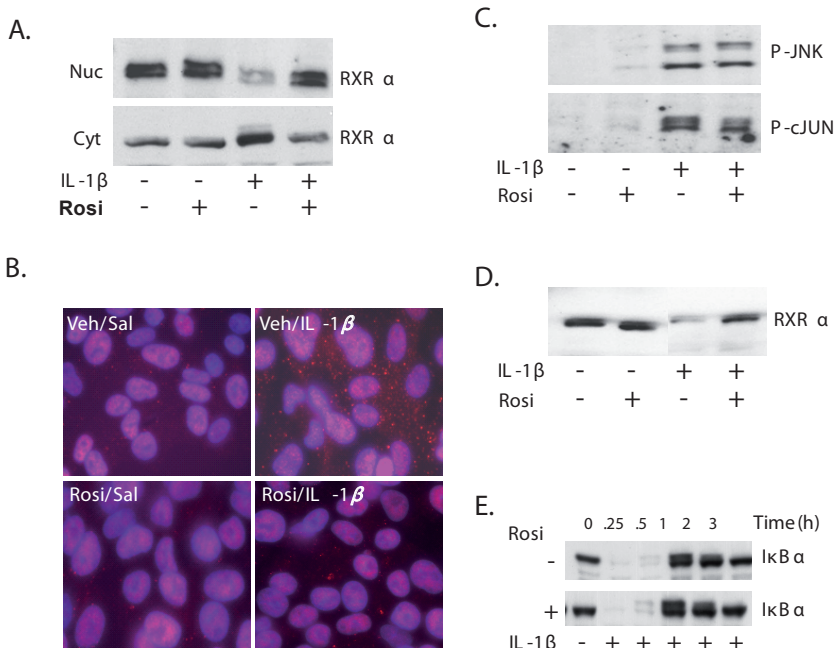

Figure 4. ***Rosiglitazone attenuates IL-1β -mediated RXRα nuclear export and degradation in vitro.*** HepG2 cells were pre-treated for 30 minutes with 10 μM Rosi or DMSO vehicle, followed by treatment with IL-1β (10 ng/ml) or vehicle control (0.0001% BSA in PBS) for 30 minutes. (A) Nuclear (Nuc) and cytosolic (Cyt) extracts were analyzed by immunoblotting with antibodies to RXRα to determine the effects of Rosi on subcellular localization of RXRα in the presence of IL-1β. (B) Immunofluorescence analysis of saline or IL-1β-treated HepG2 cells, pre-treated with vehicle or Rosi. The cells were stained with Alexa-Fluor-labeled antibody detecting RXRα, DAPI-staining of the nuclei, and the merged images are shown. (C) Nuclear protein levels of P-JNK (upper panel) or P-c-JUN were determined at 30 and 60 minutes of IL-1β treatment, preceeded by pre-incubation with Rosi. (D) Whole cell extracts (WCEs) were probed with RXRα antibodies to determine the effect of Rosi on protein levels of RXRα in total cell extracts, after IL-1β treatment. (E) Total cell lysates were prepared from HepG2 cells treated with DMSO or Rosi, prior to treatment with IL-1β from 0 – 3 hours. The samples were analyzed by immunoblotting with IκBα antibodies.

dependent degradation of RXRα is unlikely due to a global, non-specific interference of proteasomal activity by Rosi, since Rosi had no effect on IL-1β-dependent degradation of prototypic proteasome target, IkBa 840 (**Figure 4E**). These results demonstrate that Rosi prevents cytokine-mediated nuclear export of the central NR, RXRα, sequestering it in the nucleus, resulting in attenuation of suppression of hepatic genes during inflammation.

Discussion

Induction of the negative hepatic APR by inflammation is characterized by suppression of hepatic genes, resulting in broad defects in liver function. Effective treatment for inflammation-induced pathogenesis of liver diseases is lacking, and warrants continued exploration into novel mechanisms for therapeutic intervention. Agonists for PPAR family members have anti-inflammatory properties, although any role for direct effects on hepatocyte function is unknown. This study demonstrates that the PPARγ agonist, Rosiglitazone, attenuates the effects of inflammation on hepatic gene expression. The protective action of Rosi is in part mediated by blocking inflammation-mediated nuclear export of RXRα, the common and essential heterodimer partner for type II NRs [10]. Rosi pretreatment of mice led to a marked inhibition of the suppressive effects of LPS on target gene expression in liver, correlating with a mechanism involving retention of RXRα in the nucleus and maintenance of nuclear binding activities of RXRα-containing heterodimer pairs. Rosi had no effect on cytokine expression, suggesting a direct effect on cytokine-mediated cell signaling events in hepatocytes rather than the possibility of an indirect action by inhibiting cytokine-production by KCs. In IL-1β-treated HepG2 cells, short-term exposure to Rosi markedly attenuated IL-1β-mediated nuclear export of RXRα. Taken together, Rosi potentially and directly interferes with inflammation-based cell signaling pathways in liver cells.

One component of our initial working hypothesis was that Rosi blocked KC activation by LPS. This appears to be a minor player, since liver cytokine RNA and serum cytokine levels were unchanged by Rosi pre-treatment, raising the possibility that Rosi can act directly on hepatocytes. Rosi blocked inflammation-mediated RXRα nuclear export both *in vivo* and *in vitro* (Figures 2 and 4) without affecting JNK activation, as illustrated by comparable levels of P-JNK and P-cjun regardless of the presence of Rosi. This suggests that there is an effect of Rosi on phospho-JNK targeting of RXRα, or on the RXRα protein itself, rendering it less accessible to phosphorylation by activated JNK. There are several possibilities, involving either PPARγ-dependent or PPARγ-independent mechanisms. The anti-inflammatory effects of Rosi are unlikely to require Rosi-activated PPARγ-dependent gene expression, since short-term 30 minute pre-treatment in HepG2 cells blocked IL-1β's effects on RXRα. Rosi's effects may still involve PPARγ, perhaps via Rosi-activated PPARγ sequestering of RXRα in PPARγ:RXRα heterodimers, altering RXRα's conformation, or the amount of non-dimerized RXRα in the nucleus accessible to activated JNK. Another possible role for Rosi and PPARγ involves direct association with other nuclear regulators, as has been reported whereby PPARγ associates with JunD in hepatic stellate cells to decrease JunD binding to the AP-1 site [31]. Rosi-liganded PPARγ may associate with c-fos, c-Jun, JunD or other AP-1-binding proteins, thus preventing one or more of these factors to bind to the AP-1 promoter element. Since P-cJun levels were comparable in Rosi/LPS and Veh/LPS nuclear extracts, PPARγ

binding to c-Jun is unlikely, however, association of PPARγ to other AP-1-binding factors remain to be explored.

There are several possible PPARγ-independent mechanisms for Rosi's anti-inflammatory actions [19, 32, 33]. In peritoneal macrophages derived from PPARγ conditional knockout mice, Rosi has both PPARγ-dependent and independent effects [34]. There is indirect evidence that some PPARγ-independent effects of Rosi might be mediated by the activation of PPARδ [39]. Further studies will determine roles for PPARγ in mediating the effects of Rosi.

Rosi attenuated the effects of LPS on hepatic genes without reducing expression of the cytokines IL-1β, TNFα and IL6. This was surprising, since PPARγ ligands inhibit production of inflammatory cytokines in monocytes and macrophages in culture, and macrophage-derived liver-resident KCs are the main producers of cytokines *in vivo* [18, 20]. Furthermore, a specific PPARγ agonist inhibited LPS-induced TNFα production in cultured rat KCs, although any effect on mouse KCs remains to be determined [22]. Although the entire array of cytokines was not evaluated, the lack of effect on these critical and sentinel cytokines involved in hepatic APR indicates that the mode of action of Rosi in attenuating the effects of LPS does not appear to be at the level of inhibiting non-parenchymal cell expression of cytokines, nor excess production of anti-inflammatory cytokines. The lack of effect on cytokine levels in LPS-treated mice might, overall, be a positive attribute to Rosi, since cytokine-activated pathways are involved in the hepatic regenerative response, which, in the setting of liver cell damage, should likely be preserved to enhance overall healing from injury.

Recent studies demonstrate that the therapeutic effects of PPARγ ligands are not limited to their use as insulin-sensitizers, as many of these agents have beneficial effects in conditions associated with cardiovascular diseases and inflammation [33]. Animal models of liver cell damage and fibrosis are attenuated with PPARγ agonists [35], while a pilot study in patients with steatohepatitis have shown that Rosi improved the histology and laboratory abnormalities associated with this disease [23, 36]. Given the current safety profile of this agent, it is tempting to consider Rosi and other PPARγ-agonists as potential anti-inflammatory agents for clinical trials in liver diseases where inflammation plays a role in pathogenesis.

Overall, we conclude that the PPARγ agonist, Rosi attenuates the effects of inflammation on hepatic gene expression by preventing the nuclear export of the central NR, RXRα. RXRα, as an obligate heterodimer with other class II NRs, regulates the expression of a broad array of genes involved in important physiological processes in the liver, many of which are impaired during the negative hepatic APR. Thus, Rosi may have a role in counteracting the pathophysiology of inflammation in chronic and acute liver disease.

Acknowledgements

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CHAPTER 4

The LXR-agonist T0901317 attenuates endotoxin-induced changes in hepatobiliary transporter gene expression in mice

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Abstract

Background & Aims: During sepsis-associated cholestasis, circulating endotoxins (e.g. LPS) activate resident liver macrophages, Kupffer cells, leading to pro-inflammatory cytokine production, activation of hepatocellular cell-signaling cascades and rapid suppression of hepatobiliary transporter expression. We sought to determine if LPS-associated suppression of transporter genes in mice could be attenuated by T0901317, a liver X receptor (LXR) agonist, recently shown to exert anti-inflammatory actions in LPS-stimulated peritoneal macrophages.

Methods: In vivo, mice were gavage-fed T0901317 (1-100mg/kg/day) for three days prior to intraperitoneal LPS injection. In vitro, mouse and rat primary hepatocytes were treated with TNFα or IL-1β after pre-treatment with varying doses of T0901317. Transporter and cytokine expression were assessed by real-time PCR and immunoblotting, plasma cytokine levels by Luminex assay, and nuclear transcription factors by EMSA and immunoblotting.

Results: In vivo, T0901317 attenuated LPS-induced suppression of 3 transporter genes--Na/taurocholate co-transporting polypeptide (Ntcp), multidrug resistance-associated protein 2 (Mrp2) and Mrp3, while up-regulation of multidrug resistance gene 1b (Mdr1b) was blocked. These effects were differentially reflected in protein expression with partially preserved Mrp2. Surprisingly, cytokine expression was not reduced by T0901317 pre-treatment, suggesting that T0901317 has direct anti-inflammatory activities in hepatocytes. In vitro, cytokine-induced suppression of Mrp2 expression, and nuclear NF-κB binding, were both attenuated by T0901317 pre-treatment.

Conclusions: LXR agonist T0901317 attenuates the effects of LPS on hepatic transporter gene expression *in vivo*, most likely via direct, cytokine-independent signaling pathways in hepatocytes. LXR agonists may provide a novel and effective means to reduce the deleterious consequences of hepatic inflammation.

Introduction

Sepsis-associated cholestasis is a frequently occurring clinical phenomenon in both adults and children, particularly in premature infants [1-3]. The putative mechanism underlying this inflammation-induced cholestasis involves endotoxin-mediated (e.g., LPS) activation of cell signaling pathways, primarily in the resident liver macrophages, commonly referred to as Kupffer cells. LPS-activated Kupffer cells produce pro-inflammatory cytokines, including TNFα, IL-1β and IL-6. These, in turn, activate hepatocellular signaling pathways that change expression of many genes involved in bile formation, mainly affecting those centrally responsible for hepatobiliary transport [4]. In rodent models, endotoxemia leads to down-regulation of bile acid transporters at RNA and protein levels, as well as to alterations of their subcellular localization and functional activity [5]. Among the best studied hepatobiliary transporters that respond to endotoxemia at transcriptional and post-transcriptional levels are the basolateral Na^+/taurocholate co-transporting peptide (Ntcp, Slc10A1), the canalicular bile salt exporting protein (Bsep, Abcb11) and the multi-drug resistance related protein 2 (Mrp2, Abcc2) [6-14]. The latter is primarily involved in transport of di-anionic bile salt metabolites and glutathione [15, 16], whose secretion into the canalicular lumen along with that of bile acids exported by Bsep provides the driving force for bile formation in rodents.

A central role of Kupffer cells and their release of pro-inflammatory cytokines in the cascade leading to reduced transporter gene expression has been shown in various rodent models of sepsis-associated cholestasis. In vivo, inactivation or depletion of Kupffer cells not only reduced whole liver cytokine expression and secretion [17], but also attenuated the hepatocellular responses to endotoxin challenge [12, 18-20]. Moreover, treatment of primary hepatocytes with medium derived from LPS-treated Kupffer cells mimicked *in vivo* LPS-responses, while LPS itself had no apparent direct effect on hepatocytes, while addition of anti-cytokine antibodies to conditioned medium led to reduction of hepatocellular LPS-responses [12]. This indicates that cytokines are indeed key mediators of this process, which was confirmed by *in vivo* studies in several models, in which administration of anti-TNFα and anti-IL-1β antibodies reduced LPS-induced effects on transporter expression and nuclear binding activity of key transcription factors [21, 22]. Conversely, treatment of rodents with TNFα or IL-1β mimicked the effects of LPS treatment [6, 21, 23].

Better understanding of the inflammatory cascade leading to cholestasis has provided several potential targets for intervention. These include members of the nuclear receptor (NR) superfamily, which have been shown to serve as key regulators of hepatic transporter gene expression and to be centrally involved in inflammatory pathways [24-27]. Two type II members of the NR superfamily have recently been identified as potential anti-inflammatory targets—the peroxisome proliferator-activated receptor (PPAR)-γ and the liver X receptor (LXR). Recent evidence suggests that rosiglitazone, an agonist of the PPARγ (Nr1c3), acts as an anti-inflammatory agent

in liver, and can interfere with endotoxin-mediated suppression of hepatobiliary transporter genes [28]. LXR has two isoforms, LXRα and LXRβ (Nr1h3 and Nr1h2, respectively). LXRα is primarily expressed in the liver, intestine, kidney and adipose tissue, while LXRβ is ubiquitously expressed, albeit at a lower level [29]. The natural ligands for LXR are oxysterols and LXR is primarily involved in control of cholesterol and fatty acid homeostasis, as it serves as a sterol sensor with feed-forward characteristics. Upon activation, LXR enhances the reverse transport of cholesterol, cholesterol esterification and biliary cholesterol excretion, as well as conversion of cholesterol to bile acids in rodents [30].

Recently, it was found that activation of LXR has potent anti-inflammatory effects in macrophages in mouse models of atherosclerosis and dermatitis [31, 32]. Furthermore, Joseph et al. showed that LPS injection in LXRα/β double knock-out mice led to increased hepatic expression of the genes encoding inducible nitric oxide synthase (iNOS) and TNFα, as well as to increased plasma levels of IL-6 [31]. Considering the crucial role of Kupffer cells, the resident hepatic macrophage population, in the pathogenesis of inflammation-associated cholestasis and that these cells express both LXR isoforms in rats [33], we regarded LXR a potentially attractive target for pharmacological intervention in the inflammatory cascade leading to cholestasis. In this study, we examined the effects of the synthetic LXR agonist, T0901317, as an anti-inflammatory agent in liver, employing both *in vivo* and *in vitro* models of sepsis-associated cholestasis.

Materials and methods

Animals and treatments

Eight week old, male C57BL/6 (Charles River, Laboratories Inc., Wilmington, MA) mice were housed at our facility at constant room temperature, humidity and light-dark cycle, and had free access to both water and standard rodent chow (Picolab Rodent Diet 20, Purina Mills, St. Louis, MO) throughout experiments. Animal protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

Mice were administered T0901317 (1-100 mg/kg/d, Cayman Chemical, Ann Arbor, MI) or equi-volume amounts of vehicle (corn oil) by gavage for three consecutive days. Over this range of doses, T0901317 had previously been shown to dose-dependently increase liver phospholipid transfer protein in mice [34]. Fifteen minutes after the third dose, mice were injected intraperitoneally with a non-lethal dose (2 μg/g bodyweight) of LPS (Salmonella typhimurium, Sigma-Aldrich, St. Louis, MO) or vehicle (0.9% saline) alone, as described previously [35]. Mice were sacrificed at indicated time-points after brief inhalation anesthesia by isoflurane. Livers were excised, weighed and snap-frozen in liquid nitrogen. Blood was collected at time of sacrifice in EDTA-containing vials. Liver tissue and plasma were stored at -80°C until further use.

Primary hepatocyte culture

Primary hepatocytes were isolated from mouse and rat using an in situ collagenase-perfusion method. Mouse and rat hepatocytes were isolated according to protocols described previously [36, 37] and purified using multiple centrifugation steps (purity > 99%, viability > 80%). Mouse hepatocytes were plated on Primaria plates (BD Biosciences, Franklin Lakes, NJ) and rat hepatocytes on collagen-coated plates in William's medium E containing fetal bovine serum (10%), penicillin/streptomycin (100 U/L), glutamine/gentamycin (50 mg/L). After 3-4 hours, medium was changed to serum-free medium. After 16-24 hours, medium was changed again. After 90 minutes, cells were pre-treated with T0901317 (0.01-20 μM) or vehicle (DMSO, 0.25% final concentration) alone for 30-60 minutes, after which TNFα (20 ng/ml) or vehicle (0.1% bovine serum albumin in phosphate buffered saline) alone was added. Cells were harvested at indicated time-points.

Quantitative Real-Time PCR

RNA was isolated from frozen mouse livers using the RNeasy Midikit (Qiagen, Valencia, CA) according to manufacturer's instructions with the addition of on-column RNase-free DNase digestion. RNA was isolated from cultured hepatocytes using Tri-reagent (Sigma, St. Louis, MO) according to manufacturer's instructions. RNA-concentrations were assessed by spectrophotometry or RiboGreen RNA Quantitation kit (Molecular Probes, Inc., Eugene, OR). RNA-integrity was confirmed by gel electrophoresis. RNA was reversed transcribed using the PROSTAR First Strand RT-PCR kit (Stratagene, La Jolla, CA) or Moloney-murine leukemia virus reverse transcriptase (Sigma) according to manufacturers' instructions. cDNA was stored at -20°C until further usage. RNA-expression levels were determined using Taqman real-time PCR method with the ABI Prism 7000 Gene Detection system (Applied Biosystems, Foster City, CA) as described previously [28, 35]. Target gene expression was normalized to cyclophilin expression for each individual sample. Primer and dual-labeled probes (5'-FAM, 3'-TAMRA) were synthesized by Sigma Genosys (The Woodlands, TX), Synthegen (Houston, TX) or Eurogentec (Seraing, Belgium). Sequences are shown in Tables 1a (mouse) and 1b (rat). Primers/probe sets for cyclophilin and iNOS were newly designed using Primer Express software (Applied Biosystems). Those sets not shown in Table 1 were obtained directly from Applied Biosystems (Assay-on-Demand).

Plasma analysis

Plasma levels of IL-1β, IL-6 and TNFα were determined simultaneously using xMAP technology (Luminex Corporation, Austin, TX) with a commercially available kit (Linco Research, St.Charles, MO).

Table 1 Primers and probe sequences for mouse (A) and rat (B) genes

Table 1a. Mouse primers and probe sequences

Gene	Acc. nr.	Forward primer	Reverse primer	Probe	Ref.
Cyclophilin	XM_130275	CAGATCGAG-GGATCGATTCAG	TCACCACTTGACAC-CCTCATTC	CTCCTCCACATTGGA-GACAAGAGATGCA	
Ntcp	AB003303	ATGACCACCTGCTC-CAGCTT	GCCTTTGTAG-GGCACCTTGT	CCTTGGGCATGAT-GCCTCTCCTC	72
Bsep	NM_021022	CTGCCAAGGAT-GCTAATGCA	CGATGGCTAC-CCTTTGCTTCT	TGCCACAGCAATTT-GACACCCTAGTTGG	72
Mrp2	NM_013806	GCTGGGAGAAAT-GGAGAATGTC	GACTGCTGAG-GGACGTAGGCTA	TGGGCATATCAC-CATCAAGGGCTCC	73
Mrp3	BC048825	TCCCACTTTTCG-GAGACAGTAAC	ACTGAGGACCTT-GAAGTCTTGGA	CACCAGTGTCAT-TCGGGCCTATGGC	74
Mdr1b	M14757	GCTGGACAAGCT-GTGCATGA	TGGCAGAATACT-GGCTTCTGCT	CTTCCCCTCTTGATGCT-GGTGTTTGGAAAC	75
Srebp1c	NM_011480	GGAGCCATGGATT-GCACATT	CCTGTCTCAC-CCCCAGCATA	CAGCTCATCAACAAC-CAAGACAGTGACTTCC	72
IL-1b	NM_008361	CAACCAACAAGT-GATATTCTCCATG	GATCCACACTCTC-CAGCTGCA	CTGTGTAATGAAAGACG-GCACACCCACC	76
TNFa	NM_013693	CATCTTCTCAAAAT-TCGAGTGACAA	TGGGAGTAGACAAG-GTACAACCC	CACGTCGTAGCAAAC-CACCAAGTGGA	76
sIL-1Ra	M57525	CTCCTTCTCATCCT-TCTGTTTCATT	GCATCTTGCAG-GGTCTTTTCC	AGAGGCAGCCT-GCCGCCCTT	28

Table 1b. Rat primers and probe sequences

Gene	Acc. nr.	Forward primer	Reverse primer	Probe	Ref.
Cyclophilin	NM_031793	CAGATCGAG-GGATCGATTCAG	TCACCACTTGACAC-CCTCATTC	CTCCTCCACATTGGA-GACAAGAGATGCA	
Mrp2	NM_012833	GACGACGATGAT-GGGCTGAT	CTTCTCATGGCCAAG-GAAGCT	CCCACCATGGAG-GAAATCCCTGAGG	77
iNOS	NM_012611	CTATCTCCATTCTAC-TACTACCAGATCGA	CCTGGGCCTCAGCT-TCTCAT	CCCTGGAAGAC-CCACATCTGGCAG	

Elecreophoretic mobility shift assay (EMSA)

Nuclear extracts from liver tissue were prepared using a method described by Timchenko et al. [38] with some modifications [28]. Nuclear extracts from primary hepatocyte cultures were prepared using the method described by Hoppe-Seyler et al. [39] with some modifications [40]. Protein concentration of nuclear extracts was determined using BCA protein assay (Pierce, Rockford, IL). EMSAs were performed using 5-10 µg of nuclear extract protein according to the previously described protocol [28]. Briefly, nuclear extracts were incubated with 2.5 x 10^4 cpm of radiolabeled probe. For competitor-studies, a 100-fold excess of cold specific and non-specific oligonucleotides were added immediately preceding the addition of the probe. Samples were incubated on ice for 30 minutes and electrophoresed through a non-denaturing 6% polyacrylamide gel at 4°C. Gels were dried on filter paper at 80°C for 35-40 minutes and autoradiographed or analyzed using a phosphorimager (Cyclone, PerkinElmer, Boston, MA).

Immunoblotting

Liver total membrane fractions were prepared using a protocol modified from Klett et al.[41]. Briefly, ~100mg of liver tissue was homogenized in ice-cold buffer (5mM Hepes, 250mM sucrose, pH 7.4) containing protease-inhibitors (Complete (Roche, Almere, the Netherlands), 1mM PMSF, 1mM DTT) and phosphatase-inhibitors (1mM activated Na3VO4, 1mM NaF). After 15 minute incubation, homogenates were centrifuged at 200 x g during 5 minutes. The supernatant was re-centrifuged at 1,500 x g for 5 min. The supernatant of this second spin was then subjected to ultracentrifugation at 100,000 x g during 60 minutes. The resulting pellet was subsequently resuspended in lysis-buffer (10mM Tris, 2mM EDTA, 2mM EGTA, 1.0% Triton X-100, 0.5M NaCl, 0.25% deoxycholate) with protease- and phosphatase-inhibitors, incubated for 30 minutes on ice and finally centrifuged at 16,000 x g for 10min. The resulting supernatant containing the solubilized membranes was snap-frozen and stored at -80°C until further use. Whole cell lysates were prepared from primary hepatocytes using the following protocol. Hepatocytes were washed with ice-cold PBS and incubated with the same lysis buffer described above for 30 minutes on ice. Plates were scraped, lysates were spun 20,000 x g for 5 minutes and supernatants were used for further analysis. Protein content of total membrane fractions and cell lysates was determined by BCA protein assay.

Twenty-five µg of membrane fractions or cell lysates were separated by SDS-PAGE (7.5% or 12% gel respectively), transferred to a nitrocellulose membrane and incubated with antibodies for Ntcp[42], Mrp2[43] (both gifts from Dr. Bruno Stieger, University Hospital, Zurich), IκBα (sc-371, Santa Cruz Biotechnology, Santa Cruz, CA) or phospho-NF-κB p65 (#3031, Cell Signaling Technology, Beverly, MA), followed by species-specific secondary antibodies. Detection was performed either Tropix substrate (Applied Biosystems) and the Image Station 2000R (Kodak, Rochester, NY) or SuperSignal West Pico substrate (Pierce) and the Gel-Doc XR (Bio-Rad, Hercules, CA)

Statistical analysis

Statistical analysis of results was performed using SPSS 14.0 (SPSS Inc., Chicago, IL). Data are reported as mean ± S.D. Due to the relatively small sample-sizes, non-parametric tests were used: Kruskal-Wallis H-test followed by pair-wise comparisons with the Mann-Whitney test. P-values <0.05 were considered significant.

Results

Pre-treatment with T0901317 attenuates LPS-effects on hepatic gene expression in mice in a dose-dependent and gene-specific manner

We determined the effects of T0901317 (1-100mg/kg/day) on LPS-mediated regulation of 5 hepatobiliary transporter genes and the LPS/LXR responsive gene encoding the sterol regulatory element binding protein-1c (Srebp1c). Alterations in basal RNA levels by LPS of each of these genes were attenuated to variable degrees, in a dose-dependent fashion, for each individual gene.

Among the most severely affected genes was Ntcp. Eight hours after LPS administration, Ntcp gene expression was profoundly suppressed (to ~13% of control levels) (**Figure 1A**), while pre-treatment with T0901317 significantly attenuated this LPS-induced suppression in a dose-dependent manner. Ntcp gene expression rose to 40-45% of control (vehicle-treated) levels at the highest doses of T0901317 (50 and 100 mg/kg/day).

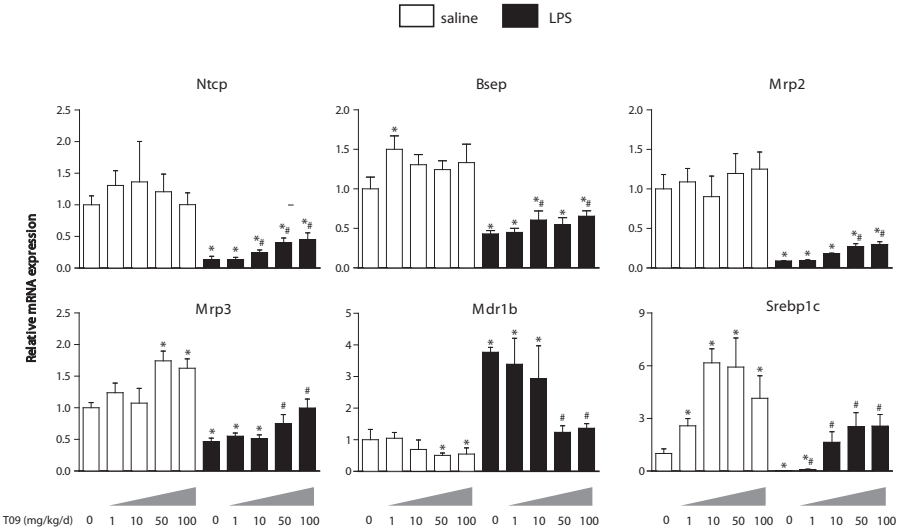

Figure 1. ***T0901317 attenuates LPS-induced alterations of hepatic transporter and Srebp1c RNA levels.*** Mice were treated with T0901317 (1-100 mg/kg/d) or vehicle alone for 3 days. Fifteen minutes after the third dose, mice received LPS (2 μg/g BW) or saline alone by intraperitoneal injection. Mice were sacrificed after 8 hours. RNA expression was determined by Taqman quantitative real-time PCR and normalized to cyclophilin gene expression for each individual animal (N = 4 per group). Vehicle/saline expression was set to 1. Error bars denote SD. Differences were analyzed pair-wise using the Mann-Whitney test: * $P < 0.05$ vs. vehicle/saline; #, $P < 0.05$ vs. vehicle/LPS (only analyzed vs. other LPS-groups).

Mrp2, Mrp3 (Abcc3) and Bsep gene expression were all suppressed by LPS-treatment, albeit to different extents (**Figure 1B-D**). Mrp2 expression showed a similar pattern as that of Ntcp, i.e. LPS-induced suppression to 8% of control levels, which rose more than 3-fold to 30% in mice pre-treated with the highest dose of T0901317. (**Figure 1C**.) Mrp3 gene expression on the other hand returned to control levels at the highest T0901317 dose. This, however, appeared to be the result of a substantial direct effect of T0901317 on Mrp3 gene expression (**Figure 1D**).

Interestingly, T0901317 exhibited anti-inflammatory effects on hepatic genes activated by LPS. T0901317 pre-treatment prevented up-regulation of the expression of the multi-drug resistance-1b (Mdr1b, Abcb1) gene. Mdr1b, one of two rodent homologues of the human MDR1 (ABCB1) gene, is up-regulated after LPS-treatment of rats [10, 44]. LPS-treatment led to a 4-fold up-regulation of Mdr1b expression in mice, which was dose-dependently reduced by T0901317 pre-treatment, to the point that Mdr1b expression was indistinguishable from control levels at doses of 50 and 100 mg/kg/day (**Figure 1E**). Interestingly, higher doses of T0901317 also significantly reduced Mdr1b gene expression in saline-injected animals, suggesting that its basal expression is regulatable by this compound.

Taken together, these results indicate that T0901317 interferes with the inflammatory signaling that leads to altered hepatic gene expression during endotoxemia in a dose-dependent and gene-specific manner.

Pre-treatment with T0901317 distinctly affects LPS-mediated suppression of Ntcp and Mrp2 protein levels

To determine whether the effects of T0901317 pre-treatment on LPS-altered hepatic transporter gene expression were also reflected by changes at the protein level, Mrp2 and Ntcp protein abundances were analyzed using immunoblotting. LPS-treatment led to suppressed expression of both transporter proteins, although the degree of LPS-induced suppression of protein expression is somewhat less than that seen for each transporter gene's RNA (**Figure 2**) .

Mrp2 protein levels (**Figure 2**, bottom panel) are induced by T0901317 pre-treatment and this effect persists in LPS-treated mice, indicating that the transcriptional effects are translated to the protein level. On the other hand, Ntcp protein levels are reduced by T0901317 pre-treatment both in saline-injected and LPS-injected mice (**Figure 2**, top panel). Thus, the higher Ntcp gene expression in T0901317-pre-treated LPS-injected mice compared to LPS-injected controls does not correspond to higher Ntcp protein levels, suggesting that post-transcriptional effects are at play. It appears that T0901317 has minimal effect on basal Ntcp and Mrp2 gene expression, but leads to changes in Ntcp and Mrp2 protein levels that suggest a distinct, post-translational target of action of T0901317. Thus, the combined effects on Ntcp and Mrp2 RNA and protein levels by T0901317 in the LPS model are complex, but appear to work favorably together to enhance adaptation.

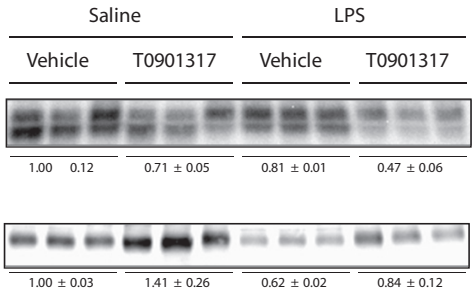

Figure 2. ***T0901317 attenuates LPS-induced suppression of Mrp2 protein levels, but decreases Ntcp protein levels.*** Mice were treated with T0901317 (100 mg/kg/d) or vehicle alone for 3 days. Fifteen minutes after the third dose, mice received LPS (2 μg/g BW) or saline alone by intraperitoneal injection. Mice were sacrificed after 8 hours. Protein expression was determined by immunoblotting. Relative band densities are given as mean ± SD (N = 3 per group) compared to vehicle/saline group.

Treatment of mice with T0901317 leads to increased liver weight

Liver weight increased after T0901317-treatment compared to vehicle-treatment in a dose-dependent manner. Liver weight in vehicle-treated mice was 51.4 mg liver/g bodyweight, while liver weight in mice treated with 100mg/kg T0901317 was 87.6 mg liver/g bodyweight (both groups saline-injected, $P < 0.05$). This agrees with previous reports and most likely related to enhanced lipogenesis [45-47]. Of note, liver weight was not different between saline and LPS-injected mice receiving the same dose of T0901317.As anticipated, T0901317 treatment led to the induction of the established LXR target gene, Srebp1c (**Figure 1F**), a key regulator of de novo fatty acid synthesis [48].Thus, mice pre-treated with T0901317 appear to follow the expected physiological effects of this compound, indicated that the livers of these mice were sufficiently enriched with T0901317.

Attenuation of LPS-effects by T0901317 is <u>not</u> mediated by reduced hepatic cytokine expression

Given the role for cytokine-induced pathways in suppressing transporter gene expression in liver, and the reported suppression of macrophage-mediated cytokine gene expression by LXR agonists in cell culture, it was relevant to first determine if the effects on transporter gene expression were potentially mediated by *in vivo* effects of T0901317 on liver cytokine gene expression. Thus, gene expression of 6 LPS-induced cytokines was measured at both 4 (peak) and 8 hours after LPS in livers from mice pre-treated with either vehicle or T0901317.

Surprisingly, hepatic gene expression of IL-1β, TNFα and IFNγ was elevated to similar levels in vehicle and T0901317 pre-treated mice at both 4 (**Figure 3A, C, D**) and 8 hours after LPS-injection (data not shown) even at doses that clearly reduced effects of LPS-signaling on transporter gene expression. Moreover, LPS-induced

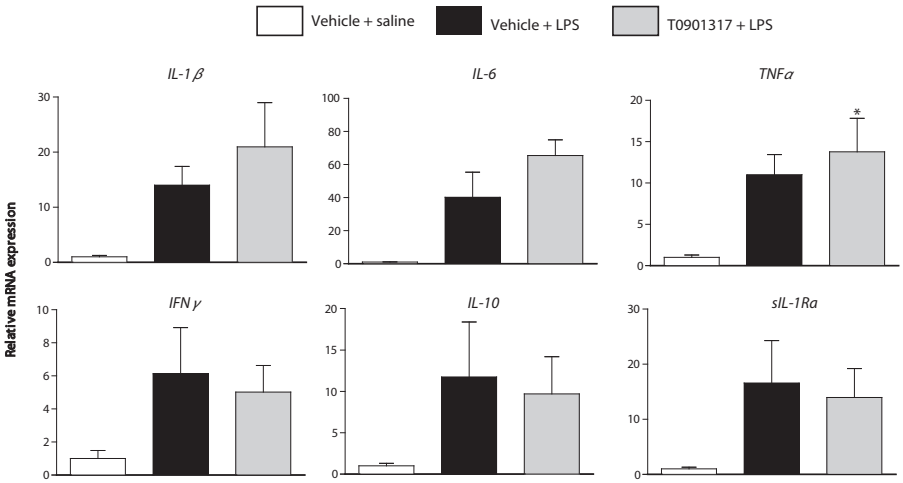

Figure 3. ***T0901317 does not reduce LPS-induced cytokine RNA levels.*** Mice were treated with T0901317 (50mg/kg/d) or vehicle alone for 3 days. Fifteen minutes after the third dose, mice received LPS (2 μg/g BW) by intraperitoneal injection or saline alone and were sacrificed after 4 hours. Total RNA was isolated from liver tissue and reverse transcribed. Cytokine gene expression was determined by Taqman quantitative real-time PCR and normalized to cyclophilin gene expression for each individual animal. N=5 per group. Vehicle/saline expression was set to 1. Error bars denote SD. Differences were analyzed pair-wise using the Mann-Whitney test. Expression levels of all cytokines were significantly higher in both LPS-injected groups than in the saline-injected group. *, $P < 0.05$ vs. vehicle/LPS.

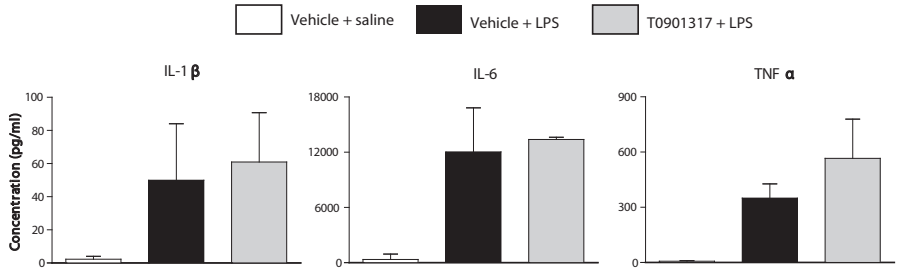

Figure 4. ***T0901317 does not reduce LPS-induced plasma levels of pro-inflammatory cytokines.*** Mice were treated with T0901317 (50mg/kg/d) or vehicle alone for 3 days. Fifteen minutes after the third dose, mice received LPS (2 μg/g BW) by intraperitoneal injection or saline alone. Mice were sacrificed after 4 hours and blood was collected. Cytokine (IL-1β, TNFα, IL-6) levels were determined by Luminex assay. N=5 per group. Vehicle/saline expression was set to 1. Error bars denote SD. Differences were analyzed pair-wise using the Mann-Whitney test. Expression levels in both LPS-injected groups were significantly higher than in the saline-injected group.

IL-6 expression was even higher in T0901317-pre-treated mice compared to vehicle-treated controls (**Figure 3B**). Of note, T0901317 alone did not lead to changes in cytokine gene expression in saline-injected animals (data not shown).

To address potential effects through anti-inflammatory cytokines or modulation of pro-inflammatory cytokine action, we also determined expression of the anti-inflammatory cytokine IL-10 and the secreted form of the IL-1 receptor antagonist (sIL-1Ra) (**Figure 3E-F**). The latter was recently shown to be up-regulated in THP1-monocytic cells by PPARγ-agonists [49]. Gene expression of both IL-10 and sIL-1Ra were, however, not affected by T0901317 pre-treatment.

Although hepatic cytokine gene expression after LPS-treatment has previously been shown to correspond well with actual cytokine release [17], we also measured serum cytokine protein levels as a more physiologically relevant metric of Kupffer cell activation. Analysis of plasma levels of IL-1β, IL-6 and TNFα showed that plasma levels of these cytokines corresponded well with their hepatic gene expression levels and that T0901317 did not reduce circulating protein levels of these three pro-inflammatory cytokines (**Figure 4A-C**). Hence, the effects of T0901317 on hepatic transporter gene expression cannot be attributed to effects on cytokine levels in these mice. Taken together, these results suggest that T0901317 does not exert its anti-inflammatory effects through changes in hepatic cytokine expression—rather, these effects appear to occur either through other inflammatory mediators or, perhaps, via direct effects on hepatocytes.

T0901317 blocks cytokine-induced alterations of primary hepatocyte gene expression

To examine the down-stream consequences of the direct hepatocellular anti-inflammatory effects of T0901317, we determined its effects on transporter gene expression in primary rodent hepatocytes after treatment with TNFα or IL-1β. In primary rat hepatocytes, an well-studied model of Mrp2 gene regulation [12, 50], TNFα and IL-1β suppressed Mrp2 gene expression by 44% and 32%, respectively, while pre-treatment with T0901317 dose-dependently reversed this (**Figure 5A, B**). This effect, however, may also in part be the result of direct induction of Mrp2 by T0901317 *in vitro* or a repression of endogenous activation of cell signaling pathways in cultured primary hepatocytes, since Mrp2 expression in rat hepatocytes pre-treated with T0901317 alone was also increased. Expression of iNOS, another inflammation-regulated gene, was highly induced after cytokine treatment. This induction was strongly and dose-dependently reversed by T0901317 (**Figure 5C, D**), indicating that T0901317 inhibits inflammatory signaling in hepatocytes.

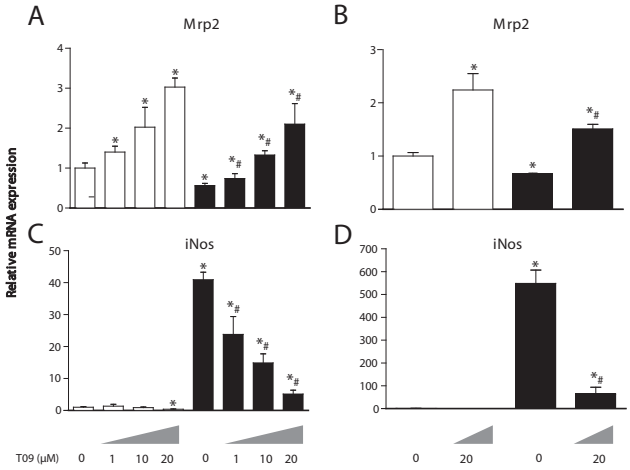

Figure 5. ***T0901317 attenuates effects of pro-inflammatory cytokines TNFα and IL-1β on primary rat hepatocyte Mrp2 and iNOS gene expression.*** Primary rat hepatocytes were treated with T0901317 (1-20 μM) for 1 hour prior to treatment with TNFα (20 ng/ml) or IL-1β (10 ng/ml). Cells were harvested after 12 hours. RNA isolation and cDNA synthesis were performed as described in Materials and Methods. Mrp2 (A, B) and iNOS (C, D) gene expression was determined by real-time quantitative RT-PCR. Target gene expression levels were normalized to cyclophilin expression levels for individual samples. Vehicle/saline expression was set to 1. Error bars denote SD. Differences were analyzed pair-wise using the Mann-Whitney test: * $P < 0.05$ vs. vehicle/saline; #, $P < 0.05$ vs. vehicle/cytokine (only analyzed vs. other cytokine-treated-groups).

T0901317 reduces TNFα-induced NF-κB binding activity in primary mouse hepatocytes

To explore possible direct anti-inflammatory hepatocellular effects of T0901317, we examined its effects on TNFα-treated hepatocytes. Since NF-κB is involved in the inflammation-mediated induction of both Mdr1b gene expression *in vivo* (**Figure 1E**, [51]) and iNOS gene expression by cytokines (**Figure 5**, [52, 53]), we focused our studies on T0901317's effects on TNFα induction of NF-κB. GW3965, a potent synthetic LXR agonist, has previously been shown to have LXR-dependent anti-inflammatory effects in cultured mouse macrophages, mediated by interference with NF-κB signaling [31, 54], yet the precise mechanism of action remains unknown. Thus, using the model of cultured mouse hepatocytes, TNFα-treatment led to increased nuclear NF-κB binding activity, which was substantially inhibited by short-term pre-treatment with T0901317 (**Figure 6A**). Interestingly, pre-treatment with T0901317 also led to slightly reduced binding activity in cultured hepatocytes not stimulated with TNFα. The effect of T0901317 on NF-κB binding by TNFα is dose-dependent (**Figure 6B**). Neither dissociation of NF-κB from a complex with the inhibitor of κB-α (IκBα), nor NF-κB phosphorylation, was targeted by T0901317

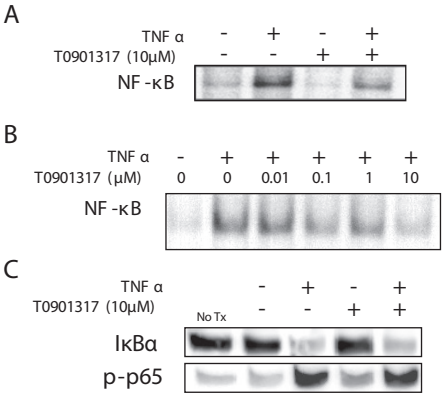

Figure 6. ***T0901317 dose-dependently inhibits TNFα-mediated induction of NF-κB binding activity in primary hepatocytes.*** Primary mouse hepatocytes were treated with T0901317 for 30 minutes prior to treatment with TNFα (20 ng/ml). Nuclear extracts were prepared after 8 hours. Nuclear extract protein was incubated with a radiolabeled oligonucleotide containing a consensus NF-κB binding site, electrophoresed through a 6%-non-denaturing polyacrylamide gel and imaged using a phosphorscreen. A representative assay of three experiments is shown. Effect of T0901317 pre-treatment of hepatocytes on (LPS-induced) NF-κB binding (A) and its dose-dependency (B), immunoblots with whole cell lysates with IκBa degradation and p65 phosphorylation (C). N.B. "No Tx" represents lanes with nuclear extracts from hepatocytes not treated with either vehicle (DMSO) or saline.

signaling (**Figure 6C**). Thus, there is a T0901317-mediated reduction of the availability of NF-κB for binding to its cognate DNA recognition sites, perhaps by altering the macromolecular transcription complexes in the nucleus in ways to ultimately mediate its transrepressive and gene regulatory effects [55].

Discussion

This study establishes the ability of the LXR-agonist, T0901317, to attenuate effects of endotoxemia on hepatic transporter gene expression in mice. This capacity of T0901317 to modulate the effects of LPS is dose-dependent and gene-specific. This protective effect is (in part) reflected at the protein level as well. Surprisingly, it appeared that the actions of the LXR-agonist were not mediated by reduction of cytokine gene expression or secretion into the bloodstream of treated mice. Coincident with the attenuation of LPS' effects on transporter gene expression, was a reduction in NF-κB signaling in liver. This suggests that, at least in part, the anti-inflammatory properties of T0901317 work directly in hepatocytes, perhaps by T0901317-LXR directed suppression of the macromolecular transcriptional machinery, including those involved in NF-κB activation.

The experimental set-up used allowed us to directly examine the potential of T0901317 as an anti-inflammatory agent in liver, since the consequences of a single

dose of LPS are primarily due to endotoxemia per se without extensive confounding due to initiation of adaptive mechanisms within hepatocytes. Furthermore, the relatively low doses of LPS used in this study did not lead to major hepatocellular damage, indicated by the lack of elevated alanine aminotransferase levels (ALT) (data not shown).

The most surprising result from this study was that the attenuation of LPS-induced changes in transporter gene expression by T0901317 occurred without affecting either gene expression or secretion of pro-inflammatory cytokines IL-1β, IL-6 and TNFα. The level of attenuation of inflammation-mediated gene expression by this NR agonist is substantially similar to the degree of inhibition mediated by the PPARγ-agonist rosiglitazone on LPS-mediated suppression of transporter genes, although the mechanism of action of these two NR ligands are distinct, since the T0901317 compound, unlike rosiglitazone, did not alter the LPS-mediated subcellular localization of RXRα (data not shown) [28]. Together, these data strongly suggest that neither NR ligand works through inhibiting non-parenchymal cell cytokine expression, but via direct, and likely distinct, intracellular targets within hepatocytes. Although implied by work in macrophage cell culture, it appears that the effects of both NR ligands seen *in vitro*, do not correlate with effects *in vivo* on Kupffer cells. These results reveal potential novel opportunities to interfere with sepsis-associated cholestasis, and inflammation-mediated effects on liver function as a whole.

Previously, LXR had been shown to interfere with NF-κB signaling in cultured macrophages [31, 54, 56]. Suppression of inflammation-induced matrix metalloproteinase-9 (Mmp9) gene expression by LXR-agonists T0901317 and GW3965 was found to be mediated by inhibition of NF-κB activity. This suppression was absent in macrophages derived from LXRα/β null-mice [54]. In contrast to our data, these authors did not find reduced NF-κB binding activity upon LXR-activation. We can only speculate on the cause of this discrepancy, but it may be related to differences in the cell-types studied (macrophage vs. hepatocyte), the type of inflammatory stimulant used (LPS vs. TNFα) and duration of treatment. The doses of T0901317 used in our *in vitro* experiments were higher that those used by Castrillo et al. [54], but corresponded to actual hepatic concentrations after oral dosing [57].

The molecular mechanisms underlying the interaction between LXR and NF-κB signaling remain to be elucidated. Multiple mechanisms could be at play, for instance competition for co-factors as suggested by Terasaka et al. [56] or induction or regulation of additional mediators. The latter concept is supported by two recent publications showing that LXR-agonists induce factors that are able to enhance macrophage survival in different settings [58, 59]. LXR-activation could perhaps also affect expression of anti-inflammatory mediators in a similar fashion. Thirdly, LXR could also directly interact physically with NF-κB. This principle had already been described to be the mechanism underlying the anti-inflammatory effect of PPARγ [60], but was very recently shown to be effective in LXR-mediated suppression of inflam-

matory signaling in cultured macrophages as well by Ghisletti et al. [55]. Transrepression of inflammatory gene expression was shown to occur by ligand-dependent SUMOylation of PPARγ and LXR and subsequent inhibition of co-repressor complex removal and thus inhibition of gene expression [55]. It was also shown that, although the general principle of transrepression by LXR and PPARγ may be comparable, the actual SUMOylation pathways are different [55]. The latter may also explain (in part) differential effects of T0901317 and rosiglitazone on hepatic transporter gene expression after LPS [28]. Whether or not T0901317 induced SUMOylation of LXR is playing a role in altering the broad transcriptional programs induced by LPS remains to be determined.

There are few *in vivo* studies on the mechanistic targets for anti-inflammatory effects of LXR agonist but, recently, Wang et al. [61] reported that pre-treatment of rats with GW3965 attenuated liver injury induced by co-administration of LPS and peptidoglycan. This effect was associated with reduced TNFα plasma levels, which appears to contradict our findings. However, co-administration of LPS and peptidoglycan is known to cause extensive liver injury as can be deduced from the profound increase in ALT levels as well as histological evidence of focal hepatocellular injury [61]. Notably, as in our studies with T0901317, GW3965 pre-treatment did not affect serum IL-6 and IL-10 levels in this study, suggesting that the *in vivo* anti-inflammatory effects of LXR agonists may be more diverse and distinct form those identified in cell culture studies 61.

Multiple studies have shown that structurally unrelated LXR-ligands, both natural and synthetic, possess anti-inflammatory effects in macrophages as well as keratinocytes and that these are dependent on LXR expression [31, 32, 54]. Preliminary results using the LXR-agonist GW3965 showed that this compound also inhibits iNOS up-regulation in hepatocytes. However, cytokine-induced Mrp2 suppression was not reversed by GW3965, which suggests that agonist functionality of T0901317 and GW3965 are distinct when it comes to Mrp2 regulation or that T0901317 affects Mrp2 expression through LXR-independent mechanisms. Activation of the pregnane-X-receptor (PXR) by T0901317 may be the likely contributor to this effect since Mrp2 is a known PXR target [62] and it was recently shown that T0901317, but not GW3965, can activate both LXR and PXR [63, 64].

In addition, T0901317 has also been implicated as potential agonist of the farnesoid-X-receptor (FXR) [65]. It is, however, unclear whether FXR-activation played a role in the current study: while Bsep expression was modestly induced by T0901317, expression of the small heterodimer partner (SHP), another classical target gene of FXR, was actually suppressed (data not shown). Thus, it is not likely that FXR is a relevant target of T0901317.

One intriguing finding is the reduction of TNFα-induced NF-κB DNA binding in cells treated with T0901317 and the question whether or not this is related to its anti-inflammatory effects in the absence of altered hepatic cytokine expression. Since

many of the hepatic transporter genes are themselves activated by RXR-containing heterodimers [22], one can envision several molecular targets in this pathway where reduced NF-κB signaling may directly or indirectly increase RXR-mediated gene expression. First, NF-κB and RXR bind to each other and mutually repress their abilities as transcriptional activators [66, 67] so that any potential sequestration of NF-kB would derepress any interactions with RXR. Indirectly, if T0901317 induces binding of SUMOylated LXR to corepressors , this may allow for coactivators to be more available for RXR-containing heterodimers. Investigations of either of these possibilities are avenues for future research.

The induction of hepatic steatosis by T0901317, previously described [45-47], probably precludes clinical use of this particular compound. The anticipated arrival of LXR-agonists with less pronounced lipogenic effects might allow for more specific modulation of inflammatory processes [68]. Moreover, if the apparent lack of effect at the level of Kupffer cells were due to pharmacokinetic mechanisms rather than an inherent insensitivity to T0901317, then targeting of LXR-agonists to these cells would be a feasible alternative to circumvent steatotic effects. Wang et al. [61] showed that activation of isolated rat Kupffer cells can indeed be suppressed by the other synthetic LXR-agonist GW3965.

Cholestasis can be the result of inflammatory signaling, yet cholestatic liver disease by itself can also be considered as the cause of recurrent endotoxemia [2, 4]. This indicates that inflammation and cholestasis are intertwined phenomena and implies that treatments aimed at suppressing the response to endotoxemia may also be beneficial in cholestatic liver disease in general. Previously, various strategies have been employed in attempt to modulate the hepatic inflammatory response cascade leading to cholestasis. These were aimed at different levels of this cascade and included, amongst others, Kupffer cell depletion or inactivation [12, 18-20], use of glucocorticoids to inhibit cytokine secretion [13, 69, 70], and administration of anti-cytokine antibodies [21, 22]. Although of use in experimental settings, this has of yet not led to clinically effective and accepted therapies in addition to removal of endotoxin-sources by effective antibiotic treatment. Therefore, there remains a dire need for anti-inflammatory treatments that are effective and safe under cholestatic conditions. Intriguingly, Uppal et al. recently found a protective role of LXR against bile acid toxicity and cholestasis per se [71]. Although this was found to be limited to female mice for yet to be clarified reasons, it suggests that it may actually be LXR-agonists that fulfill this role of the desired anti-cholestatic and anti-inflammatory agent.

In conclusion, the presented study shows that the synthetic LXR-agonist T0901317 possesses anti-inflammatory characteristics that attenuate the effects of endotoxemia on hepatic transporter gene expression in mice, most likely through a Kupffer cell-independent mechanism. This is also partially reflected by altered transporter protein expression. This suggests that alternative routes can be taken in attempt to modulate the hepatic inflammatory response leading to cholestasis. Further optimi-

zation of treatment regiments, e.g. through the addition of other nuclear receptor modulators (e.g. rosiglitazone), the use of selective LXR-agonists or cell-specific targeting, may provide us with even more potent modulators of hepatic inflammation and ultimately lead to new treatment strategies for inflammation-induced cholestasis.

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CHAPTER 5

Liver X receptor agonist T0901317 attenuates the inflammatory response in primary rat Kupffer cells

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Abstract

Background/Aims: Kupffer cells (KCs), liver resident macrophages, play important roles in the pathogenesis of many liver diseases, including inflammation-induced cholestasis (IIC). Liver X receptor (LXR) can modulate the macrophage inflammatory response. LXR-agonist T0901317 has been shown to attenuate suppression of hepatobiliary transporter expression in an *in vivo* IIC-model (lipopolysaccharide (LPS)-injection in mice). T0901317, however, appeared to act preferentially on hepatocytes, which also led to the unwanted induction of lipogenesis. We, therefore, sought to determine whether KCs were responsive to anti-inflammatory effects of T0901317 *in vitro*.

Methods: Primary rat KCs were treated with T0901317 and LPS. Inflammatory responses were assessed by mRNA expression and protein secretion of several pro- and anti-inflammatory cytokines (Il-6, Il-1β, Tnfα, Il-10). Effects of T0901317 and LPS in KCs were compared to those in mouse macrophage cell-lines and those obtained with dexamethasone.

Results: T0901317 attenuated inflammatory response in KCs as well as in macrophage cell-lines of murine origin. In KCs, T0901317 consistently attenuated Il-6 response, but it was less potent than dexamethasone.

Conclusions: KCs are responsive to anti-inflammatory effects of T0901317 *in vitro*. KC-targeted LXR-activation is an attractive strategy to intervene in IIC by selectively modulating the KC inflammatory response, while avoiding unwanted lipogenic effects of LXR-activation in hepatocytes.

Introduction

Kupffer cells (KCs) play important roles in liver (patho)physiology [1, 2]. As the liver resident macrophages, KCs provide an effective barrier against potentially harmful threats coming directly from the gastrointestinal tract or from elsewhere in the body. KCs are also important in prevention and repair of liver injury [3, 4] and in liver regeneration [1]. However, KCs may act as double-edged swords, since their activation can also have detrimental effects on the liver. This is evident from KC involvement in the pathogenesis of a spectrum of conditions, e.g., alcoholic liver disease [5], ischemia-reperfusion injury [6], fibrosis [4], hepatotoxicity and subsequent tumor progression/growth [7].

KCs are also involved in the pathophysiology of inflammation-induced cholestasis (IIC) [8, 9]. IIC is a frequently occurring clinical phenomenon, e.g. in the setting of sepsis [10]. Both *in vivo* and *in vitro* studies have demonstrated that KC activation by circulating endotoxins or other inflammatory mediators leads to the production and release of the pro-inflammatory cytokines. These, in turn, will act on hepatocytes, ultimately leading to the reduced expression and function of hepatobiliary transporters that contribute to the inflammation-induced reduction in bile flow, i.e. cholestasis [8-10].

Progress in unraveling molecular mechanisms contributing to IIC has not yet led to effective treatment strategies. Considering their important role in the pathogenesis of IIC, KCs appear as attractive targets for pharmacological interventions. One plausible strategy is activation of the liver X receptor (LXR, Nr1h3/2), which has been identified as modulator of inflammatory response in macrophages [11-13] and is expressed in KCs [14].

Previously, we have demonstrated the protective effects of LXR-agonist T0901317 in an animal model of IIC, i.e., lipopolysaccharide (LPS)-injected mice (Mulder et al. (unpublished data)). T0901317 attenuated LPS-induced changes in transporter expression, but appeared to act at least in part KC-independently, since cytokine production and release were not reduced upon treatment. Unfortunately, this apparently preferential effect of T0901317 on hepatocytes led to unwanted induction of lipogenesis. One plausible strategy to interrupt the inflammatory cascade by LXR-activation while avoiding hepatocellular lipogenic effects, would be KC-targeting provided that KCs would be responsive to LXR-agonists.

To directly address the responsiveness of KCs to the anti-inflammatory actions of T0901317, we now examined the effects of T0901317 *in vitro* in LPS-treated primary rat KCs and compared these to those in two mouse macrophage cell-lines, i.e. IC-21 and RAW264.7. Our results indicate that T0901317 selectively attenuates the LPS-induced inflammatory response in KCs with Il-6 induction being consistently suppressed. This indicates that KC-targeted LXR-activation may provide a new strategy to intervene in IIC, while avoiding unwanted hepatocellular effects of lipogenesis.

Materials and methods

Chemicals & Reagents

T0901317 was purchased from Cayman Chemical (Ann Arbor, MI). Lipopolysaccharide (Salmonella typhimurium), 9cis retinoic acid and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO). All cell culture supplies were purchased from Gibco/Invitrogen (Breda, the Netherlands) unless otherwise stated.

Animals

Male Wag/Rij rats (200-250g, Harlan, Horst, The Netherlands) and C57Bl6/J mice (20-25g, Charles River, Maastricht, the Netherlands) were housed at our facility at constant room temperature, humidity and light-dark cycle, and had free access to water and standard rodent chow. All procedures with animals were approved by the Animal Welfare Committee of the University of Groningen.

Cell culture

IC-21 and RAW264.7 cells were obtained from ATCC/LGC Promochem (Teddington, United Kingdom). IC-21 cells were cultured in modified RPMI1640 containing 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate, 4500mg/L glucose and 1500mg/L sodium bicarbonate (ATCC/LGC Promochem). RAW264.7 cells were cultured in RPMI1640 and Hepa1_6 cells in DMEM. All media contained penicillin/streptomycin (100U/ml and 100µg/ml respectively) and growth media contained 10% fetal bovine serum too (FBS, Gibco/Invitrogen).

Primary rat KCs were isolated under semi-sterile conditions after collagenase perfusion [15], followed by centrifugation and counterflow elutriation as described previously [16]. KCs were cultured in 24-well plates at a density of 0.75x106 cells per well in RPMI-1640 supplemented with 20% heat-inactivated FBS, 2mM L-glutamine, 100IU/ml penicillin, and 100µg/ml streptomycin. Primary rat hepatocytes were isolated and cultured as described in **Chapter 4**. Hepatocytes were plated on collagen-coated plates in William's medium E (WME) containing 10% FBS. Four hours after plating, plating medium was changed to serum-free WME.

All cells were cultured in a humidified 5% CO2/95% air atmosphere and 37°C environment.

Cell treatments

(Pre)treatment of primary cells was started approximately 24 hours after isolation. KCs and macrophage cell-lines were pre-treated with the different compounds for 0-24 hours before LPS-treatment. Doses of LPS used were 100ng/ml (RAW264.7) and 1µg/ml (IC-21 and KC), which had been shown to elicit a strong inflammatory response. At the end of treatments, medium was collected, centrifuged at 16,000 x g

for 5 minutes to remove cellular debris and stored at -80°C until further usage. After treatments and removal of medium, cells were washed twice with ice-cold PBS.

Conditioned medium experiments

In conditioned medium (CM) experiments, primary rat hepatocytes were treated with medium taken from KCs, IC-21 cells or RAW264.7 cells. Control experiments showed that partial replacement (½-½ v/v) of WME medium by either fresh RPMI or CM derived from unstimulated macrophages did not lead to an inflammatory response (as evidenced by stable expression of inducible nitric oxide synthase (iNos) gene).

Gene expression

Total RNA was isolated from cells or liver tissue using Tri-reagent (Sigma) according to manufacturer's instructions. RNA yield was determined using either RiboGreen RNA Quantitation kit (Molecular Probes, Inc., Eugene, OR) with RNA integrity being confirmed by gel electrophoresis, or using the RNA Nano Labchip with the Agilent 2100 Bioanalyzer (Agilent Technologies Netherlands, Amstelveen, the Netherlands). RNA was reverse transcribed using M-MLV reverse transcriptase (Sigma). cDNA was stored at -20°C until further usage. Gene expression was analyzed using Taqman real-time PCR method with the ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Expression of target genes was normalized to cyclophilin expression for each individual sample. Primer and dual-labeled probes (5'-FAM, 3'-TAMRA) were Eurogentec (Seraing, Belgium). Primer/probe sequences for Tnfα, Il-1β, Il-6 and mouse Lxrβ were newly designed using Primer Express software (Applied Biosystems) and are shown in Table 1. Other sequences used were published previously [17-22] or presented in **Chapter 4**.

Cytokine secretion

Medium Il-1β, Il-6, Tnfα and Il-10 concentrations were determined simultaneously using xMAP technology (Luminex Corporation, Austin, TX). Commercially available kits for mouse and rat samples were used according to manufacturer's instructions (Linco Research, St.Charles, MO).

Statistical methods

Statistical analysis of results was performed using SPSS 14.0 (SPSS Inc., Chicago, IL) and BrightStat (www.brightstat.com) [23]. Data are reported as mean ± S.D. Due to the relatively small sample-sizes, non-parametric tests were used: Mann-Whitney U-test (MW) or Kruskal-Wallis H-test followed by Conover pair-wise comparisons (Conover). P-values <0.05 were considered significant.

Table 1. Newly designed primer and probe sequences used for Taqman real-time PCR.

Gene	Species	Acc nr	Forward primer	Reverse primer	Probe
Tnfα	M/R	NM_013693 (M) NM_012675 (R)	GTA GCC CAC GTC GTA GCA AAC	AGT TGG TTG TCT TTG AGA TCC ATG	CGC TGG CTC AGC CAC TCC AGC
Il-1β	M	NM_008361	ACC CTG CAG CTG GAG AGT GT	TTG ACT TCT ATC TTG TTG AAG ACA AAC C	CCC AAG CAA TAC CCA AAG AAG AAG ATG GAA
	R	NM_031512	ACC CTG CAG CTG GAG AGT GT	TTG ACT TCT ATC TTG TTG AAG ACA AAC C	CCC AAA CAA TAC CCA AAG AAG AAG ATG GAA AAG
Il-6	M/R	NM_031168 (M) NM_012589 (R)	CCG GAG AGG AGA CTT CAC AGA	AGA ATT GCC ATT GCA CAA CTC TT	ACC ACT TCA CAA GTC GGA GGC TTA ATT ACA
Lxrβ	M	NM_009473	AAG GAC TTC ACC TAC AGC AAG GA	GAA CTC GAA GAT GGG ATT GAT GA	CTT CCA CCG TGC AGG CTT GCA G

M = mouse, R = rat

Results

KCs and mouse macrophage cell-lines express Lxr/Rxrα and respond similarly to LXR-activation and LPS.

We compared nuclear receptor (NR) mRNA expression, effects of T0901317 treatment and the inflammatory response in primary KCs to two different mouse macrophage cell-lines, i.e., IC-21 and RAW264.7. Both cell-lines have previously been used as *in vitro* substitutes for Kupffer cells [24-26], while RAW264.7 cells have been shown to be responsive to the anti-inflammatory effects of LXR-agonists [11, 12].

Basal mRNA expression of NRs Lxrα, Lxrβ and retinoid X receptor-α (Rxrα) were measured in KCs and RAW264.7 cells (**Figure 1A**). To account for the fact that these cells were derived from different species (KCs rat, RAW264.7 mouse), mRNA expression in cells was compared that in whole liver tissue. Similar expression patterns were observed for KC and RAW264.7 cells relative to whole liver expression (Lxrβ > Lxrα > Rxrα), but levels were higher in primary KCs. Of note, the absolute mRNA expression of Lxrα in KC and RAW264.7 cells is actually higher than that of Lxrβ (~8-fold). The discrepancy between the relative and absolute expression levels is due to the fact that Lxrα is the predominant Lxr-isoform in hepatocytes [27], which constitute the majority of cells in the liver [28].

To determine whether T0901317 is able to activate LXR in the three cell-types, we assessed the expression of Abcg1 in T0901317-treated Kupffer cells and macrophages. ABCG1/Abcg1 has been shown to be an LXR-target in both human and mouse macrophages [29, 30]. In all three cell-types, T0901317 induced Abcg1 expression (**Figure 1B**) indicating that T0901317 entered the cells in sufficient amounts to activate an LXR-target gene.

Next, we compared the inflammatory response in the three cell-types. **Figure 1C-D** shows a similar Tnfα-response to LPS for the three cell-types. The inflammatory response was also similar when examined down-stream of macrophages and KCs, using conditioned medium (CM) to mimick the *in vivo* setting.

We focused on the genes iNos and multidrug resistance associated protein (Mrp)-2, whose expression has been shown to be sensitive to pro-inflammatory cytokines (**Chapter 4**). CM derived from LPS-treated KC, IC-21 and RAW264.7 cells led to increased expression of iNos (**Figure 1E**) and a decreased expression of Mrp2 (**Figure 1F**). Of note, the responses elicited by CM derived from LPS-treated cells exceeded those of control-CM supplemented with LPS, indicating that the similarity of these effects are not merely due to direct effects of LPS on the primary hepatocytes.

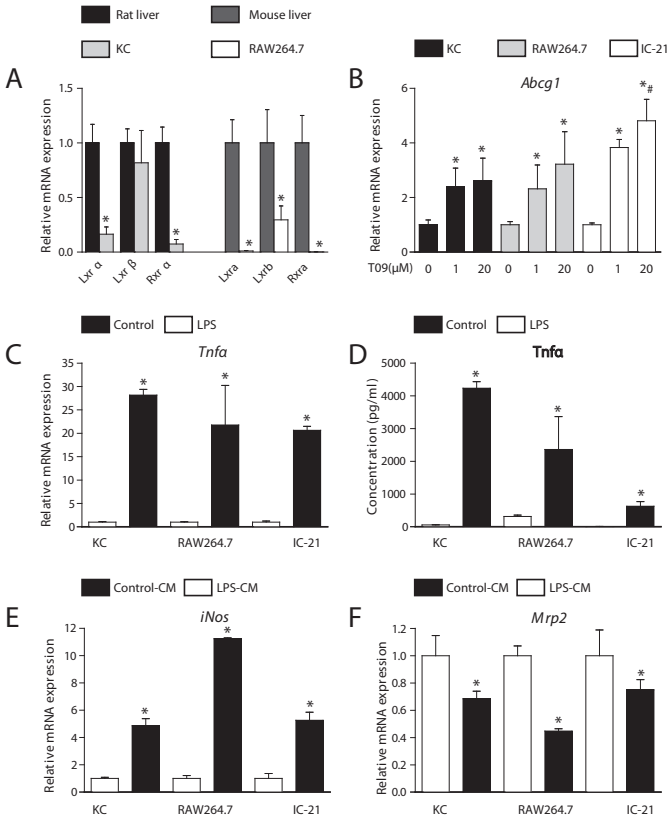

Figure 1. ***KCs and mouse macrophage cell-lines express Lxr/Rxrα and respond similarly to LXR-activation and LPS.*** *(*A) Basal NR mRNA expression in KCs and cell-lines was compared by real-time PCR. Expression levels are expressed relative to whole liver expression in the appropriate species. *P<0.05 cell vs. liver (MW). (B) KCs and cell-lines were treated with LXR-agonist T0901317 (T09) for 24 hours and expression of LXR-target gene Abcg1 was measured. *P<0.05 vs. 0μM T09, #P<0.05 vs. 1μM T09 (Conover). (C) Tnfα mRNA expression and (D) Tnfα protein secretion by KCs and cell-lines after LPS-treatment for 3 hours. *P<0.05 vs. control (MW). (E) iNOS and (F) Mrp2 mRNA expression in primary rat hepatocytes treated with KC or cell-line derived conditioned medium. *P<0.05 vs. control-CM (MW). Data are presented as mean ± SD.

These results show that KCs can respond similarly to mouse macrophage cell-lines with regard to T0901317 and LPS. Reciprocally, data indicate that these cell-lines are suitable substitute for KCs to optimize experimental conditions to study anti-inflammatory properties of T0901317.

LXR-agonist T0901317 attenuates inflammatory gene response in IC-21 and RAW264.7 cells

Initially, we analyzed the effects of T0901317 on the inflammatory response of IC-21 and RAW264.7 cells. Since mRNA expression of all three cytokines was highly induced in IC-21 and RAW264.7 cells at three hours after LPS administration (data not shown), we used this time point for our experiments. Based on preliminary experiments, cells were pre-treated for 20-24 hours with different doses of T0901317. Pre-treatment with T0901317 dose-dependently inhibited mRNA expression of Il-6 and Il-1β at 3 hours after LPS-treatment in both IC-21 and RAW264.7 cells, while Tnfα was only inhibited in RAW264.7 cells (**Figure 2A-C**). Of note, pre-treatment with T0901317 at 10μM resulted in an intermediate level of inhibition compared 1μM and 20μM.

The effects of T0901317 on LPS-induced cytokine mRNA expression were in part translated into reduced cytokine secretion: at 24 hours after LPS-treatment, Il-6 and Il-1β concentrations in medium of LPS-treated RAW264.7 cells were markedly suppressed by T0901317 pre-treatment (**Figure 3**). Of note, despite reduced Tnfα mRNA expression after three hours, medium Tnfα concentrations were not different after 24 hours. These results indicate that T0901317 has anti-inflammatory effects in these macrophage cell-lines, but that these effects are cytokine-specific and cell-line-dependent.

T0901317 pre-treatment differentially modifies Kupffer cell inflammatory response and much less potently than dexamethasone

Pre-treatment of KCs with T0901317 according to the scheme used for the cell-lines differentially reduced cytokine mRNA expression and secretion (**Figure 4A-C**). LPS-induced Il-6 mRNA expression and secretion was reduced after pre-treatment with low dose T0901317, but this suppressive effect was less when a high dose was used (**Figure 4A**). Unlike that of Il-6, Il-1β mRNA expression was not affected by T0901317, but its secretion slightly reduced with a higher dose T0901317 (**Figure 4B**). While Tnfα mRNA expression was reduced only at low dose T0901317, this was not translated into reduced secretion (**Figure 4C**). The cause for the discrepancy between cytokine mRNA expression and secretion of Il-1β and Tnfα is unclear, but may reflect effects of T0901317 on other (post-transcriptional) processes.

To determine both the sensitivity of KC to anti-inflammatory signaling and to gauge the potency of T0901317 as an anti-inflammatory agent, we compared the effects of T0901317 to those of dexamethasone, a well-known and widely used anti-

inflammatory agent. Pre-treatment with dexamethasone led to a much stronger suppression of both LPS-induced mRNA expression and secretion of the three cytokines (**Figure 4A-C**), indicating that KC are sensitive to anti-inflammatory treatment.

In summary, T0901317 has anti-inflammatory effects in KC, but these effects are cytokine-selective and much less potent compared to dexamethasone. T0901317 predominantly affects Il-6 expression and secretion.

T0901317 minimally enhances LPS-induced Il-10 secretion by Kupffer cells

The anti-inflammatory potential of T0901317 in KC can also be examined by determining the secretion of anti-inflammatory mediators, e.g., Il-10. Il-10 is a well-known anti-inflammatory cytokine and its induction is considered as one of the anti-inflammatory effects of glucocorticoids [31]. Pre-treatment of KC with T0901317 only led to a minimal increase in Il-10 secretion, while at high dose T0901317 did not affect Il-10 secretion (**Figure 5**). Dexamethasone, on the other hand, led to strong induction of Il-10 secretion. These results again indicate that the T0901317 has cytokine-selective effects in KCs.

RXRα-ligand 9cRA acts synergistically with T0901317 in suppressing LPS-induced Il-6 mRNA expression in IC-21 cells but does not increase maximal inhibition

Since T0901317 was less potent compared to dexamethasone, we examined whether addition of a ligand of RXRα, the obligate heterodimer partner for class 2 nuclear receptors [32], could enhance the effects of T0901317 to achieve effects comparable to dexamethasone. RXRα-ligands have previously been shown to possess anti-inflammatory effects in primary Kupffer cells [33, 34]. The two compounds indeed acted synergistically in attenuating the upregulation of Il-6 mRNA expression in IC-21 cells, while 9cRA alone was not effective (**Figure 6**). The maximal inhibition of the induction of Il-6 mRNA expression was, however, not greater than that of high dose T0901317 alone.

This indicates that addition of 9cRA to T0901317 potentiates the effects of T0901317 needed, but that there is a maximal achievable level of inhibition, which remains lower than that of dexamethasone. Hence, we did not further pursue this approach in KCs.

Discussion

This study shows that primary KCs are responsive to the anti-inflammatory effects of the LXR-agonist T0901317. Pre-treatment with T0901317 reduced pro-inflammatory cytokine expression and secretion by KCs, but these effects were cytokine-selective and Il-6 expression was most consistently affected. These results indicate that KC-targeted LXR-activation by T0901317 may provide a new strategy

to intervene in the cascade leading to IIC, while avoiding undesired hepatocellular LXR-activation.

In this study, we used two different mouse macrophage cell-lines to compare the effects of T0901317 in KCs. Both IC-21 and RAW264.7 cells had been used previously as *in vitro* KC substitutes [24-26] and were shown to behave similarly to KCs in our hands. Additional experiments showed that these three cell-types not only responded similarly to T0901317 and LPS, but also to other physiological relevant stimuli (e.g., bile acids (data not shown)), and that their relative cytokine secretion profile was strikingly similar to that seen *in vivo* (Il-6 > Tnfα >> Il-1β) [35]. Although KCs and macrophage cell-lines behave similarly in CM-experiments, this approach could unfortunately not be employed to examine the downstream functional effects of inhibition of the KC inflammatory response by T0901317 due to the carry-over of T0901317 in CM to primary hepatocytes. As the latter have previously been shown to be responsive to the anti-inflammatory actions of LXR-agonists [36] [and **Chapter 4**], the carry-over precludes CM-experiments with hepatocytes that are not both Lxrα and Lxrβ-deficient, to analyze the functional effect of KC inhibition.

Our current results strongly suggest that the lack of effects of T0901317 on KC inflammatory response seen *in vivo* was not due to an intrinsic unresponsiveness of KCs to the anti-inflammatory effects of T0901317. Although the cause of the preferential effect on hepatocytes remains to be determined and may be due to the pharmacokinetics properties of T0901317, our results imply that KC-targeted LXR-activation is an attractive strategy to explore. Using this strategy the hepatic inflammatory response could potentially be modulated, while avoiding the unwanted lipogenic effect of LXR-activation in hepatocytes [37, 38], which remains the major hurdle in the therapeutic application of LXR-agonists [39]. Various pharmacological tools could be employed to achieve KC-targeted LXR-activation, e.g. through packaging in large (multilamellar) liposomes [40] or coupling to mannosylated albumin [41].

During the course of this study, Wang et al. reported on the beneficial anti-inflammatory effects of a different synthetic LXR-agonist, i.e., GW3965, in *in vivo* and *in vitro* models of hepatic inflammation [42]. Although these authors used a different *in vivo* model (intravenous administration of a different LXR-agonist to rats followed by treatment with LPS and peptidoglycan), they found that GW3965 affected cytokine expression patterns in KCs *in vitro* selectively too [42]. This cytokine-selective effect of LXR(-activation) was previously shown in whole mouse liver [11] and primary peritoneal macrophages [13]. Despite the recent discovery of the anti-inflammatory actions of ligand-activated LXR (i.e., though inhibition of the co-repressor release a repressed inflammatory gene promoter [43], the molecular basis for the observed selectivity remains to be elucidated. Although one might expect a more universal effect of LXR-agonists on inflammatory signaling, one has to bear in mind that the inflammatory response is extremely complex and involves many different intracellular routes as well as auto-/paracrine processes with specific temporal character-

istics. The net anti-inflammatory effect of LXR-activation *in vivo* may therefore be unpredictable.

KC-derived Il-6 has been shown to be an initiator of the acute phase response [44] as well as a controller of this response and as such protect the liver against chronic injury [45, 46]. Il-6 may thus be the effector molecule that makes KCs act as double-edged swords. Il-6 is generally considered to be involved in the pathophysiology of IIC as was shown in both septic (e.g., LPS) and aseptic (e.g., turpentine) models [47-49]. Considering the consistent effects of T0901317 on Il-6 expression and secretion by KCs *in vitro*, KC-targeted T0901317 is an attractive therapeutic strategy, at least in the setting of acute inflammation. This, obviously, remains to be confirmed *in vivo*.

Over the last decade, several NRs were shown to have anti-inflammatory properties [9]. Amongst these NRs is, besides LXR, the peroxisome proliferator activated receptor (PPAR)-γ (Nr1c3), which was [50, 51]. Although original studies were mainly performed in primary macrophages aimed at modulation of atherosclerotic processes, agonists of these NRs have since then been used in attempt to modulate a whole range of other conditions in which macrophages appear to play a central role in the pathogenesis, such as IIC [9]. We previously showed that PPARγ-agonist rosiglitazone attenuates inflammation-induced suppression of RXRα-dependent gene expression [52].

In conclusion, primary KCs are responsive to the anti-inflammatory effects of LXR-agonist T0901317, albeit cytokine-selectively. Along with previous studies showing the efficacy of LXR-agonists in various animal models of inflammatory conditions as potentially effective compounds, this provides further support to continue to explore and optimize the therapeutic application of LXR-agonists in these conditions. Considering the importance of KCs in the pathogenesis of a wide-spectrum of liver diseases, KC-targeting of LXR-agonists may provide us with a new and elegant method to intervene in these disease-states, while limiting adverse effects associated with hepatocellular LXR-activation.

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CHAPTER 6

Dysregulation of biliary cholesterol secretion during inflammation in mice

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In preparation

Abstract

Background: Inflammation-induced cholestasis (IIC) is frequently observed. Pro-inflammatory cytokines play key roles in IIC pathophysiology by activating various signaling pathways leading to altered expression of hepatocellular transporters. Limited information is available about the regulation of biliary cholesterol secretion during inflammation. We aimed to determine whether inflammation-induced suppression of the canalicular cholesterol halftransporters Abcg5/8 mRNA expression is associated with impaired biliary cholesterol secretion and if so, to elucidate and modulate underlying mechanisms.

Methods: Effects of inflammatory signaling on hepatic Abcg5/8 mRNA/protein expression and on basal and bile salt-stimulated bile formation were determined in lipopolysaccharide (LPS)-treated mice by real-time PCR, Western blotting and gallbladder cannulation with or without tauroursodeoxycholate (TUDCA) infusion, respectively. Mechanisms of altered mRNA expression were analyzed using promoter-reporter constructs containing the mouse or human Abcg5/8 intergenic region (IGR) transiently transfected into human hepatoma cell lines. DNA-binding activity of the liver X receptor (LXR) was assessed by electrophoretic mobility shift assay. In attempt to preserve Abcg5/8 mRNA expression, mice were pre-treated with LXR-agonist T0901317.

Results: LPS-treatment of mice led to a reduced Abcg5/8 expression and was associated with a reduced biliary cholesterol secretion under basal conditions and during TUDCA-stimulation. LPS lowered secretory rate maxima of bile salts and cholesterol. Promoter-reporter assays failed to identify a role for IGR in reduction of Abcg5/8 mRNA expression during inflammation. Indirect evidence for a mediating role of LXR was provided by reduced DNA-binding of nuclear extracts to the canonical LXR binding element. Pretreatment of mice with LXR-agonist T0901317 prior to LPS-injection led to (partially) preserved Abcg5/8 mRNA expression.

Conclusions: Inflammation leads to dysregulation of biliary cholesterol secretion. Although this process appears to be transcriptionally regulated and may involve impaired LXR-transactivation of Abcg5/8, the exact pathophysiological mechanism remains to be elucidated. LXR-activation may provide a means to reduce the inflammation-induced dysregulation of cholesterol secretion.

Introduction

Inflammation-induced cholestasis (IIC) is a frequently observed clinical phenomenon, particularly in children [1-3]. Pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, play key roles in the pathophysiology of IIC [1]. These cytokines can be produced locally by Kupffer cells, i.e., resident liver macrophages, upon their activation or reach the liver from the systemic circulation [4]. Upon binding to their respective receptors on hepatocytes, cytokines activate various signaling pathways leading to altered expression of hepatocellular transporters such as those for bile salts (sodium-taurocholate co-transporting polypeptide (Ntcp, Slc10a1) and bile salt export pump (Bsep, Abcb11), organic anion transport proteins (Oatps, Slc21a)), bilirubin (multidrug resistance-associated protein (Mrp)-2, Abcc2) and drugs/toxins (multidrug resistance P-glycoprotein (Mdr)-1b, Abcb1) [5].

Biliary cholesterol secretion is an important route for elimination of excess cholesterol from the body [6]. The identification of the heterodimeric transporter Abcg5/8 as a cholesterol transporter in 2000 provided an actual molecular mechanism for biliary cholesterol secretion [7, 8]. The genes encoding these two half-transporters are located on the human chromosome 2p21 [7, 8] and the murine chromosome 17 [9]. These genes are oriented in a head-to-head configuration and separated by a relatively short intergenic region (IGR), which acts as a bi-directional promoter in humans [10]. In mice, however, it is presumed not to act as a minimal promoter [9], although it appears to contain several putative transcription factor binding sites. Abcg5/8 mRNA expression is regulated by the oxysterol activated liver X receptor (LXR, Nr1h3/2) [11], but this does not appear to be directly mediated by a cis-acting LXR-response element in the IGR [7, 10].

Besides LXR, several other nuclear receptors (NRs), ligand-activated transcription factors, have been shown to modulate Abcg5/8 expression, including the liver receptor homologue-1 (Nr5a2) and the farnesoid X receptor (Nr1h4){12. More recently, Sumi et al. showed the importance of hepatocyte nuclear factor (HNF)-4α (Nr2a1) as a key regulator of ABCG/Abcg5/8 mRNA expression, acting in concert with GATA-transcription factors GATA4 and/or GATA6 [13]. This regulation of Abcg5/8 expression by HNF4α may also in part explain the tissue-specific expression of these half-transporters as HNF4α is an important transcription factor for hepatocyte and enterocyte differentiation [14, 15].

Currently, limited information is available about the regulation of canalicular cholesterol transport during inflammation. Khovidhunkit et al. reported a profound down-regulation of Abcg5/8 mRNA expression in mice upon lipopolysaccharide (LPS)-administration [16], but the pathophysiological relevance as well as the underlying mechanism remained to be elucidated. In the present study, we aimed to determine whether the suppressive effect of LPS on Abcg5/8 mRNA expression is also associated with a reduced biliary cholesterol secretion and if so, how transcriptional regulation of the genes might be affected by inflammatory signaling.

Methods

Animals & treatment

Eight to ten week old male C57BL/6J mice (Charles River Laboratories, Maastricht, the Netherlands) were housed at our facility at constant room temperature, humidity and light-dark cycle, and had free access to both water and standard mouse chow (Arie Blok, Woerden, the Netherlands) throughout experiments. Animal protocols were approved by the Institutional Use and Care of Animal Committee of the University of Groningen.

Mice were injected intraperitoneally with LPS derived from Salmonella typhimurium (0.5-7.5 mg/kg bodyweight, Sigma-Aldrich, St. Louis, MO) or vehicle (0.9% saline) alone and sacrificed at indicated time points after brief inhalation anesthesia by isoflurane. Livers were excised, weighed and snap-frozen in liquid nitrogen. Blood was collected at time of sacrifice in lithium-heparin-containing vials and centrifuged at 1500 x g for 5 minutes. Tissues and plasma were stored at -80°C until further use.

For a separate experiment, mice were pre-treated with LXR-agonist T0901317 (in corn oil by gavage) for three consecutive days (0-100mg/kg/day), received an intraperitoneal injection with LPS (2mg/kg) 15 minutes after the third dose of T0901317 and were sacrificed after 8 hours.

Gallbladder cannulation and bile salt infusion

In subgroups of mice, bile production and bile composition was determined at 24 hours after LPS-injection. To this end, bile salt infusion experiments were performed as described previously [17]. Briefly, mice were anaesthesized by an intraperitoneal injection with a mix of fentanyl (0.16mg/kg), fluanison (5mg/kg) and diazepam (12.5mg/kg). After jugular vein catheterization, common bile duct was ligated and the gallbladder was cannulated. Bile was collected during 15-minute time periods, starting at the moment of complete passage of the cannula. After two "basal" bile collections, tauroursodeoxycholate (TUDCA, CalBiochem/Merck Biosciences, Darmstadt, Germany) infusion via the jugular vein was started at rates of 150-300-450-600 nmol/min (increasing after every second 15min collection). During the experiments, mice were kept in a humidified incubator. After the fourth bile collection at 600 nmol/min, mice were killed by exsanguination (via abdominal inferior vena cava). Livers were excised, weighed and stored. Bile production was determined gravimetrically (assuming 1mg = 1µl) and bile samples were stored at -20°C until further analysis. Flow and secretion rates were corrected for pre-LPS bodyweight, as LPS-treatment induced anorexia and (thus) weight loss.

Analysis of bile and plasma constituents was performed as described previously [17]. Of note, for the analyses of bile, collection 1 was omitted, since the composition of the fraction may have been influenced by the amount of bile present in the gallbladder at the moment of cannulation.

Gene expression

Total RNA was isolated from mouse tissues or cells using Tri-reagent (Sigma) according to manufacturer's instructions. Concentrations of isolated RNA were determined photometrically (NanoDrop Technologies, Wilmington, DE) and integrity was confirmed by gel electrophoresis. RNA was reverse transcribed using Moloney-murine leukaemia virus reverse transcriptase (Sigma) according to manufacturer's instructions. cDNA was stored at -20°C until further usage. Gene expression was analyzed using Taqman real-time PCR method with the ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Expression of target genes was normalized to 18S or cyclophilin expression for each individual sample. Primer and dual-labeled probes (5'-FAM, 3'-TAMRA) were Eurogentec (Seraing, Belgium). Primer/probe sequences were published previously [18-21] or were presented in previous chapters.

Isolation of liver plasma membrane fractions and Western blotting

Plasma membrane fractions were prepared from three to four mouse livers according to the method previously described [22]. Enrichment was determined by comparing Na-K-ATPase and alkaline phosphatase activity in liver homogenates and isolated plasma membrane fractions [22]. The enrichment in Na-K-ATPase and alkaline phosphatase activity were approximately 20- and 15-fold.

Western blotting was performed as described previously using ten µg of plasma membrane protein [22]. Primary antibody used to detect Abcg5 [23] was a gift from Dr Bert Groen, while those used for Ntcp [24], Bsep [25] and Mrp2 [26] were all gifts from Dr Bruno Stieger. The antibody used for Mdr2 (Abcb4) is commercially available (Santa Cruz, SC 58-221).

Cloning of mouse Abcg5/8 intergenic region (IGR)

The mouse IGR was amplified by PCR using DyNAzyme EXT DNA polymerase (Finnzymes OY, Espoo, Finland) using the following primers: 5'-ccatggctagcaggaagcaaa-3' and 5'-gccatgaccagtgctgtttgtgc-3'. The amplified 367bp element covers both putative transcriptional start sites [10]. PCR product was gel purified using Zymoclean purification kit (Zymo Research Corp, Orange, CA) according to manufacturer's instructions and bluntly inserted in the Sma1-digested (Roche) luciferase reporter vector pGL3 (Promega, Madison, WI). This generated constructs containing the mIGR in both orientations.

Cell culture and transient transfection experiments

Human hepatoma HepG2 cells and Hep3B cells were obtained from ATCC/LGC Promochem (Teddington, United Kingdom) and cultured in DMEM containing 10% fetal bovine serum, 100U/l penicillin and 100µg/l streptomycin. Cells were kept at 37°C with an ambient CO2 of 5%. All cell culture supplies were purchased from Gibco/Invitrogen (Breda, the Netherlands).

HepG2 and Hep3B cells were transiently transfected with a pGL3-reporter construct containing the human ABCG5/8 intergenic region in ABCG8 orientation [10]. Lipofectamine 2000 (Invitrogen) was used as transfection reagent according to manufacturer's instructions with 1µg reporter construct DNA per well of 12-well plate along. Transfected cells were treated with 1µg/ml lipopolysaccharide, 10ng/ml human IL-1β (R&D Systems, UK) or 20ng/ml mouse Tnfα (R&D Systems) after 24 hours. After another 24 hrs, transfected cells were lysed and luciferase activities were determined according to manufacturer's protocol (Promega).

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed using 10 µg of nuclear extract protein according to the previously described protocol [27]. Nuclear extracts were incubated with 2.5 x 104 cpm of radiolabeled probe containing the canonical DR4 element. For competitor-studies, a 100-fold excess of cold specific or non-specific oligonucleotides (containing an AP-1 binding site) were added immediately preceding the addition of the probe. Samples were incubated on ice for 30 minutes and electrophoresed through a non-denaturing 6% polyacrylamide gel at 4°C. Gels were dried on filter paper at 80°C for 35-40 minutes and autoradiographed.

Statistical methods

Statistical analysis of results was performed using SPSS 14.0 (SPSS Inc., Chicago, IL) and BrightStat (www.brightstat.com) [28]. Data are reported as mean ± S.D. In case of relatively small sample-sizes ($N \leq 6$), non-parametric tests were used, i.e., Mann-Whitney U-test (MW) or Kruskal-Wallis H-test followed by Conover pairwise comparisons (Conover). Bile flow-bile salt and cholesterol-bile salt profiles were analyzed using multiple linear regression analysis (with or without logarithmic transformation of bile salt output). P-values <0.05 were considered significant.

Results

Mouse liver Abcg5/8 mRNA expression is dose- and time-dependently suppressed after LPS treatment

LPS treatment led to a reduced mRNA expression of both half-transporters Abcg5 and Acbg8. Dose-dependency of LPS-induced suppression of Abcg5/8 expression is shown in **figure 1A**. The maximal suppression of mRNA expression was found

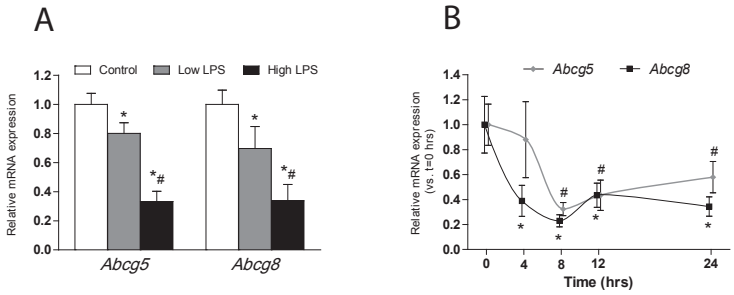

Figure 1. ***Dose- and time-dependency of Abcg5/8 mRNA expression in mouse liver after LPS-administration.*** Mice were injected intraperitoneally with saline +/- LPS. (A) Mice were injected with 0, 0.5 or 7.5mg/kg LPS (resp. Con, Low LPS and High LPS) and sacrificed after 24hrs. (B) Mice were injected with 5mg/kg LPS and sacrificed at indicated time points. Data are presented as mean ± SD. (A) *P < 0.05 compared to Con, # P < 0.05 vs. Low LPS (Conover). (B) (A) *P < 0.05 vs. Abcg5 at 0hrs, # P < 0.05 vs. Abcg8 at 0hrs (Conover).

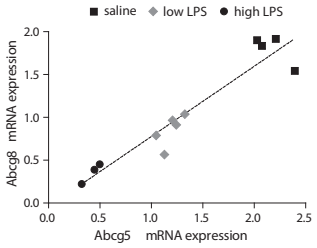

Figure 2. ***Correlation of Abcg5 and Abcg8 mRNA expression in mouse liver after LPS-administration.*** Mice were injected intraperitoneally were injected with 0, 0.5 or 7.5mg/kg LPS and sacrificed after 24hrs. Hepatic Abcg5/8 mRNA expression was determined by Taqman real-time PCR. mRNA expression levels are shown for individual samples. Pearson correlation 0.952 (P<0.05).

to occur at approximately 8 hours after LPS administration (intermediate dose) for both genes, but suppression persisted for at least 24 hours (**Figure 1B**). Interestingly, mRNA expression levels of both half-transporters remain tightly coupled after LPS treatment (**Figure 2**).

LPS-treatment reduces hepatic Abcg5 protein expression

We determined whether the effects of LPS on Abcg5 mRNA expression were translated at the protein level to account for potential post-transcriptional effects on Abcg5/8 protein expression as were recently shown by Sabeva et al.[29]. We used the intermediate dose of LPS (5mg/kg) and chose the 24hr after LPS time point to account for an anticipated delay between transcriptional and translational effects. As shown in **Figure 3**, LPS treatment reduced Abcg5 protein expression. Protein

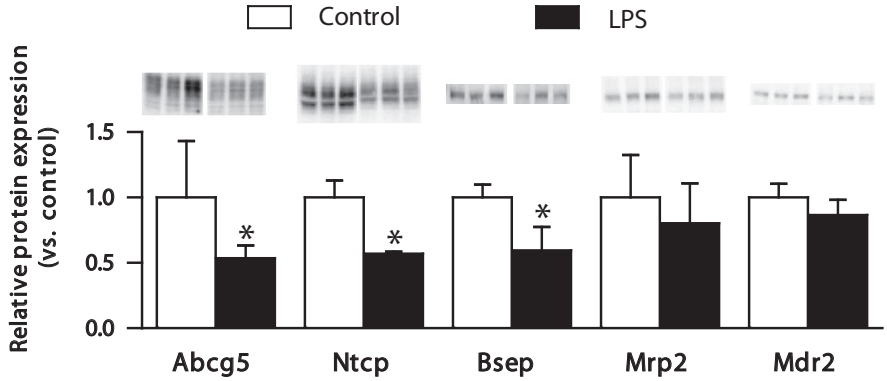

Figure 3. ***Hepatobiliary transporter protein expression after LPS-administration.*** Mice were injected with saline +/- LPS (5mg/kg) intraperitoneally and sacrificed after 24hr. Transporter protein expression was by Western blot using plasma membrane enriched fractions. Protein levels in control livers were set at 1. Representative Western blots are shown in top panels. Data are presented as mean ± SD. * $P < 0.05$ vs. control (MW).

expression of the bile salt transporters Ntcp and Bsep were also reduced in accordance to earlier reports, while Mrp2 and Mdr2 protein levels were not significantly affected. These results indicate that the effect of LPS on Abcg5 mRNA expression translates into altered protein levels.

LPS-administration suppresses TUDCA-stimulated bile flow through inhibition of bile salt secretion – To further examine the functional consequences of LPS-induced suppression of Abcg5/8 expression, we performed gallbladder cannulations and measured bile flow and the hepatobiliary secretion of bile constituents under basal conditions and during stimulation of bile production by intravenous TUDCA administration. Twenty-four hours after LPS-injection (5mg/kg), basal bile flow (fractions 1 and 2) was not significantly affected (**Figure 4A**). However, TUDCA-stimulated bile flow was significantly suppressed in LPS-treated mice (**Figure 4A**).

Since bile flow is largely driven by bile salt secretion [30, 31], it is not surprising that bile salt secretion was affected by LPS in a similar manner as bile flow (**Figure 4B**). Interestingly, bile salt-induced choleresis (i.e., bile flow/bile salt secretion) and bile salt-independent bile flow (i.e., Y-intercept of bile salt secretion vs. bile flow relationship) were not affected significantly at 24hrs after LPS (**Figure 4C**). These observations indicate that LPS primarily affects the secretion of bile salts rather than subsequent water and solute transport. This is further illustrated by the increased plasma bile salt concentrations in LPS-treated mice after maximal TUDCA-stimulation (**Figure 4C** (insert)). Since biliary secretion of phospholipids is also driven by bile salt secretion, it was not surprising to find that biliary phospholipid secretion was suppressed during (maximal) TUDCA-stimulation (**Figure 4D**).

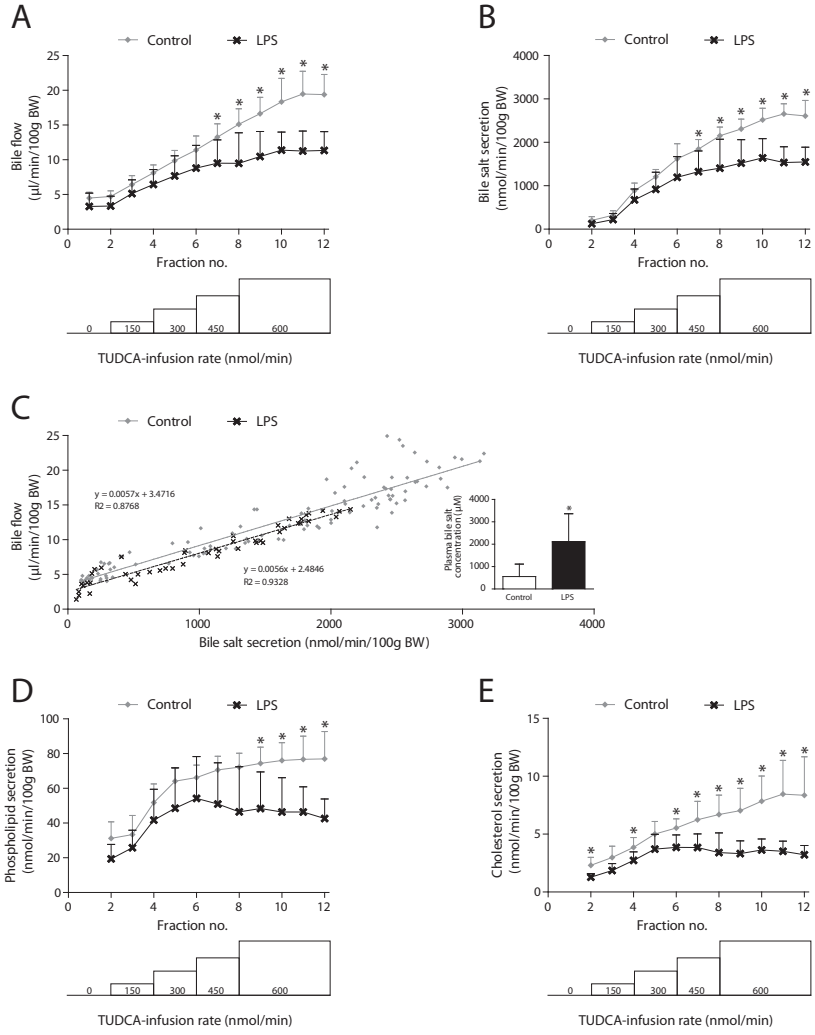

Figure 4. ***Effects of LPS-administration on basal and TUDCA-stimulated bile flow, biliary secretion of bile constituents, bile salt-induced choleresis and accumulation of bile salts in plasma during TUDCA-infusion.*** Mice were injected intraperitoneally with saline or 5mg/kg LPS. After 24hrs, bile flow was analyzed after gall bladder cannulation. Bile was collected in 12 fractions of 15min each. After two basal collections, bile flow was stimulated by intravenous TUDCA-infusion at increasing rates (bottom panel). (A) Bile flow rate was determined gravimetrically. (C) Bile salt-induced choleresis was assessed by linear regression analysis. (C, insert) Plasma bile salt concentrations and biliary secretion of (B) bile salts, (D) phospholipids and (E) cholesterol were assessed by methods described previously [17].

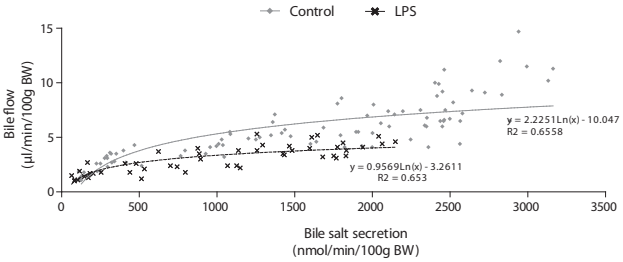

Figure 5. ***Relationship between cholesterol and bile salt secretion after LPS-administration.*** Mice were injected intraperitoneally with saline or 5mg/kg LPS. After 24hrs, bile flow was analyzed after gall bladder cannulation. Bile flow was stimulated by intravenous TUDCA-infusion at increasing rates (0-600nmol/min). The effect of LPS on bile salt-induced cholesterol secretion rate was determined using multiple linear regression analysis (after Ln-transformation of bile salt secretion rate). LPS-administration led to a significant reduction of slope of regression line: control 2.23 vs. LPS 0.96 ($P<0.05$).

LPS-administration suppresses both basal and TUDCA-stimulated biliary cholesterol secretion

Biliary cholesterol secretion was also reduced by LPS (**Figure 4E**). Unlike bile flow and bile salt secretion, cholesterol secretion was already reduced under basal conditions (fraction 2) and during lower TUDCA-infusion rates (fractions 4, 6). Furthermore, the extent of suppression of TUDCA-stimulated cholesterol secretion appeared to be greater than that of bile flow and bile salt secretion. This indicates that cholesterol secretion is more sensitive to LPS-treatment, which is illustrated by the relationship between cholesterol and bile salt secretion rates (**Figure 5**). LPS-administration led to a reduced cholesterol/bile salt ratio. This indicates that the effects of LPS seen on cholesterol secretion are, indeed, in part bile-salt "independent".

Inflammatory signaling does not suppress the promoter activity of the ABCG5/8 intergenic region

Considering the persistent coupling of Abcg5/8 mRNA expression levels under different conditions (**Figure 2**, also reported by others [32], we hypothesized that the relatively small IGR (~200bp) confers the LPS-sensitivity *in vivo*. The IGR has been shown to contain several functional cis-acting elements, including binding sites for Hnf4α/Gata4/6 [13] and the forkhead transcription factor FoxO1 [33], but also several other putative elements, including an NF-κB binding site [10] (**Figure 6**). We cloned the mIGR and inserted it bluntly into a pGL3-reporter construct. This yielded constructs that contained the mIGR in both orientations. The cloned fragment had a length of 367-bp and covered both transcriptional start sites. Transfection of these constructs into the human hepatoma cell lines HepG2 and Hep3B yielded consistently low luciferase activities (**Figure 7A-B**). Transfection of a pGL3-construct containing the cytomegalovirus promoter (CMV-construct) yielded high luciferase activity indicating that cells were effectively transfected in these experiments

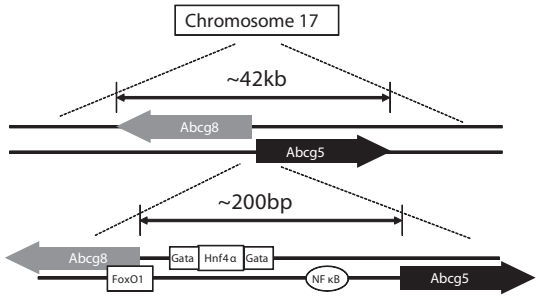

Figure 6. ***Genetic organization of mouse Abcg5/8 genes.*** The mouse Abcg5/8 genes are located on chromosome 17 in head-to-head configuration and encoded on opposite strands. The intergenic region contains several functional (rectangles) and putative binding sites (ovals). bp, base pair; kb, kilo-base pair (= x 1000bp)

(**Figure 7A-B**). Of note, transfection of both mIGR-constructs into mouse hepatoma cell line Hepa1_6 yielded luciferase activities that barely exceeded background (data not shown). These results suggest that the mIGR is, at most, a weak promoter.

Since the human IGR (hIGR) shares approximately 74% homology with the mIGR including several important cis-acting elements [10, 13, 33] and has stronger promoter activity in HepG2 and Hep3B cells than the mIGR (**Figure 7A-B**), we assessed whether the hIGR is responsive to inflammatory signaling. A pGL3-construct containing the hIGR in ABCG8 orientation was transiently transfected into both human hepatoma cell lines, which were treated with LPS or the cytokines (human IL-1β or mouse Tnfα). Neither LPS nor the pro-inflammatory cytokines suppressed the activity of the hIGR-containing construct (**Figure 8A-B**). Induction of inflammatory signaling

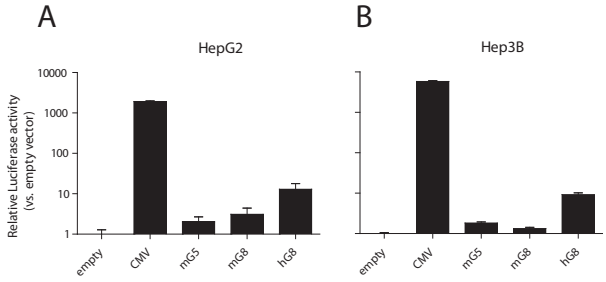

Figure 7. ***Basal activity of mouse and human ABCG5/8 intergenic region (IGR) containing reporter constructs after transient transfection in human hepatoma cell-lines.*** The mouse IGR was cloned and inserted into pGL3-reporter vector in both Abcg5 (mG5) and Abcg8 (mG8) orientation. The IGR-reporter constructs were transiently transfected into (A) HepG2 and (b) Hep3B cells. As controls, empty pGL3-vector (empty) and pGL3-vectors containing the human IGR in ABCG8 orientation (hG8) 10 or the cytomegalovirus promoter (CMV) were transfected. Luciferase activity of the empty vector was set at 1.

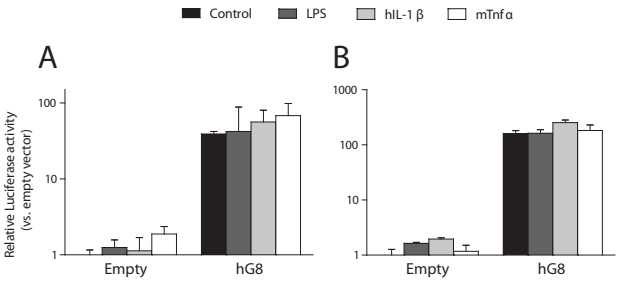

Figure 8. ***Effects of inflammatory signaling on transcriptional activity of human ABCG5/8 intergenic region (hIGR) containing reporter construct.*** A pGL3-vector containing the hIGR in ABCG8 orientation (hG8) was transiently transfected into (A) HepG2 and (b) Hep3B cells. Cells were treated with saline ± LPS (1μg/ml) or pro-inflammatory cytokines (hIL-1β (10ng/ml) or mTnfα (20ng/ml)) for 24hrs. As controls, empty pGL3-vector (empty) were transfected. Luciferase activity of the empty vector (treated with saline) was set at 1.

by both cytokines was confirmed by increased activity of the CMV-construct (data not shown), which is known to be responsive to such signaling (own observations and previous reports [34]. These results suggest that the suppression of Abcg5/8 expression *in vivo* is not is not mediated by the mIGR.

LPS-treatment reduces binding of hepatic nuclear extracts to a canonical DR4-element

Another potential mechanism of inflammation-induced suppression of Abcg5/8 mRNA expression is through interference with transcription factors whose binding sites are outside of the mIGR, i.e., enhancers. Although there is a multitude of potential transcription factors regulating Abcg5/8 mRNA expression through such enhancers, the primary candidate is LXR, since this nuclear receptor has been shown to regulate Abcg5/8 mRNA expression [11], while mIGR does not appear to contain an LXR response element [35]. Moreover, LPS-treatment of mice has previously been shown to lead to a rapid decrease in nuclear levels of the obligate

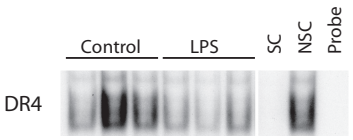

Figure 9. ***Effects of LPS-injection on DNA-binding of hepatic nuclear extracts to the canonical DR4 element.*** Mice were injected intraperitoneally with saline (control) or 2mg/kg LPS (LPS). Liver nuclear extracts were prepared and EMSA was performed using radiolabeled oligonucleotides containing the canonical DR4 element. For competitor studies, 100-fold excess of cold competitors were added to nuclear extracts prior to radiolabeled probe. SC, specific competitor, NSC, non-specific competitor. Probe, free probe without addition of nuclear extract.

heterodimer partner of LXR, i.e., the retinoid X receptor (RXR)-α (Nr2b1), and subsequently reduced nuclear binding to a canonical direct repeat (DR)-4 element [36]. LXR:RXRα heterodimers are known to have high affinity for such DR4 elements [37]. Using EMSA, we semi-quantitatively confirmed that LPS-treatment led to reduced DR4 DNA-binding (**Figure 9**).

Pre-treatment with LXR-agonist T0901317 attenuates LPS-induced suppression of Abcg5/8 mRNA expression

To determine whether the effects of LPS on hepatic Abcg5/8 expression could be prevented by prior LXR activation, we determined the effects of pre-treatment of mice with the LXR-agonist T0901317 for three consecutive days on LPS-induced effects on Abcg5/8 expression. As expected, pre-treatment with T0901317 induced Abcg5/8 expression and this effect persisted after LPS-injection leading to (near) normal Abcg5/8 mRNA expression in mice pre-treated with higher doses of T0901317 (**Figure 1**0). This suggests that T0901317 may be used to maintain hepatic Abcg5/8 expression in the setting inflammatory signaling.

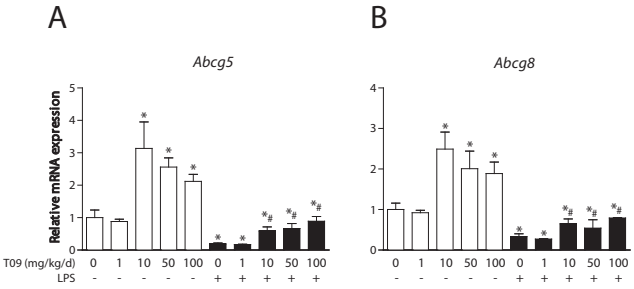

Figure 10. ***Pre-treatment with LXR-agonist T0901317 partially preserves Abcg5/8 mRNA expression after LPS-injection.*** Mice were pre-treated with increasing doses of T0901317 (T09, 0-100mg/kg/d) for three consecutive days, treated with LPS (2mg/kg) 15minutes after third dose of T0901317 and sacrificed eight hours later. (A) Abcg5 and (B) Abcg8 mRNA expression was assessed by Taqman real-time PCR. Data are presented as mean ± SD. *P < 0.05 compared to con/vehicle, #P < 0.05 vs. LPS/vehicle (Conover).

Discussion

In this study, we show that LPS-treatment of mice leads to a reduced mRNA and protein expression of Abcg5/8 that are associated with a reduced biliary cholesterol secretion. The latter occurs under basal conditions as well as during TUDCA-induced stimulation of bile formation. The exact pathophysiological mechanism of reduced Abcg5/8 mRNA expression, however, remains unclear. Based on our promoter-reporter assays, it appears that suppressed activity of the IGR is not responsible for this effect. One potential mechanism, for which indirect evidence is

provided, involves reduced LXR-transactivation of these genes. Pretreatment of mice with LXR-agonist T0901317 prior to LPS-injection led to (partially) preserved Abcg5/8 mRNA expression.

Our results of dose- and time-dependent suppression of Abcg5/8 mRNA expression by LPS closely resemble those presented recently by Khovidhunkit et al.[16]. This study adds to their report that hepatic inflammation is also associated with reduced protein expression of Abcg5 as well as with reduced biliary cholesterol secretion. The importance of confirming that suppressed Abcg5/8 mRNA expression also translates into reduced protein expression, is underlined by the recent report that showed that Abcg5/8 protein expression is also regulated at the post-transcriptional level[29]. Unfortunately, we could not directly confirm Abcg8 protein expression due to the lack of an anti-Abcg8 antibody. Abcg5 protein content in plasma membrane fractions is, however, very likely a proper reflection of the functional Abcg5/8 transporter complex, since heterodimerization of these two half-transporters is obligatory for proper trafficking to the canalicular membrane[38, 39]. In addition to post-transcriptional regulation, Abcg5/8-independent mechanisms may also cause a discrepancy between Abcg5/8 mRNA expression and biliary cholesterol secretion. Several reports have indicated that Abcg5/8-independent routes for biliary cholesterol secretion must exist[17, 40]. Plösch et al. showed that biliary cholesterol secretion was induced by high-cholesterol feeding independently of Abcg5/8 mRNA induction[17]. Although this could also be explained by post-transcriptional regulation, the authors suggested that there is either a functional reserve capacity of Abcg5/8 or an alternative transport route for biliary cholesterol secretion[17]. Further evidence for existence of an alternative route was recently provided by Groen et al., who showed that, in mice, deficiency of the canalicular transporter Atp8b1 (a phosphatidylserine flippase also known as Fic1) increased biliary cholesterol secretion regardless whether these mice were Abcg8-deficient or not[40]. The exact mechanism of this alternative route, however, remains to be characterized in detail.

In our model of analyzing IIC at 24 hours after LPS-treatment, cholestasis (as defined by reduced bile flow and accumulation of bile salts in plasma) is only revealed upon stimulation of bile formation by high TUDCA-infusion rates. This suggests that the normal bile salt secretory capacity of the mouse liver exceeds regular physiological demands, especially when one bears in mind that LPS leads to a significant reduction in Bsep protein expression. This considerable reserve capacity to maintain bile salt secretion rate has been reported previously[41]. Nevertheless, LPS-treatment did reduce the secretory rate maximum for bile salts by approximately 40% (**Figure 4B**)[42], which has not been described previously. This indicates that the reduced Bsep protein levels upon LPS-treatment do have pathophysiological impact. LPS-treatment did not significantly affect bile salt-independent bile flow. This does not appear to agree with previous reports[43, 44], but may very well be due to different species used and different durations of LPS-treatment.

Biliary cholesterol secretion is stimulated by bile salt secretion, requires Mdr2-mediated phospholipid secretion and is mediated at least in part by Abcg5/8 [45]. Our results show that LPS-treatment leads to a reduction in biliary cholesterol secretion that cannot be attributed solely to reduced bile salt secretion, since the basal cholesterol secretion rate and the bile salt-induced cholesterol secretion are both lower (Figures 4E and 5). This bile-salt independent effect of LPS on cholesterol secretion coincides well with the reduced Abcg5 protein expression, indicating that expression of Abcg5/8 may very well become the rate-controlling after LPS-treatment. Since Mdr2 protein expression and basal phospholipid secretion were not suppressed, the LPS-effects are most likely not mediated by reduced phospholipid secretion.

Transcriptional regulation of Abcg5/8 has remained a topic of ongoing investigation. We initially hypothesized that Abcg5/8 mRNA expression would be suppressed by effects of inflammatory signaling on the mIGR. The mIGR had been reported not to act as a minimal promoter [9], but recently functional cis-acting elements have been identified that bind transcription factors Hnf4α/Gata4/6 [13] and FoxO1 [33]. Expression and activity of Hnf4α is known to be suppressed in response to inflammatory signaling via multiple mechanisms [5]. Currently, it is not known how FoxO1 in hepatocytes is affected by inflammatory signaling. FoxO1 phosphorylation status and subsequent subcellular localization is regulated (partially) by insulin signaling and subsequent intracellular signal transduction via the PI-3K/AKT pathway [33]. Hence, it was conceivable that inflammation affects FoxO1 activity, for instance through interference with normal signal transduction or indirectly through altered insulin release/sensitivity. Our promoter-reporter studies, however, failed to identify a role for the IGR in suppression of ABCG/Abcg5/8 mRNA expression. The mIGR was found to be, at most, a weakly active promoter, when transfected into human and mouse hepatoma cells. This may, however, be due to the lack of essential (mouse-specific) transcription factors and coactivators in these cells. Interestingly, both mouse hepatoma cells and primary hepatocytes have a very low endogenous Abcg5/8 mRNA expression (data not shown) suggesting cellular dedifferentiation. The latter may explain the lack of activity of the mIGR in the promoter-reporter studies and also the higher activity of the hIGR in HepG2 cells, which have a much higher endogenous ABCG5/8 expression. Rather than through interference with *in vivo* activators of Abcg5/8 mRNA expression, we had also hypothesized that inflammatory signaling might also induce transrepressive factors. A potential mediator for the latter mechanism is NF-κB. This transcription factor is highly induced upon inflammatory stimuli and is able to exert both transactivating and transrepressing effects on gene transcription [46, 47]. Our results, however, did not support the concept of induced transrepression, at least not for the hIGR.

LPS-treatment did lead to reduced binding of whole liver nuclear extracts to the canonical DR4 elements. This is in close agreement with previous reports [27, 36, 48] and provides at this point the only molecular clue how inflammatory signaling can lead to reduced Abcg5/8 mRNA expression. Since LXR-RXRα heterodimers preferen-

tially bind to DR4 elements [37], one is inclined to assume that these EMSA results are conclusive. However, despite the well-established role of LXR in the regulation of their mRNA expression [11], an LXR response element in the vicinity of the Abcg5/8 genes remains to be identified.

Besides transcriptional regulation of mRNA expression, one has to bear in mind that effects on mRNA stability can also lead to dramatically altered mRNA expression levels. Regulation of mRNA stability can be mediated by so-called AU-rich elements (AREs) in 3'-untranslated region (UTR) of transcripts [49]. These AREs are, for instance, known to play a role in the regulation of cytokine mRNA expression [49], but also in that of Cyp7a1, the enzyme mediating the first step of the "classic" pathway of bile acid synthesis [50]. In silico analysis of the mouse Abcg5/8 3'-UTRs revealed three AUUUA elements in the Abcg8 transcript, but none of these three AREs was located in the nonamer that is supposedly required for mRNA breakdown, i.e., UUAUUUA(A/U)(A/U) [51]. The Abcg5 transcript did not contain any AREs in its 3'-UTR. Although ARE-mediated mRNA breakdown cannot be ruled out as potential regulating mechanism for Abcg8 mRNA expression, the differences between the 3'-UTRs of the two transcripts and the close coupling of expression suggests that this is not the main mechanism involved.

Sepsis-associated cholestasis is probably the best known example of IIC, but many other conditions that involve an inflammatory component can alter hepatobiliary transport systems [5]. This study shows that biliary cholesterol secretion is affected by inflammatory signaling. This will contribute to the general disturbance of whole body cholesterol metabolism during chronic inflammatory events (reviewed by Khovidhunkit et al. [52] and Esteve et al. [53]). Although this may be beneficial during the initial host defense against various (infectious) insults, it is assumed that chronic inflammation-induced alterations of cholesterol metabolism are profoundly harmful and will contribute to the development of atherosclerosis [52].

In conclusion, this study shows that inflammation suppresses the expression of Abcg5/8 at the mRNA and protein levels and that this is associated with a reduced biliary cholesterol secretion both under basal conditions and during maximal induction of the process by TUDCA-infusion. The molecular mechanisms of reduced Abcg5/8 mRNA expression remain unclear, although interference with normal transactivation by LXR is plausible. This transactivation by LXR may also provide a strategy to maintain hepatic Abcg5/8 expression during inflammatory conditions, although the unwanted induction of hepatic lipogenesis as a result of aspecific LXR-activation, must be circumvented first. The results of this study not only expand our knowledge of the pathophysiology of IIC beyond the scope of dysregulated transport of bile salts and bilirubin, but also indicate how (generalized) inflammatory conditions may affect cholesterol homeostasis.

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CHAPTER 7

General discussion & future perspectives

General discussion

The aim of the work described in this thesis was to examine possibilities of intervention in the pathogenesis of cholestasis and ensuing liver disease with the use of NR ligands. Cholestatic liver disease comprises a heterogeneous group of conditions that, relatively frequently, affects infants and children. Only a limited number of patients can be treated curatively and most current treatments are symptomatic. Patients afflicted by progressive cholestatic liver disease are generally bound for liver transplantation, although the time until end-stage liver disease can very widely. Despite increasing success rates of liver transplantation, this procedure is still associated with significant morbidity and mortality. Thus, there remains a need for new therapeutic strategies that will help to effectively treat cholestatic liver disease. Effectiveness in this regard would be defined as an improved prognosis for young patients with cholestatic liver disease, which includes an improved quality of life, a reduction in the dependency on liver transplantation and an improved, short-term and long-term outcome of liver transplantation.

Nuclear receptors and inflammation-induced cholestasis

Considering their important role in bile salt homeostasis and protection against bile salt toxicity, NRs as ligand-activated transcription factors are attractive targets for pharmacological intervention in the pathogenesis of cholestatic liver disease [1, 2]. More recently identified functions of NRs, most noticeably the anti-inflammatory actions of several family members, have further expanded the spectrum of possible applications of NR-ligands. With regard to cholestatic liver disease, this includes the conditions characterized by IIC. In **chapter 2**, the dual role of NRs in IIC was reviewed. NRs are involved in the pathogenesis of IIC, but are also involved in the adaptive responses to IIC and may provide targets to intervene in the underlying inflammatory cascade. The latter concept was studied in next three chapters. Pretreatment with the synthetic PPARγ ligand rosiglitazone (**chapter 3**) or the synthetic LXR ligand T0901317 (**chapter 4**) was shown to attenuate the effects of inflammatory signaling on hepatobiliary transporter down-regulation in LPS-injected mice. Although these ligands were found to exert their effects via different mechanisms, they both appeared to act Kupffer cell (KC)-independently. The latter was surprising, since both PPARγ and LXR had previously been reported to inhibit the inflammatory response in macrophages. In our studies, these ligands did not appear to inhibit this response in the resident liver macrophage, i.e., KCs, but to act directly on the hepatocyte. In case of T0901317, the direct hepatocellular action creates a serious problem, since hepatocellular LXR-activation induces de novo lipogenesis leading to massive hepatic steatosis. This effect has precluded the clinical application of currently available LXR-agonists. To determine whether the apparent lack of effect of T0901317 on KC-activation *in vivo* was due to inherent irresponsiveness of these cells, *in vitro* studies with primary rat KCs were performed (**chapter 5**). These revealed that KCs are sensitive to the anti-inflammatory effects of T0901317, although

these effects are cytokine-selective and much less potent than those of well-known anti-inflammatory drug, dexamethasone. Based on these results, it was concluded that KC-targeted delivery of LXR-agonists may provide a new approach to (selectively and subtly) intervene in the inflammatory cascade leading to IIC, while avoiding the detrimental, hepatocellular lipogenesis.

Besides the delivery of intestinal delivery of bile salts, bile formation is an important process to eliminate endogenous and exogenous toxic products from the body, including cholesterol. Bile has long been thought to be the only excretion route for cholesterol from the body, either as free cholesterol or catabolized to bile salts. Thus far, it was not known how this excretion route was affected by inflammation, although it had been shown that the canalicular cholesterol transporter Abcg5/8 was down-regulated at the mRNA level. In **chapter 6**, it was shown that this down-regulation by inflammatory signaling was also associated with reduced biliary cholesterol secretion, although the molecular mechanism remains to de clarified. These results suggest that inflammation-induced disturbances in cholesterol metabolism are probably in part mediated via effects on biliary cholesterol secretion.

Inflammation and cholestasis

The link between inflammation and clinical cholestasis has been known for a long time [3, 4] and affects everyday pediatrics, since the presence of an infection is an important consideration in the differential diagnosis of neonatal jaundice [5, 6]. Moreover, the severity of clinical/biochemical cholestasis has been shown to correlate with the severity of septicaemia in adult critical care patients [7]. Although this emphasizes the relevance of clinical cholestasis as a symptom, it is less obvious when inflammation leads to cholestatic liver disease. This is important information as it has consequences with regard to the need to intervene in the process, but also to the need to follow-up on patients after IIC has "resolved". To the best of our knowledge, there is currently no information available on the long-term hepatic sequelae of IIC. However, it may not be too far-fetched to suspect that severe inflammatory insults on the neonatal liver may have long-term effects. This holds especially true, when one considers the growing body of evidence that more subtle perinatal disturbances in nutritional, metabolic en hormonal status are already associated with diseases later in life [8]. Thus, further investigation into potential long-term hepatic dysfunction seems warranted.

Besides being a cause of cholestasis, inflammatory signaling can also be involved in continuing the disease process in cholestatic liver disease. As illustrated in **Figure 1**, cholestasis itself can lead to inflammatory signaling, thus creating a vicious cycle [3]. This process will most likely be further propagated once liver injury/damage occurs through further aggravation of inflammatory signaling [9].

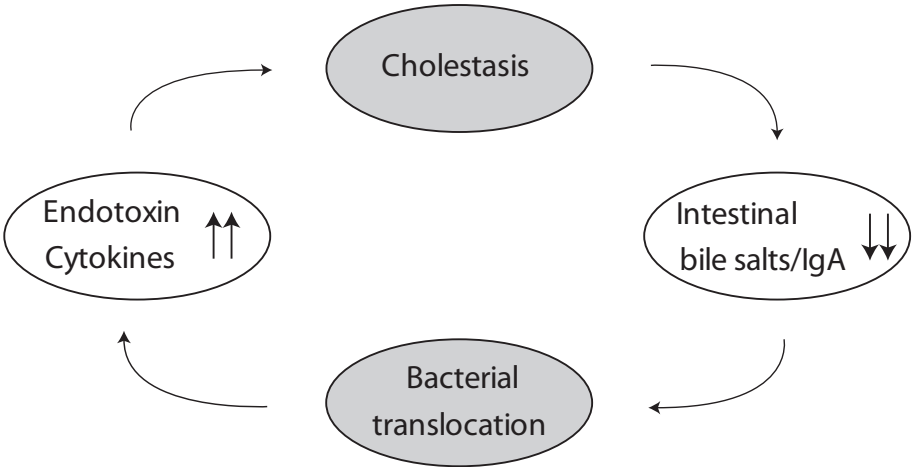

Figure 1. ***Vicious circle of cholestasis and inflammation (adapted from Trauner et al.*** [3]***).*** IgA, immunoglobulin A.

Drawbacks of LXR and PPARγ-ligands

Inherent to their nature of ligand-activated transcription factors, NRs provide a platform for pharmacological interventions for many diseases, including cholestatic liver diseases. NRs, however, modulate the activities of many, often controlling steps in a multitude of physiological processes. It is therefore to be expected that attempts to modulate NR activity in one cell type will lead to unwanted and/or detrimental side-effects elsewhere.

As already mentioned several times, the major side-effect of LXR-agonists and therefore, the main hurdle to overcome prior to their clinical use is the induction of hepatic lipogenesis and the resulting steatosis and hypertriglyceridemia [10]. This has thus far precluded their clinical application, although early animal studies revealed a great potential with regard to their use in treatment of atherosclerosis [11-13]. In **chapter 2**, several possible approaches are described that can be taken in attempt to circumvent this detrimental lipogenic side-effect of LXR-agonists.

In contrast to the LXR-agonists, synthetic PPARγ agonists are already in clinical use. The class of thiazolidinediones, comprising of clinically applied rosiglitazone and pioglitazone, have been used as "insulin sensitizers" in the treatment of type 2 diabetes for more than ten years [14]. The results of animal studies suggest that the clinical application of PPARγ-agonists could be expanded to other conditions, including atherosclerosis and various types of cancer [15]. The beneficial effects of PPARγ-agonists on carcinogenesis, however, do not appear to be universal [16]. Furthermore, several other findings have raised concerns about the safety of this group of drugs. Long-term rosiglitazone treatment in type 2 diabetes patients was reported

to be associated with an increased incidence of myocardial infarction [17]. Thiazolidinediones have also been shown to be associated with an increased fracture risk in women due to bone loss [18]. The underlying mechanisms appear to involve both impaired osteoblastogenesis [19, 20] and increased osteoclastogenesis [21]. This drawback of PPARγ-agonists becomes even more relevant when one considers the fact that cholestasis also leads to reduced uptake of vitamin D, another important regulator of bone mineralization. This may render cholestatic liver disease patients even more vulnerable to fractures.

FXR and cholestatic liver disease

Although FXR was not studied in the context of this thesis, it is worthwhile to discuss how modulation of its activity could be used as an approach to treat cholestatic liver disease. As previously stated, intrahepatocellular bile salt concentrations are tightly regulated and the bile salt-sensing FXR plays a central role in this regulation. The importance of FXR in this process is illustrated by the impaired tolerance to FXR-deficient mice to bile acid feeding [22]. The efficacy of bile salt homeostasis is illustrated by the beneficial effects of bile acid supplementation in the treatment of several bile salt synthesis defects. Due to this supplementation, the endogenous synthesis of bile acid intermediates is strongly suppressed and the production of these toxic compounds thus limited [23, 24]. Although it has not been proven formally, FXR is probably crucial is this process.

With the advent of the synthetic, non-bile salt FXR-ligand, GW4064 [25], investigators were able to initiate FXR-signaling in the absence of raised bile salt levels. Liu et al. found this compound to be hepatoprotective in two rat models of intrahepatic and obstructive cholestasis, i.e., α-naphthylisothiocyanate-treatment and bile duct-ligation, respectively [26]. These authors concluded that FXR-activation could be useful in treatment of cholestatic liver disease [26]. Others, however, have expressed serious doubts about this conclusion [27, 28]. These doubts were not only based on concerns regarding the experimental models used and missing data, but also on conflicting findings that FXR-deficient mice were protected against liver injury after bile duct ligation [28, 29].

Although one has to bear in mind that FXR activation is not necessarily the functional opposite of FXR-deficiency (see below), the main conclusion drawn from these conflicting results is that one should again make a clear distinction among the different types of cholestasis based on the pathophysiological mechanism. In the case of obstructive cholestasis, one can expect that initially "healthy" hepatocytes will respond adequately to increased bile salt retention. One component of this response will be an increase in canalicular bile salt transport, mediated (partially) via BSEP, but this response will subsequently face "the obstruction" leading to subsequent damage to the bile ducts / liver [29]. Hence, it is not surprising that FXR-deficiency was found to be protective in obstructive cholestasis, although this was probably

in part the result of FXR-independent up-regulation of the basolateral efflux transporter Mrp4 (Abcc4) [28, 29]. This suggests that FXR-antagonists may be of benefit in obstructive cholestasis, although one needs to be aware of such compounds may also dis-inhibit bile salt import and synthesis and suppress the induction of OSTα/β efflux pathway, which is known to be regulated by FXR [30, 31].

In contrast to obstructive cholestasis, hepatocellular cholestasis is characterized by reduced bile formation due to aberrant hepatocellular function. In this case, FXR-activation may be useful through increasing canalicular bile salt export via BSEP and basolateral efflux via OSTα/β as well as inhibition of bile salt import via NTCP and synthesis (analogously to treatment of bile salt synthesis defects). However, the efficacy of this approach will be dependent on whether intracellular FXR-signaling is intact, since it may also be (affected by) the cholestatic cause, e.g., inflammatory signaling (**Chapter 2**) or FXR-antagonism [32]. Very interestingly, it was very recently reported that FXR and its activation can also inhibit inflammatory signaling in the liver [33].

Although the above suggests that the preferred type of FXR-modulation (i.e., activation or inhibition) is always obvious, this is often not the case in daily practice. As stated earlier, obstructive cholestasis will induce a certain degree of hepatic inflammation, especially when cellular damage occurs. It is a priori difficult to predict what would be the desired/wanted modulation of FXR-activity.

When one considers applying FXR-modulators in cholestatic liver disease, one clearly needs to take other effects of FXR in non-parenchymal liver cells and outside the liver also into account. In hepatic stellate cells, FXR has, in conjunction with PPARγ and SHP, been shown to inhibit fibrogenesis [34-36]. Since portal fibrosis is frequently observed in cholestatic liver disease [37], FXR-activation may thus have additional benefits. In the intestine, FXR is protective against bacterial translocation during experimental obstructive cholestasis [38] and, in enterocytes, FXR-activation reduces intestinal uptake of bile salts and thus increases fecal excretion, while FGF15/Fgf19 production is induced, leading to further suppression of hepatic bile salt synthesis [39]. Intestinal FXR-activation therefore seems to be beneficial in general.

Although previously merely regarded as "soaps" required to drive bile formation and to facilitate intestinal lipid digestion and absorption, bile salts are increasingly regarded as signaling molecules that have multiple effects outside of the "enterohepatic cycle" [40, 41]. FXR mediates many, but not all, of these effects of bile salts. Use of FXR-modulators will thus have some "unexpected" effects as well, e.g., altered triglyceride and glucose metabolism.

Treating cholestatic liver disease with NR ligands – realistic expectations

Cholestatic liver disease can have many different causes. Some types can be treated very well whereas others progress to end-stage liver disease and are only effectively

treated by liver transplantation. The latter group consists mainly of hepatocellular forms and those obstructive forms unsuitable for surgical intervention. In spite of the proven effectiveness of treatment of some bile salt synthesis defects, it does not seem realistic to expect that the use of NR-ligands will provide instant cures for most currently incurable conditions. The primary goal, therefore, should probably be to focus on modulation of disease processes, e.g., through inhibition of ongoing insults or through boosting of adaptive mechanisms.

Attenuation of the disease process will probably improve quality of life for most patients in the pre-transplant phase. Furthermore, the benefit of being able to delay the progression to end-stage liver disease will allow for growth of pediatric patients, optimalization of their nutritional status and elective rather than emergency transplantations. All of these factors have been shown be positively correlated with the outcome of pediatric liver transplantation [42-44]. In some patients, e.g., PNAC patients, slowing of the disease progression may also be able to avoid transplantation indefinitely, as they may pass the parenteral-nutrition dependent phase.

Intervention in progressive cholestatic liver disease is ideally based on a solid understanding of the pathophysiological mechanisms [45]. This is not limited to the primarily causative mechanisms, but also those that are responsible for disease progression. To illustrate the importance of the latter, one can consider the variable natural course of post-Kasai biliary atresia. Although the majority of patients survives with their native liver for more than 10 years, some suffer from rapid loss of liver function [46, 47]. Understanding the causes of and/or risk factors for this remarkable difference will help to develop treatment regimes more tailored to individual patients.

General experimental concerns

Overestimation of the importance of transcriptional regulation

In the past decade, transcriptional regulation of hepatobiliary transport systems, bile salt homeostasis and detoxification pathways has been studied intensively. It has provided a molecular understanding of various processes that had been known for many years. Examples of "explained" processes include, besides the negative feedback of bile salts on their own synthesis, the induction of the stress-responsive Mdr1b gene [48] and the induction of bilirubin conjugation by phenobarbital [49]. The identification of nuclear receptors as important regulators of many of these processes has provided the opportunity to intervene pharmacologically [1]. However, a major issue in current research is that mRNA expression data are often directly translated into anticipated functional/physiological effects. This approach, however, overestimates the importance of transcriptional regulation and can be potentially deceiving. This is illustrated by discrepancies between mRNA and protein expression/localization of several genes/proteins that were discussed in this thesis, i.e. Abcg5/8 [50], RXRα [51] and pro-inflammatory cytokines [52]. Therefore, it remains important to

confirm functional significances at the protein level and ideally even beyond that level (i.e., physiological experiments and fluxomics approaches).

Interpretation of data derived from knock-out animal models

It is hard to imagine current day biomedical research without the availability of knock-out (KO) models, as they appear to be the mainstay of formal proof of "factor-dependency". The general importance of these models is probably best illustrated by the fact that the Nobel Prize for Physiology or Medicine 2007 was awarded the investigators, who developed the technique to generate KO mice [53]. Although ongoing improvements in the methodologies used have provided more elegant variants (e.g., conditional, tissue-specific, hypomorph), KO models have several disadvantages. Most of these disadvantages are well known and apply to the use of KO models in general, e.g., engagement of compensatory responses and unintentional disruption of adjacent genes or regulatory elements. However, some are particularly relevant to NR-KO models. Several NRs, including LXR, also regulate gene transcription while they are in an unliganded state, e.g., through recruitment of corepressor complexes [54]. Genetic deficiency of the NR can then lead to reduced repression, i.e., de-repression, which actually leads to enhanced expression [55, 56]. Furthermore, this de-repression is not a universal effect, but a promoter-specific effect involving other regulatory elements [57]. The relevance of these observations for interpretation of NR-KO data is that can help explain discrepancies in the effects seen in genes regulated by the same NR in KO of this particular NR. However, it, more importantly explains why effects of NR deficiency cannot be incautiously interpreted as effects that would be achieved by an NR-antagonist. Thus, a NR-KO is not the functional opposite of an NR-agonist.

Zonation: functional heterogeneity amongst hepatocytes

Since hepatocytes form the majority of liver cells both in number and mass and are actively engaged in protein synthesis [58, 59], mRNA and protein expression in whole liver homogenates can be regarded as a reasonable reflection of the expression pattern of an "average" hepatocyte. However, considering the important functional heterogeneity amongst hepatocytes located in different acinar zones [58], this average hepatocyte probably does not exist. This warrants cautious interpretation of data dealing with intracellular "cascades", since components of such cascades need to be present in the same cell. Laser-dissection techniques or immunohistochemical imaging can aid in determining whether all of the components are actually located within the same cell. Although the concept of zonation was already introduced in the field of hepatobiliary transport over 25 years ago [60, 61], it has received only sparse attention since [62]. It will be particularly interesting to determine whether the expression of transporters matches with their regulators.

Future perspectives

Using the results described in this thesis as a starting point, several different directions can be taken to further clarify the pathogenesis of IIC and to examine the potential use of NR-ligands to intervene in this process. Firstly, we have used an animal model in which mice were injected with a single dose of LPS. Although widely used as septic inflammatory model, it is clearly different from real clinical sepsis [63]. It will therefore be important to determine whether rosiglitazone and T0901317 are also effective in other models of sepsis (e.g., bacterial infection, cecal-ligation-and-puncture) and non-septic inflammation (e.g. turpentine-injection, genetically engineered mice with constitutively active inflammatory signaling). Moreover, in our experiments, mice were pre-treated with the NR-ligands while in the anticipated clinical setting anti-inflammatory drugs would be given after initiation of the inflammatory process. Considering the effects of inflammation of NR function (**Chapter 2**), the latter may be profoundly affected making it difficult to make predictions on the effectiveness of NR-ligands as therapeutic modulators. Therefore, the effect of NR ligands should be examined after initiation of the inflammatory cascade.

As mentioned in **Chapter 5**, KC-targeted LXR-activation may provided a means to intervene in the inflammatory cascade while avoiding, the unwanted hepatocellular effects. This will either require the use of pharmacological approaches to target the LXR ligands to the KCs or the discovery/development of KC-selective modulators of LXR.

Considering the potential beneficial effects of FXR-agonists in the treatment of hepatocellular cholestasis (see above), it is worthwhile to examine the effects of FXR-agonists in IIC. Will these preserve bile salt secretion and thus bile flow? Will they modulate inflammatory response? Ligands of others NRs involved in bile salt metabolism, i.e., VDR, PXR and CAR, could also be employed in attempt to intervene in the cascade leading to IIC.

Concluding remarks

Although many aspects of the pathogenesis of cholestatic liver disease have been unraveled over the past decades and the effects of several pharmacological interventions are now better understood, there remains a need for new pharmacological approaches to treat this group of conditions. The further exploration of pathogenesis continues to be necessary in order to identify new therapeutic targets [37, 45]. The use of NR ligands remains attractive, but requires further investigation of their effects in different, more clinically relevant animal models and optimization of treatment regimes with regard to anticipated cell-specific side-effects.

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Appendices

English summary

The liver performs many different functions. Besides detoxification of waste products and their subsequent elimination, the liver plays a central role in whole body metabolism and produces a wide range of proteins and other substances involved in e.g., blood coagulation, immunological responses and hormonal actions. Another important function of the liver is the formation of bile. Bile is produced by liver cells ("hepatocytes") and secreted into a specialized network of minute canaliculi which merge into larger ducts and eventually into the common bile duct. The latter drains into the small intestine. Bile contains a high concentration of bile salts. These detergents ("soap-like") are required for optimal digestion and absorption of nutrients by the intestine, most importantly for fats and fat-soluble nutrients (e.g. vitamins A, D, E and K). Besides bile salts, bile also contains waste products which can be excreted via the feces.

Impairment of bile formation or bile flow is called "cholestasis", which has been derived from the Greek words χολη (= "bile") and στασις (= "stoppage"). Cholestasis can be caused in the liver cells ("hepatocellular cholestasis") or in the bile drainage system ("obstructive cholestasis"). The consequences of cholestasis is a shortage of bile salts within the intestine and accumulation of bile components in liver and the rest of the body. The former will cause maldigestion and malabsorption of fats and fat-soluble nutrients and possibly nutritional deficiencies. The latter is exemplified by the emergence of jaundice. Bilirubin, a breakdown product of red blood cell hemoglobin, is normally excreted into bile, but can accumulate in the skin and sclera during cholestasis.

As already mentioned, bile salts are detergents. Similarly to their ability to solubilize dietary fats (or lipids) in the intestine, bile salts can also solubilize lipids in the walls of liver cells. This can lead to liver cell injury and death, ultimately leading to impaired liver function (i.e., reduced bile formation, protein synthesis and detoxification). The latter can be defined as cholestatic liver disease.

The above illustrates that bile salts can act as double-edged swords. Bile salts are on one hand indispensable for absorption of essential nutrients, but are on the other hand potentially harmful for the liver and the body. Therefore, local concentrations of bile salts need to be tightly controlled. Several mechanisms exist to provide such a level of control. One mechanism is transcriptional regulation, i.e. regulation of the expression of genes encoding proteins which are involved in bile salt transport and synthesis. Research over the past decade has shown that nuclear receptors (NRs) play an important role in the transcriptional regulation of bile salt homeostasis. NRs are proteins whose activity as transcription factors is regulated by binding of specific compounds (so-called "ligands"). Hence NRs are also called "ligand-activated transcription factors". Well-known examples of NRs are the classic hormone receptors, e.g. those for glucocorticoids or sex hormones. More recently, it has become clear that the NR family is larger and also includes receptors which regulate intracellular

processes. The farnesoid X receptor has been identified as an NR whose activity is regulated by binding of bile salts. When the concentration of bile salts within the liver cell increases, FXR will be activated, which leads to the increased expression of genes involved in bile salt export and the reduced expression of those genes involved in bile salt import and synthesis. This response protects the liver cell against an overload of potentially toxic/harmful bile salts. FXR acts as a "thermostat" keeping the intracellular bile salt concentration within a normal range. Besides FXR, several other NRs regulate the expression of genes involved in bile salt homeostasis and bile formation, e.g. the liver X receptor (LXR) and the peroxisome proliferators-activated receptors (PPARs).

As previously stated, cholestasis has various causes, which can be roughly divided into two categories, i.e. hepatocellular causes and obstructive causes. Examples of cholestatic liver disease in children are biliary atresia and parenteral nutrition-associated cholestasis (PNAC). In biliary atresia, bile ducts are obliterated early in infancy by a thus far not entirely clarified process. Recent evidence suggests that it involves an immunological response against the bile ducts provoked by a (viral) insult. PNAC can occur when patients are fed intravenously, but the exact mechanism behind this condition remains to be clarified too. PNAC is known to occur more frequently in children, specifically in premature infants and in infants with a short bowel (due to congenital intestinal anomalies and/or (subsequent) surgery). Several types of treatments for cholestatic liver disease are available, both pharmacological and surgical, depending on the nature of the type of disease. Unfortunately, not all patients benefit (permanently) from these treatments and many of them are eventually bound for liver transplantation. Although transplantation can be life-saving, the number of suitable donors is limited and the procedure remains associated with significant morbidity and mortality. Thus, there remains a need for new treatment strategies. In the studies described in this thesis, the potential application of NR-ligands to intervene in one specific type of cholestatic liver disease, i.e. inflammation-induced cholestasis (IIC), was investigated.

In **chapter 2**, the link between NRs and IIC is reviewed. The mechanisms behind bile formation and NR-action are described. Besides their role as "thermostats" within liver cells, NRs are also crucial for basal expression of various transporter proteins and, therefore, for normal bile formation. Since inflammatory processes, e.g., a bacterial infection such as sepsis or a urinary tract infection, can lead to reduced NR-activity, it comes as no surprise that IIC is (in part) mediated by reduced NR-function. However, the feature of NRs that their activity is regulated by ligands, offers the opportunity to search for and design new ligands that can potently and selectively induce NR-activity. Such ligands could be applied therapeutically. One example would be the use of an FXR-ligand. Such a ligand could be used to boost FXR activation in an earlier stage, i.e., at lower bile salt concentrations. Protective mechanisms might therefore be engaged prior to the emergence of (permanent) liver cell injury.

Several NRs have also been shown to possess anti-inflammatory actions, e.g., PPARγ and LXR. These actions were initially demonstrated in macrophages, which are monocyte-derived immune cells. A sub-group of macrophages resides permanently in the liver. These so-called Kupffer cells (KCs) are known to play an important role in the inflammatory cascade leading to IIC, since they secrete an array of inflammatory mediators (cytokines) in response to various infectious/toxic stimuli. These cytokines subsequently act on liver cells and inhibit bile formation. Based on the assumption that suppression of the inflammatory response in KCs by PPARγ- or LXR-ligands would inhibit the cascade leading to IIC, we performed several animal an cell-culture studies, which are described in **chapters 3-5**. As an animal model for IIC, we injected mice with lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, which provokes a strong inflammatory response in the liver. As a result of LPS-injection, the gene expression of various proteins involved in bile salt transport and bile formation is suppressed. In **chapter 3**, our studies with PPARγ-ligand rosiglitazone are described. Pre-treatment with this compound was shown to partially inhibit the cascade leading to IIC, but this effect did not appear to occur at the level of the KCs. Rosiglitazone appeared to act within the liver cells and was shown to preserve the nuclear levels of the NR retinoid X receptor (RXR)-α. RXRα is a special NR, since it is the obligate partner for other NRs to be active. The rapid export of RXRα from the nucleus is one mechanism of reduced transporter gene expression in response to inflammatory stimuli. Preservation of nuclear RXRα levels after LPS by rosiglitazone pretreatment was associated with partially preserved hepatic gene expression. In **chapter 4**, it was shown that pre-treatment of mice with the LXR-ligand, T0901317, also leads to partial preservation of hepatic gene expression after LPS-injection. This effect also appeared to occur within the hepatocytes, but was not associated with preserved RXRα levels. T0901317 was shown to suppress the effects of another, but not NR, transcription factor, i.e., NF-kB. The drawback of T0901317, however, is the unwanted effect of induction of fatty acid synthesis, which leads to the generation of a massively fatty liver. Considering this side-effect and the apparent lack of an anti-inflammatory effect of T0901317 in KCs, we next investigated whether it would be possible to inhibit the inflammatory response of these cells using T0901317. These studies are described in **chapter 5**. Kupffer cells were isolated from rat liver and treated with LPS. The subsequent inflammatory response could be partially inhibited by pre-treatment with T0901317. Although this anti-inflammatory effect was less potent and more cytokine-selective than that of the well-known anti-inflammatory drug, dexamethasone, our results indicate that KC-specific LXR-ligands or general LXR-ligands which are pharmacologically targeted at KCs are of potential use as a therapeutic strategy in IIC.

Besides affecting the transport of bile salts, inflammatory processes also affect the transport of other bile constituents, e.g., cholesterol. In **chapter 6**, we describe that inflammation signalling also leads to reduced cholesterol transport from hepatocytes into bile. The underlying mechanism also appears to involve a transcriptional

suppression, since the gene expression of the half-transporters Abcg5 and Abcg8 is suppressed, but the exact mechanism remains to be elucidated. Again, reduced activity of NRs (i.e., LXR and hepatocyte nuclear factor-4α) may be a central mediating mechanism.

In conclusion, this thesis shows that NRs can play a mediating a role in the generation of cholestasis, most noticeably of IIC, but that modification of their activity using new ligands may also provide new treatment strategies for cholestatic liver diseases. Although additional investigations remain necessary to further explore these new applications of NR-ligands, they may eventually answer to a dire clinical need for new treatment options. Hopefully, this will lead to an improved outcome of children with cholestatic liver disease.

Nederlandse samenvatting

De lever heeft veel en zeer uiteenlopende functies. Naast het ontgiften van afvalstoffen en de verwijdering hiervan uit het lichaam speelt de lever een centrale rol in de stofwisseling en maakt ze een scala aan stoffen betrokken bij o.a. de bloedstolling, afweerreacties en hormoonsystemen. Een andere zeer belangrijke functie is de productie van gal. Door de levercellen ("hepatocyten") geproduceerde gal wordt via een gespecialiseerd systeem van galweggetjes en galwegen naar de dunne darm geleid. Gal bevat hoge concentraties van zogenaamde galzouten. Dit zijn zeepachtige stoffen, die noodzakelijk zijn voor een optimale vertering en opname van voedingsstoffen door de darm, m.n. van vetten en vet-oplosbare stoffen (bijv. vitamines A, D, E en K). Naast galzouten bevat gal ook afvalstoffen die langs deze route uiteindelijk via de ontlasting uitgescheiden kunnen worden.

Wanneer de productie van gal door de lever of de afvloed hiervan naar de darm gestoord is, spreekt men van "cholestase". Dit woord is afgeleid van de Griekse woorden χολη (= "gal") en στασις (= "stilstaan"). De oorzaak hiervan kan zowel in de levercellen zelf ("hepatocellulaire cholestase") als in de afvoerende galwegen ("obstructieve cholestase") gelegen zijn. Als gevolg hiervan ontstaat er een tekort aan galzouten in de darm, dat zal leiden tot een verstoorde opname van vetten en vet-oplosbare stoffen en mogelijk tot een tekort aan (essentiële) voedingsstoffen. Daarnaast zullen stoffen die normaal gesproken in de gal uitgescheiden worden, zich nu in de lever en vervolgens ook in de rest van het lichaam ophopen. Het meest bekende gevolg hiervan is het ontstaan van geelzucht, waarbij bilirubine (een afbraakproduct van hemoglobine uit rode bloedcellen) zich in de huid en in het oogwit ophoopt. Zoals gezegd zijn galzouten zeepachtige stoffen. Net zoals zij in staat zijn om in de darm vetten te doen "oplossen", kunnen zij dit, mits de ophoping voldoende is, ook doen in de lever zelf. Hierdoor kunnen levercellen beschadigd raken en te gronde gaan. Dit leidt vervolgens weer tot een vermindering van de functie van de lever als geheel, o.a. leidend tot verminderde productie van gal en eiwitten en verminderde ontgiftingsactiviteit. Men kan dan spreken van cholestatische leverziekte.

Bovenstaande maakt duidelijk dat galzouten twee gezichten hebben. Aan de ene kant zijn ze onmisbaar voor de opname van essentiële voedingsbestanddelen, maar aan de andere kant zijn ze ook potentieel schadelijk voor de lever/het lichaam. Daarom dient de lokale concentratie van galzouten nauwkeurig gereguleerd te worden. Dit gebeurt op verschillende manieren, waarvan *transcriptionele regulatie* er één is. Transcriptie is het proces, waarbij een gen (DNA) wordt "afgelezen". Hierbij wordt mRNA gevormd dat vervolgens weer wordt vertaald in een eiwit met een bepaalde fysiologische functie. Levercellen kunnen zichzelf beschermen door de transcriptie van genen die coderen voor eiwitten betrokken bij het transport van galzouten of de aanmaak hiervan te reguleren. Onderzoek gedurende het afgelopen decennium heeft aangetoond dat nucleaire receptoren (NRs) een belangrijke rol spelen bij deze transcriptionele regulatie van de galzouthuishouding. NRs zijn eiwitten, die door

binding van meer of minder specifieke stoffen (zgn. liganden) "aan" gezet kunnen worden om genen tot expressie te laten komen. Ze worden daarom ligand-geactiveerde transcriptiefactoren genoemd. Bekende voorbeelden van NRs zijn de klassieke hormoonreceptoren, bijv. die voor bijnierschorshormonen en geslachtshormonen. Meer recent is echter duidelijk geworden dat de familie van NRs beduidend groter is en ook receptoren omvat, die een rol spelen bij de regulering van processen binnen individuele cellen. De farnesoid X receptor (FXR) is bijvoorbeeld geïdentificeerd als NR, waarvan de activiteit gereguleerd wordt door galzouten. Wanneer de concentratie van galzouten binnen een levercel stijgt, zal geactiveerde FXR de expressie van genen betrokken bij de opname en aanmaak van galzouten remmen en tegelijkertijd die van genen betrokken bij de uitscheiding ervan te verhogen. Het netto-effect hiervan zal zijn dat de cel beschermd wordt tegen een overmaat aan galzouten. FXR fungeert dus als een "thermostaat" om de concentratie galzouten binnen de levercel binnen normale grenzen te houden. Naast FXR zijn ook andere NRs betrokken bij de regulering van genen die een rol spelen bij de galzouthuishouding en galvorming, bijv. de liver X receptor (LXR) en de peroxisome proliferator-activated receptors (PPARs).

Zoals gezegd kent cholestase diverse oorzaken, die grofweg ingedeeld kunnen worden in de eerder genoemde categorieën van hepatocellulaire en obstructieve oorzaken. Bekende voorbeelden van cholestatische leverziekten op de kinderleeftijd zijn o.a. galgangatresie (ook wel biliaire atresie genoemd) en parenterale voeding-geassocieerde cholestase. Bij galgangatresie ontstaat door een nog onopgehelderd proces kort na de geboorte een afweerreactie gericht tegen de galgangen, die vervolgens beschadigd en geblokkeerd raken en daarmee de galvloed verhinderen. Bij parenterale voeding-geassocieerde cholestase gaat de toediening van voedingsstoffen direct in de bloedbaan via een infuus (parenteraal, = "buiten de darm") gepaard met een verstoorde aanmaak van gal. Het mechanisme hierachter is vooralsnog niet opgehelderd, maar het treedt vooral op bij te vroeg geboren baby's of bij baby's met een "korte darm" t.g.v. aangeboren afwijkingen en/of operatieve ingrepen. Er bestaan verschillende behandelingsmethoden voor cholestatische leverziekten, zowel medicamenteus als chirurgisch, maar helaas hebben niet alle patiënten hier (blijvend) baat bij en is het uiteindelijk vaak noodzakelijk om over te gaan tot levertransplantatie. Hoewel dit een levensreddende ingreep kan zijn, is het aantal beschikbare donorlevers beperkt en gaat levertransplantatie nog altijd gepaard met een aanzienlijke mate van ziektelast en sterfte. Er blijft dus behoefte bestaan aan nieuwe behandelingsmogelijkheden. In de studies beschreven in dit proefschrift is onderzocht in hoeverre het mogelijk is om met gebruik van NR-liganden in te grijpen bij één type cholestatische leverziekte, namelijk ontsteking-geïnduceerde cholestase (Eng: *inflammation-induced cholestasis* (IIC)).

In **hoofdstuk 2** wordt stilgestaan bij het verband tussen NRs en IIC. De mechanismen van galvorming en de werkingsmechanismen van NRs worden beschreven. Naast hun rol als "thermostaten" binnen levercellen zijn NRs ook van belang voor

de basale expressie van verschillende transporteiwitten en dus voor de normale galvorming. Aangezien ontstekingsreacties, bijvoorbeeld op basis van een bacteriële infectie zoals sepsis of een urineweginfectie, aanleiding kunnen geven tot verminderde activiteit van NRs, is het niet verwonderlijk dat IIC (deels) wordt gemedieerd door verminderde NR-activiteit. Echter, de eigenschap van NRs dat hun activiteit gereguleerd wordt door liganden, biedt ook de mogelijkheid om stoffen te zoeken of ontwerpen, die de activiteit van NRs krachtig en selectief kunnen beïnvloeden, maar die qua chemische structuur afwijken van de natuurlijke liganden. Dergelijke stoffen zouden mogelijk gebruikt kunnen worden als medicijn. Eén voorbeeld van zo'n stof zou een niet-galzout FXR-ligand kunnen zijn. Hiermee zou FXR-activatie versterkt kunnen worden zonder dat er een verder verhoogde, en daarmee nog meer schadelijke, galzoutconcentratie hoeft te bestaan. Door de levercel daarmee al in een eerder stadium te laten denken dat de galzoutconcentratie te hoog is, kunnen mechanismen om de cel te beschermen eerder in gang gezet worden.

Verschillende NRs blijken ook ontstekingsremmende effecten te hebben, bijv. PPARγ en LXR. Deze effecten zijn voornamelijk aangetoond in zgn. macrofagen, afweercellen die ontstaan uit witte bloedcellen. Een subgroep van deze macrofagen bevindt zich permanent in de lever, zgn. Kupffercellen (KC). KCs spelen een belangrijke rol bij het ontstaan van IIC, doordat ze in reactie op verschillende infectieuze/toxische stimuli een verscheidenheid aan ontstekingsmediatoren uitscheiden. Deze zgn. cytokines, grijpen vervolgens aan op de levercellen zelf, wat leidt tot verstoorde galvorming. Gebaseerd op de hypothese dat remming van de ontstekingsreactie in KCs door behandeling met liganden van PPARγ en LXR in staat zou kunnen zijn om het ontstaan van IIC te kunnen stoppen, voerden we een aantal proefdier- en celkweekstudies uit, welke worden beschreven in **hoofdstukken 3-5**. Als model voor IIC werden muizen geïnjecteerd met lipopolysaccharide (LPS), een component van de celwand van Gram-negatieve bacteriën, die een krachtige ontstekingsreactie kan opwekken in m.n. de lever. Als gevolg hiervan wordt o.a. de genexpressie van verschillende galzouttransporteiwitten en andere eiwitten betrokken bij de aanmaak van gal geremd. In **hoofdstuk 3** worden studies met PPARγ-ligand, rosiglitazon, beschreven. Dit middel blijkt de cascade leidend tot IIC deels te kunnen remmen, maar niet op het niveau van de KCs. Het lijkt vooral te werken in de levercellen zelf en blijkt daarin de hoeveelheid van de NR retinoid X receptor (RXR)-α in de kern te behouden. RXRα is een bijzondere NR, aangezien deze noodzakelijk is voor de andere NRs om transcriptioneel actief te kunnen zijn. T.g.v. ontsteking wordt RXRα snel uit de kern verwijderd, waarmee de basale expressie van diverse genen geremd wordt. Behoud van RXRα in de kern tijdens ontsteking door voorbehandeling met rosiglitazon gaat dan ook gepaard met gedeeltelijk behoud van basale genexpressie. In **hoofdstuk 4** wordt getoond dat ook voorbehandeling met een LXR-ligand, nl. T0901317, in staat blijkt om deze genexpressies op peil te houden en dat dit ook in de levercel zelf werkt. Dit gaat echter niet gepaard met behoud van RXRα, maar T0901317 lijkt wel de effecten van een andere transcriptiefactor (geen NR), nl. NF-

κB, te remmen. Het grote nadeel van T0901317 is echter dat het als zeer ongewenste bijwerking heeft dat het de levercel aanzet om vetzuren aan te maken. Hierdoor ontstaat sterke vervetting van de lever. Vanwege deze bijwerking en het schijnbaar ontbreken van een ontstekingsremmend effect van T0901317 in de KCs werd vervolgens onderzocht of het toch mogelijk was om de ontstekingsreactie in deze cellen met T0901317 te remmen. Deze studies worden in **hoofdstuk 5** beschreven. Hiervoor werden uit rattenlevers geïsoleerde KCs in kweek gebracht en vervolgens met LPS gestimuleerd. Deze ontstekingsreactie bleek inderdaad deels geremd te kunnen worden door T0901317. Ondanks dat het effect van T0901317 minder krachtig was dan dat van dexamethason, een veel gebruikte ontstekingsremmer, geven onze resultaten aan dat KC-specifieke LXR-liganden of liganden die daar door gebruik van farmacologische technieken in afgeleverd worden, mogelijk therapeutisch ingezet kunnen worden om in te grijpen in IIC.

Naast effecten op het transport van galzouten hebben ontstekingsprocessen ook effecten op het transport van andere galbestanddelen, bijvoorbeeld cholesterol. In **hoofdstuk 6** wordt beschreven dat cholesteroltransport vanuit de levercel naar de gal ook door ontsteking geremd wordt. Hier speelt mogelijk ook een transcriptionele remming een rol bij, nl. die van de expressie van de twee "halftransporters" Abcg5 en Abcg8. Het exacte moleculaire mechanisme blijft echter nog wel onopgehelderd. Het is echter goed mogelijk dat een verminderde activiteit van o.a. de NRs LXR en hepatocyte nuclear factor-4α hier een mediërende rol bij speelt.

Tot slot kan geconcludeerd worden dat dit proefschrift laat zien dat NRs aan de ene kant een mediërende rol kunnen spelen bij het optreden van cholestase, bijvoorbeeld bij het ontstaan van ontsteking-geïnduceerde cholestase, en aan de andere kant als mogelijk farmacologische doelwit gebruikt kunnen worden bij de behandeling van cholestatische leverziekten. Hoewel verder onderzoek vanzelfsprekend noodzakelijk blijft, bieden NR-liganden het perspectief dat invulling gegeven kan worden aan de behoefte aan nieuwe behandelingsmogelijkheden voor kinderen met cholestatische leverziekten. Hopelijk kunnen de vooruitzichten van deze patiënten hiermee op termijn sterk verbeterd worden.

List of abbreviations

9cRA	9cis-retinoic acid
ABC	ATP-binding cassette (transporter)
AF	activation function (domain)
ALT	alanine aminotransferase
AP-1	activator protein-1
APR	acute phase response
AQP	aquaporin
ARE	AU-rich element
ASBT	apical sodium-dependent bile salt transporter
BA	biliary atresia
BRIC	benign recurrent intrahepatic cholestasis
BSDF	bile salt dependent flow
BSEP	bile salt export pump
BSIF	bile salt independent flow
BW	bodyweight
CA	cholic acid
CAR	constitutive androstane receptor
CBP	CRE-binding protein
CDCA	chenodeoxycholic acid
CLP	cecal-ligation and puncture
CM	conditioned medium
CMV	cytomegalovirus
CYP	cytochrome P450
DBD	DNA-binding domain
DR	direct repeat
EMSA	electrophoretic mobility shift assay.
ER	estrogen receptor
FBS	fetal bovine serum
FGF(R)	fibroblast growth factor (receptor)
FXR	farnesoid X receptor
Gal	D-galactosamine
GH	growth hormone
GR	glucocorticoid receptor
HDAC	histone deacetylase
HNF	hepatocyte nuclear factor
IFN	interferon
IGR	intergenic region
IIC	inflammation-induced cholestasis
IκB	inhibitor of κB
IL	interleukin
iNOS	inducible nitric oxide synthase
IR	inverted repeat
JNK	c-jun N-terminal kinase
KC	Kupffer cell
KO	knock-out
LBD	ligand-binding domain
LFABP	liver fatty acid binding protein
LPS	lipopolysaccharide
LRH	liver receptor homolog
LXR	liver X receptor
MDR	multidrug resistance (P-glycoprotein)
MMP	matrix metalloproteinase
MRP	multidrug resistance-associated protein
MW	Mann-Whitney U-test
NCoR	nuclear receptor co-repressor
NF-κB	nuclear factor-κB
NO	nitric oxide
NPC1L1	Niemann-Pick-1-like-1 (protein)
NR	nuclear receptor
NTCP	Na+-taurocholate co-transporting polypeptide
OATP	organic anion transporting protein
OCT	organic cation transporter
OST	organic solute transporter
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase
(P)FIC	(progressive) familial intrahepatic cholestasis
PGC	PPARγ co-activator
PPAR	peroxisome proliferator-activated re ceptor
PR	progesterone receptor
PXR	pregnane X receptor
RE	response element
RAR	retinoic acid receptor
RXR	retinoid X receptor
SD	standard deviation
SERM	selective estrogen receptor modulator
SHP	small heterodimer partner
sIL-1Ra	secreted interleukin-1 receptor antagonist
SLC	solute carrier
SMRT	silencing mediator of retinoid and thyroid hormone receptor
SNuRM	selective nuclear receptor modulator
SRC	steroid receptor co-activator
SREBP	sterol regulatory element binding protein
SULT2a1	dehydroepiandrosterone-sulfotransferase
TGF	transforming growth factor
TLR	Toll-like receptor
TNF	tumor necrosis factor
(T)PNAC	(total) parenteral nutrition associated cholestasis
TUDCA	tauro-ursodeoxycholic acid
UDCA	ursodeoxycholic acid
UTR	untranslated region
VDR	vitamin D receptor
WME	William's medium E

Dankwoord

Het is zover… KLAAR! Nadat het zo'n zes jaar een bijna onuitputtelijke bron van inspiratie, verwachtingen, uitdagingen, vermaak, zorgen en (af en toe ook) frustraties is geweest, komt er met dit "boekje" een punt te staan achter mijn promotietraject. Hoewel ik niet letterlijk stil wil blijven staan bij deze punt, is het wel goed om dit in de figuurlijke zin te doen. Graag wil ik iedereen bedanken die mij over de afgelopen jaren heeft begeleid, geholpen en vergezeld. Er zijn gelukkig velen geweest die hebben laten zien dat ze meerdere van deze drie rollen op zich konden nemen en dat de eerste twee rollen (meestal) prima samen gaan. Het traject dat ik doorlopen heb, heeft zich zowel letterlijk (Houston en Groningen) als figuurlijk (lab en kliniek) over twee continenten uitgestrekt. Hoewel dit laatste soms lastig is geweest, heb ik me hierin altijd gesteund gevoeld. Of het nu ging om een afspraak in de avonduren of het overnemen van een experiment, terwijl ik in de kliniek werkte of om het regelen van een "schrijfstage", het bleek allemaal mogelijk.

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in at IHOP in an isoflurane-induced state of headache and nausea at 11 p.m.…) I have thoroughly enjoyed our friendship and look forward to spending time together in the future. One question for you remains though: Will my *CRG*-genes be silenced from today on?

Zoals gezegd, heeft m'n promotietraject me op vele verschillende manieren bezig gehouden (en dan niet alleen tijdens kantooruren…). Ik ben me er goed van bewust dat dat soms tot een soort tunnelvisie heeft geleid, waarbij ik de wereld om me heen uit het oog verloor. Het afgelopen jaar heeft met het verlies van Harmen en Anniek mij persoonlijk, maar waarschijnlijk met mij ook vele anderen binnen ons lab, er heel hard aan herinnerd dat er meer is dan een promotie. *Beste Harmen*, toen ik in 2004 uit Houston terugkwam, was je net begonnen met je stage binnen ons lab. Over de volgende jaren heb ik je jezelf zien ontwikkelen tot een plezierige, soms schijnbaar norse, maar vooral hardwerkende collega met ook weer zo'n mooie, unieke vorm van humor. Wat heb je je moedig en kranig verzet tot op het laatst… *Beste Anniek*, je was volgens mij net begonnen, toen ik op het lab kwam en daarna ben je er de hele tijd bij geweest. Wat heb jij je, ondanks alle onzekerheden, indrukwekkend staande gehouden. Niets anders dan veel respect heb ik daarvoor.

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Jaap

Curriculum Vitae

Jaap Mulder werd op 30 maart 1978 geboren in Leeuwarden. Na het behalen van het Gymnasiumdiploma aan de CSG Liudger in Drachten in 1995 studeerde hij twee jaar op Kenyon College (Gambier, Ohio, VS). In 1997 begon hij met de studie Geneeskunde aan de Rijksuniversiteit Groningen. Als student deed hij onderzoek bij de afdelingen Klinische Farmacologie en Nefrologie (begeleiders: prof.dr. D. de Zeeuw, dr. H.L. Hillege en prof. dr. P.E. de Jong), en later bij de afdeling Kindergeneeskunde (begeleider: prof.dr. F. Kuipers). In de zomer van 2000 nam hij deel aan de Junior Scientific Masterclass (begeleiding prof.dr. T.H. The). Van december 2000 tot augustus 2001 was hij in het kader van de Wetenschappelijk Keuzestage werkzaam bij de afdeling Pediatric Nephrology aan The University of Texas Southwestern Medical Center (Dallas, Texas, VS; begeleiders M. Baum, M.D. en R. Quigley, M.D.). Het propaedeutisch (1998) en doctoraal examen (2002) werden cum laude behaald. Van 2001 tot 2003 doorliep hij zijn co-schappen in het Scheper Ziekenhuis in Emmen en het Medisch Spectrum Twente in Enschede, waarna het artsexamen in augustus 2003 werd behaald. Hierna werkte hij een jaar als Postdoctoral Fellow – M.D. bij de afdeling Pediatric Gastroenterology van Baylor College of Medicine (Houston, Texas, VS; begeleider S.J. Karpen, M.D., Ph.D.), deels gefinancierd door het Ter Meulen Fonds en de European Society for Paediatric Research. In september 2004 begon hij als Agiko bij het researchlaboratorium Kindergeneeskunde van het Universitair Medisch Centrum Groningen (promotores prof.dr. F. Kuipers en prof.dr. P.J.J. Sauer) gefinancierd door ZonMW (Agiko stipendium NR-920-03-287). Van april 2006 tot april 2007 doorliep hij het eerste jaar van de opleiding tot kinderarts in de Beatrix Kinderkliniek van het UMCG (opleider prof.dr. P.J.J. Sauer) en in april 2008 hervatte hij de opleiding in het Martini Ziekenhuis in Groningen (opleider dr. W.B. Geven).

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