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

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CHAPTER 6

DYSREGULATION OF BILIARY CHOLESTEROL SECRETION DURING INFLAMMATION IN MICE

Jaap Mulder
Henk Wolters
Nicolette Huijkman
Vincent Bloks
Jurre Hageman
Rick Havinga
Uwe J.F. Tietge
Saul J. Karpen
Ekkehard Sturm
Folkert Kuipers

Departments of 1Pediatrics and 2Laboratory Medicine, Center for Liver, Digestive and Metabolic Diseases, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands, 3Texas Children’s Liver Center/Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA, 4Department of Pediatric Gastroenterology/Hepatology, University of Tübingen, Germany

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\(^1\text{-}^3\). Pro-inflammatory cytokines, such as interleukin (IL)-1\(\beta\), IL-6 and tumor necrosis factor (TNF)-\(\alpha\), 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)\(^\text{[12]}\). More recently, Sumi et al. showed the importance of hepatocyte nuclear factor (HNF)-4\(\alpha\) (Nr2a1) as a key regulator of ABCG/Abcg5/8 mRNA expression, acting in concert with GATA-transcription factors GATA4 and/or GATA6\(^\text{[13]}\). This regulation of Abcg5/8 expression by HNF4\(\alpha\) may also in part explain the tissue-specific expression of these half-transporters as HNF4\(\alpha\) is an important transcription factor for hepatocyte and enterocyte differentiation\(^\text{[14,15]}\).

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\(^\text{[16]}\), 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 anaesthetized 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. 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 by 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 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. Enrichment was determined by comparing Na-K-ATPase and alkaline phosphatase activity in liver homogenates and isolated plasma membrane fractions. 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. Primary antibody used to detect Abcg5 was a gift from Dr Bert Groen, while those used for Ntcp, Bsep and Mrp2 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. 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. 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)**

EMSA were performed using 10 μg of nuclear extract protein according to the previously described protocol. Nuclear extracts were incubated with 2.5 x 10^4 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). Data are reported as mean ± S.D. In case of relatively small sample-sizes (N ≤ 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 Abcg8. Dose-dependency of LPS-induced suppression of Abcg5/8 expression is shown in figure 1A. The maximal suppression of mRNA expression was found
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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
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\textsuperscript{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.
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 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.
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). These results suggest that the suppression of Abcg5/8 expression in vivo 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, while mIGR does not appear to contain an LXR response element. Moreover, LPS-treatment of mice has previously been shown to lead to a rapid decrease in nuclear levels of the obligate

![Figure 8. Effects of inflammatory signaling on transcriptional activity of human ABCG5/8 intergenic region (hIGR) containing reporter construct.](image)

**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.

**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.
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heterodimer partner of LXR, i.e., the retinoid X receptor (RXR)-α (Nr2b1), and sub-
sequently reduced nuclear binding to a canonical direct repeat (DR)-4 element. LXR:RXRa heterodimers are known to have high affinity for such DR4 elements.

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 10). This suggests that T0901317 may be used to maintain hepatic Abcg5/8 expression in the setting inflammatory signaling.

**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. 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. 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. 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. Plösch et al. showed that biliary cholesterol secretion was induced by high-cholesterol feeding independently of Abcg5/8 mRNA induction. 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. 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. 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. Nevertheless, LPS-treatment did reduce the secretory rate maximum for bile salts by approximately 40% (Figure 4B), 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, 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\textsuperscript{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\textsuperscript{9}, but recently functional cis-acting elements have been identified that bind transcription factors Hnf4α/Gata4/6\textsuperscript{11} and FoxO1\textsuperscript{33}. Expression and activity of Hnf4α is known to be suppressed in response to inflammatory signaling via multiple mechanisms\textsuperscript{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\textsuperscript{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 \textit{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\textsuperscript{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\textsuperscript{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-
entially bind to DR4 elements, 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, 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. These AREs are, for instance, known to play a role in the regulation of cytokine mRNA expression, but also in that of Cyp7a1, the enzyme mediating the first step of the “classic” pathway of bile acid synthesis. 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). 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. 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. and Esteve et al.). 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.

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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