Role of nuclear receptors and hepatocyte-enriched transcription factors for Ntcp repression in biliary obstruction in mouse liver

Gernot Zollner,1 Martin Wagner,1 Peter Fickert,1 Andreas Geier,1 Andrea Fuchsbieler,2 Dagmar Silbert,1 Judith Gumhold,1 Kurt Zatloukal,3 Arthur Kaser,4 Herbert Tilg,4 Helmut Denk,3 and Michael Trauner1

1Laboratory of Experimental and Molecular Hepatology, Division of Gastroenterology and Hepatology, Department of Internal Medicine, and 2Department of Pathology, Medical University Graz, Graz, and 3University Hospital Innsbruck, Innsbruck, Austria; and 4Department of Internal Medicine III, University of Technology Aachen, Aachen, Germany

Submitted 19 July 2004; accepted in final form 26 June 2005

Zollner, Gernot, Martin Wagner, Peter Fickert, Andreas Geier, Andrea Fuchsbieler, Dagmar Silbert, Judith Gumhold, Kurt Zatloukal, Arthur Kaser, Herbert Tilg, Helmut Denk, and Michael Trauner. Role of nuclear receptors and hepatocyte-enriched transcription factors for Ntcp repression in biliary obstruction in mouse liver. Am J Physiol Gastrointest Liver Physiol 289: G798–G805, 2005. First published July 7, 2005; doi:10.1152/ajpgi.00319.2004.—Expression of the main hepatic bile acid uptake system, the Na+-taurocholate cotransporter (Ntcp), is downregulated during cholestasis. Bile acid-induced, farnesoid X receptor (FXR)-mediated induction of the nuclear repressor short heterodimer partner (SHP) has been proposed as a key mechanism reducing Ntcp expression. However, the role of FXR and SHP or other nuclear receptors and hepatocyte-enriched transcription factors in mediating Ntcp repression in obstructive cholestasis is unclear. FXR knockout (FXR−/−) and wild-type (FXR+/+) mice were subjected to common bile duct ligation (CBDL). Cholic acid (CA)-fed and LPS-treated FXR−/− and FXR+/+ mice were studied for comparison. mRNA levels of Ntcp and SHP and nuclear protein levels of hepatocyte nuclear factor (HNF)-1α, HNF-3β, HNF-4α, retinoid X receptor (RXR)-α, and retinoic acid receptor (RAR)-α and their DNA binding were assessed. Hepatic cytokine mRNA levels were also measured. CBDL and CA led to Ntcp repression in FXR+/+, but not FXR−/−, mice, whereas LPS reduced Ntcp expression in both genotypes. CBDL and LPS but not CA induced cytokine expression and reduced levels of HNF-1α, HNF-3β, HNF-4α, RXRα, and RARα to similar extents in FXR−/− and FXR+/+. DNA binding of these transcription factors was unaffected by CA in FXR−/− mice but was markedly reduced in FXR+/− mice. In conclusion, Ntcp repression by CBDL and CA is mediated by accumulating bile acids via FXR and does not depend on cytokines, whereas Ntcp repression by LPS is independent of FXR. Reduced levels of HNF-1α, RXRα, and RARα in CBDL FXR−/− mice and reduced DNA binding in CA-fed FXR−/− mice, despite unchanged Ntcp levels, indicate that these factors may have a minor role in regulation of mouse Ntcp during cholestasis.

bile acids; cytokines; bile duct obstruction; orphan nuclear receptors; cholestasis

THE MAIN HEPATOCYTOPLASMIC UPTAKE system for bile acids, the Na+-taurocholate cotransporter (Ntcp), is downregulated in various cholestatic liver diseases (41, 55, 57) and rodent models of cholestasis (12, 14, 42, 43). Cholestatic liver injury is associated with accumulation of bile acids and activation of proinflammatory cytokines (45), which may control Ntcp transcription via a complex network involving nuclear receptors and hepatocyte-enriched transcription factors. Bile acids bind to a bile acid receptor, farnesoid X receptor (FXR), which induces expression of short heterodimer partner (SHP) (20, 34). SHP in turn represses rat Ntcp expression by inhibiting transactivation by a constitutively active heterodimer complex formed by retinoid X receptor (RXRs)-retinoic acid receptor (RARs) in vitro (8). Another essential transactivator of rodent Ntcp is hepatocyte nuclear factor (HNF)-1α (27, 40), which is highly dependent on HNF-4α (25, 48). Bile acids inhibit HNF-4α-mediated transactivation of HNF-1α or might even directly reduce HNF-4α expression, which would be expected to decrease Ntcp promoter activity and gene expression (25, 51, 52). Induction of proinflammatory cytokines by bacterial lipopolysaccharide (LPS) downregulates Ntcp expression independently of bile acid by reducing the binding activities of RXRα-RARs and HNF-1α (6, 15, 32, 43). The only nuclear receptor known to directly repress Ntcp gene expression is HNF-3β (24, 38). However, its role in regulating Ntcp expression during cholestasis in vivo is unclear.

Although the individual effects of bile acids and LPS-induced cytokines on Ntcp expression have been studied in detail, the mechanisms mediating reduction of Ntcp in obstructive cholestasis remain unclear. Kupffer cell or cytokine blockade was unable to prevent Ntcp repression in rodents subjected to common bile duct ligation (CBDL) (17), suggesting that proinflammatory cytokines may not be involved. FXR-mediated SHP induction by bile acids has been proposed as a central mechanism (8), but the exact role of this pathway in mediating Ntcp repression in obstructive cholestasis has not been clarified. We therefore analyzed effects of CBDL in FXR−/− and FXR+/+ mice on Ntcp and its possible regulators and compared them with effects of a cholic acid (CA)-supplemented diet and LPS injection.

MATERIALS AND METHODS

Animals. C57/BL6 mice with targeted disruption of FXR (42) and wild-type littermates (obtained from Dr. Frank J. Gonzalez, National Institutes of Health, Bethesda, MD) were housed in a 12:12-h light-dark cycle and permitted ad libitum consumption of water and a standard mouse diet. The experimental protocols were approved by the local Animal Care and Use Committee according to criteria outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, revised 1985).

CBDL. All surgical procedures were performed under sterile conditions. The common bile duct was ligated close to the liver hilum...
immediately below the bifurcation and dissected between the ligatures as described previously (44, 56). In addition, cholecystectomy was performed after ligation of the cystic duct. Controls underwent a sham operation with exposure, but without ligation, of the common biliary duct and without removal of the gallbladder. To prevent eventual rupture of the gallbladder as a result of increased biliary pressure and subsequent biliary peritonitis, prophylactic cholecystectomy was performed as described previously (11, 13, 47, 56). The livers were excised 3 and 7 days after CBDL under general anesthesia with tribromoethanol (Avertin, Sigma-Aldrich, Steinheim, Germany; 10 mg ip) and immediately snap frozen and stored in liquid nitrogen until RNA extraction. A separate series of animals was used for isolation of liver nuclei and nuclear proteins.

**Bile acid feeding.** Mice were fed a CA-supplemented diet (1% wt/wt; Sigma Aldrich) for 7 days and compared with animals fed the control diet (12).

**Endotoxin treatment.** LPS from Salmonella typhimurium (15 mg/kg body wt; Sigma Aldrich), which was previously shown to reduce Ntcp expression, was injected intraperitoneally, and animals were harvested after 16 h as described elsewhere (56). Controls were injected with the vehicle (saline).

**RNA preparation and mRNA analysis.** Total RNA was isolated using Trizol (5) and RNA was quantified spectrophotometrically at 260 nm. The quality of total RNA was controlled by denaturing formaldehyde agarose gel electrophoresis. Ntcp, SHP, and GAPDH mRNA expression was assayed by Northern blotting as described previously (56). To control equal RNA loading, membranes were stained with ethidium bromide for 28S rRNA (10). RNase protection assays were performed using the Riboquant MultiProbe RNase protection assay system (Pharmigen, BD Biosciences, San Diego, CA) following the manufacturer’s instructions. After phenol-chloroform extraction of RNase-protected probes, samples were resolved on denaturing 350-μm polyacrylamide gels and quantified by phosphor imaging (Cyclone Storage Phosphor System, Packard Instruments, Perkin Elmer, Boston, MA). A standard curve of migration distances was plotted with the undigested probes as markers to establish the identity of respective RNase-protected bands. Band intensity was quantified as net digital light units with background subtraction using OptiQuant software (Packard Instruments, Perkin Elmer).

**Isolation of liver nuclei and preparation of nuclear proteins.** Immediately after the samples were harvested, fresh liver tissue was homogenized in 10 mM HEPES (pH 7.9), 10 mM MgCl2, 2 mM DTT, 25 mM sucrose, 0.1% Triton X-100, and 1% protease inhibitor cocktail (Sigma). After centrifugation at 800 g for 5 min at 4°C, nuclei were resuspended in the homogenization buffer, layered on the top of a 2.1 M sucrose cushion, and then centrifuged at 50,000 g for 80 min at 4°C. Isolated nuclei were resuspended in two-thirds packed nuclear volume of buffer: 20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, and 1% protease inhibitor cocktail. After centrifugation for 5 min, the resulting supernatants were diluted 1:1 with 20 mM HEPES (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, and 1% protease inhibitor cocktail. Nuclear protein content was determined by Bradford analysis.

**Electrophoretic mobility shift analysis.** To determine binding activity of liver-enriched transcription factors and nuclear hormone receptors, electrophoretic mobility shift assays (EMSA) were performed as described previously (16). Nuclear extracts (protein, 5–10 μg) were incubated on ice for 30 min with a 2 × 105 cpm 32P-end-labeled oligonucleotide probe representing the consensus binding site for HNF-1α, HNF-4α, or direct repeat 2 (DR-2, the binding site for RXRα-RARα). The following double-stranded oligonucleotide probes were used (sense strand): 5′-GGTTAATAATTCACA-3′ for HNF-1α (33), 5′-AGAGGAGAAGGGTACAGT-3′ for HNF-4α (37), and 5′-GGAGGGCTAGGAGGAGCAG-3′ for DR-2 (26). For competition assays, 100-fold molar excess of unlabeled oligonucleotides was coincubated with the labeled probe. Protein-DNA complexes were separated from unbound labeled probes by electrophore-
Fig. 1. Effects of common bile duct ligation (CBDL) on Na\(^+\)taurocholate cotransporter (Ntcp) and short heterodimer partner (SHP) mRNA expression in farnesoid receptor (FXR) wild-type (FXR\(^{+/+}\)) and FXR knockout (FXR\(^{-/-}\)) mice. Total RNA was isolated from CBDL (3 and 7 days) and sham-operated (0 days) FXR\(^{+/+}\) and FXR\(^{-/-}\) mice and analyzed by Northern blotting using specific probes for Ntcp, SHP, and GAPDH. Membranes were stained for 28S rRNA to control RNA loading. Densitometry data below blots are expressed as fold change relative to sham-operated FXR\(^{+/+}\) mice. Values are averages from 3 animals per time point in each group. In FXR\(^{+/+}\) mice, CBDL led to repression of Ntcp mRNA that was paralleled by induction of SHP mRNA. In FXR\(^{-/-}\) mice, Ntcp remained at baseline and SHP was not induced after CBDL. *P < 0.05 vs. FXR\(^{+/+}\) at 0 days.

Fig. 2. Effects of cholic acid (CA) and LPS on Ntcp and SHP mRNA expression in FXR\(^{+/+}\) and FXR\(^{-/-}\) mice. Total RNA was isolated from control and CA-fed (1% wt/wt, 7 days; A) and from LPS-injected (Staphylococcus typhimurium, 15 mg/kg body wt) and sham (saline)-treated (B) FXR\(^{+/+}\) and FXR\(^{-/-}\) mice and analyzed by Northern blotting using specific probes for Ntcp, SHP, and GAPDH. Membranes were stained for 28S rRNA to control RNA loading. Densitometry data below blots are expressed as fold change relative to FXR\(^{+/+}\) mice fed the standard diet. Values are averages from 4 animals in each group. A: CA induced SHP mRNA and downregulated Ntcp in FXR\(^{+/+}\) mice, but not FXR\(^{-/-}\) mice. *P < 0.05 vs. control FXR\(^{+/+}\). B: LPS downregulated Ntcp without inducing SHP mRNA in FXR\(^{-/-}\) and FXR\(^{-/-}\) mice. *P < 0.05 vs. control FXR\(^{+/+}\).

major primary bile acid retained during cholestasis) and treating them with LPS (for cytokine induction). These interventions do not represent models of cholestasis in mice but were chosen to clarify the influence of bile acids and cytokines on Ntcp expression. Effects of CA on Ntcp and SHP mRNA levels have been investigated previously in mice without FXR (42), but the CA diet was repeated for the present study as a positive control. As expected, CA reduced Ntcp steady-state mRNA to 23% of controls (P < 0.05) in FXR\(^{+/+}\) mice (Fig. 2A), whereas CA significantly increased SHP mRNA levels to 181% of controls (P < 0.05) in FXR\(^{+/+}\) mice, CBDL led to downregulation of Ntcp mRNA by 10.2 ± 0.33 on June 25, 2017 http://ajpgi.physiology.org/ Downloaded from
of its transactivators. However, reduced levels of HNF-1α, RXRα, and RARα did not cause Ntcp repression in CBDL FXR-/−/− mice, indicating that other pathways must be involved in Ntcp regulation. Because marked reductions in nuclear levels of these transcription factors can be predicted to result in similar reductions of specific DNA binding, EMSAs were not performed in CBDL and LPS-treated animals.

In contrast, CA had no effect on nuclear levels of HNF-1α, HNF-4α, RXRα, and RARα in FXR+/+ and FXR-/−/− mice (Fig. 5). CA significantly reduced protein levels of the Ntcp repressor HNF-3β (24, 38) in FXR-/−/− mice (Fig. 5), which could explain the increased Ntcp expression in CA-fed FXR-/-/− mice (Fig. 2A). To explore whether alterations in DNA binding activity (in the context of maintained nuclear protein levels) could contribute to Ntcp repression in CA-fed FXR+/+/+ mice (25), EMSAs were performed. Oligonucleotides representing consensus binding sites for HNF-1α, HNF-4α, and DR-2 were used for EMSA as described previously (26, 33, 37). In a previous study (8), bile acid treatment did not alter RXRα-RARα DNA binding but was repeated as a control in the present study. Analysis of nuclear extracts showed no change in binding activities of HNF-1α, HNF-4α, and RXRα-RARα in CA-fed FXR+/+/+ mice (Fig. 6). Surprisingly, in CA-fed FXR-/-/− mice, however, binding activity of these transcription factors and nuclear receptors was markedly decreased, despite increased Ntcp expression.

Recent studies demonstrated that phosphorylation of RXRα resulted in its translocation from the nucleus to the cytoplasm (18, 19, 53). Immunofluorescence microscopic demonstration of RXRα localization revealed a specific nuclear staining pattern in control and CA-fed animals (data not shown). Nuclear staining was markedly reduced after LPS administration and CBDL (data not shown). Lack of RXRα translocation from the nucleus to the cytoplasm at 3 and 7 days suggests that CBDL in combination with LPS results in a decrease, rather than a redistribution, of RXRα protein. However, low levels of RXRα in the cytoplasm might not be detectable by immunofluorescence microscopy, which is not a quantitative method.

Taken together, data from FXR-/−/− mice are not consistent with the postulated major role for HNF-1α, RARα, and RXRα in regulating Ntcp expression and strongly argue that other, more potent mechanisms must be involved.

CBDL and LPS, but not CA, induce proinflammatory cytokines in mouse liver. Because variations in the inflammatory response in the models studied here could contribute to Ntcp repression, hepatic levels of proinflammatory cytokines were compared (Table 1). Steady-state mRNA levels of IL-1β, IL-6, IFN-γ, and TNF-α, which are known regulators of bile secretory function and/or transporter expression (6, 15, 21, 23, 39), did not differ between naïve FXR+/+/+ and FXR-/-/− mice and were significantly induced in response to LPS in both genotypes. CBDL also resulted in increased levels of IL-1β and IL-6, although the extent of the increase was less than with...
mRNA levels were comparable between CBDL FXR observed 7 days after CBDL, when Ntcp FXR from FXR below blots are expressed as fold change relative to control diet-fed FXR, HNF-4/H9251, HNF-3/H9251. Densitometry data, and RAR/RH9251, RXR/RH9252/RH9251 diets for 7 days and analyzed by Western blotting using specific antibodies CA-fed, and CBDL mice.

DISCUSSION

cytokines mediate Ntcp mice. In contrast, CA feeding had no effect on cytokine mRNA mice. Values are averages from 3 animals in each group. CA did not change nuclear levels of HNF-1 mice. *Vol 289 • November 2005 • www.AJPGI.org

Fig. 5. Effects of CA on nuclear protein levels of HNF-1α, HNF-3β, HNF-4α, RXRα, and RARα in FXR+/+ and FXR−/− mice. Nuclear protein was isolated from FXR+/+ and FXR−/− mice fed control and CA-supplemented (1% wt/wt) diets for 7 days and analyzed by Western blotting using specific antibodies against HNF-1α, HNF-3β, HNF-4α, RXRα, and RARα. Densitometry data below blots are expressed as fold change relative to control diet-fed FXR+/+ mice. Values are averages from 3 animals in each group. CA did not change nuclear levels of HNF-1α, HNF-4α, RXRα, and RARα in FXR+/+ and FXR−/− mice but significantly reduced HNF-3β protein in FXR−/− mice. *P < 0.05 vs. control FXR+/+.

LPS. No significant increases of TNF-α mRNA levels were observed 7 days after CBDL, when Ntcp was still reduced. mRNA levels were comparable between CBDL FXR+/+ and FXR−/− mice, whereas Ntcp was reduced only in FXR+/+ mice. In contrast, CA feeding had no effect on cytokine mRNA levels in both genotypes.

Taken together, these findings indicate that proinflammatory cytokines mediate Ntcp repression in LPS-treated, but not CA-fed, and CBDL mice.

**DISCUSSION**

A variety of nuclear receptors (i.e., RXRα, RARα, and SHP) and hepatocyte-enriched transcription factors (i.e., HNF-1α and HNF-3β) have been proposed to be involved in regulation of rodent Ntcp gene expression (8, 27, 43). FXR-mediated SHP induction by bile acids was proposed as a central mechanism reducing Ntcp (8, 42, 56). As such, SHP reduces RXRα-RARα-mediated transactivation of the rat Ntcp promoter (8) but may also reduce HNF-1α transactivation by HNF-4α (25, 51, 52). The exact role of FXR/SHP in mediating Ntcp repression in obstructive cholestasis has been unknown. The present study demonstrates that downregulation of Ntcp in mouse liver during obstructive cholestasis requires FXR-dependent mechanisms. We further confirm the role of FXR in CA-mediated Ntcp repression but also clearly exclude the involvement of other pathways (i.e., reduced HNF-1α activity due to reduced transactivation by HNF-4α or induction of proinflammatory cytokines).

CBDL represents a model of obstructive cholestasis that is associated with elevation of serum bile acid levels and induction of inflammatory cytokines (36, 56). Increased levels of proinflammatory cytokines with concomitant reduction of nuclear levels of RXRα-RARα have been suggested to down-regulate hepatic and duodenal multidrug resistance protein-2/ATP binding cassette C2 expression in CBDL rats (7, 9), and reduced binding activities of RXRα-RARα and HNF-1α have also been linked to Ntcp repression after LPS treatment (6, 15, 32, 43). Reduced Ntcp expression in CBDL FXR+/+ mice but no change in Ntcp expression in CBDL FXR−/− mice in the present study indicates that downregulation of Ntcp in obstructive cholestasis is mediated by FXR. As expected, accumulating bile acids in CBDL induced the FXR target gene SHP in FXR+/+, but not in FXR−/−, mice. The reduction of SHP mRNA 7 days after CBDL has previously been described and may be attributed to declining levels of serum bile acids and SHP-mediated inhibition of its transcription (4, 56). SHP has been shown to reduce Ntcp promoter activity by inhibiting transactivation by RXRα-RARα without inhibiting DNA binding of this transactivator (8). Because protein levels of the SHP target RXRα-RARα were already markedly reduced after CBDL in the present study, this proposed SHP-mediated pathway cannot represent the major mechanism leading to Ntcp repression. This proposed SHP-mediated pathway is further

Fig. 6. Binding activity of postulated transactivators of mouse Ntcp promoter. Hepatic nuclear extracts were prepared from FXR+/+ and FXR−/− mice fed control and CA-supplemented (1% wt/wt) diets for 7 days and incubated with radiolabeled oligonucleotides containing consensus binding sites for HNF-1α, HNF-4α, and direct repeat 2 (DR-2, RARα-RXRα). Densitometry data below blots are expressed as fold change relative to control diet-fed FXR+/+ mice. Values are averages from 3 animals in each group. Samples in lanes labeled SC and NSC were incubated in the presence of unlabeled specific and nonspecific competitor DNA, respectively, at 100-fold molar excess. DNA binding of HNF-1α, HNF-4α, and DR-2 (RXRα-RARα) was not changed in response to CA in FXR+/+ mice but was markedly reduced in FXR−/− mice. *P < 0.05 vs. control FXR+/+.  

AJP-Gastrointest Liver Physiol • VOL 289 • NOVEMBER 2005 • www.AJPGI.org
downregulated Ntcp expression in FXR hepatocellular effects of the major primary bile acid retained exact SHP-independent mechanisms remain to be elucidated. Ntcp / H9251 cytokines may explain reduced levels of HNF-1 induced and FXR-mediated, but SHP-independent, mecha- yet unidentified mechanisms. Alternatively, other bile acid- in CBDL. However, SHP may exert its repressive effects via

has been demonstrated not only to reduce RXR -RAR repression, because their protein levels were also markedly

questioned by maintenance of Ntcp repression, despite SHP restoration 7 days after CBDL. Moreover, reductions of HNF-1α and RXRα-RARα cannot play a major role in Ntcp repression, because their protein levels were also markedly reduced in FXR -/# mice, which lack downregulation of Ntcp in CBDL. However, SHP may exert its repressive effects via yet unidentified mechanisms. Alternatively, other bile acid-induced and FXR-mediated, but SHP-independent, mechanisms may be involved. Elevated levels of proinflammatory cytokines may explain reduced levels of HNF-1α, RXRα, and RARα (1, 2, 15, 43, 48) but do not appear to be involved in Ntcp repression, because a comparable induction of proinflammato- mifepristone (MIF) and IL-1β levels of proinflammatory cytokines in mice (35). In

Table 1. mRNA expression of proinflammatory cytokines and chemokines in mouse liver

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CBDL</th>
<th>CA</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>100.0±4.9</td>
<td>784.1±65.6*</td>
<td>108.4±12.6</td>
<td>711.2±162.4*</td>
</tr>
<tr>
<td>FXR-/+</td>
<td>89.5±10.3</td>
<td>771.4±31.4*</td>
<td>106.1±2.3</td>
<td>783.0±99.9*</td>
</tr>
<tr>
<td>IL-1α</td>
<td>100±13.3</td>
<td>160.6±19.5</td>
<td>160.6±19.5</td>
<td>523±129.6*</td>
</tr>
<tr>
<td>FXR-/+</td>
<td>136.2±31.6</td>
<td>213±4.9*</td>
<td>213±4.8</td>
<td>744.8±206.7*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>100.0±13.4</td>
<td>370.8±69.9*</td>
<td>112.5±19.9</td>
<td>790.1±121.6*</td>
</tr>
<tr>
<td>FXR-/+</td>
<td>151.2±43.6</td>
<td>336.3±5.0*</td>
<td>168.5±56.3</td>
<td>983±259.2*</td>
</tr>
<tr>
<td>IL-6</td>
<td>100.0±9.5</td>
<td>419.1±112.7*</td>
<td>91.7±18.9</td>
<td>5126±1854.1*</td>
</tr>
<tr>
<td>FXR-/+</td>
<td>96.1±10.4</td>
<td>272.4±12.8*</td>
<td>78.6±12.7</td>
<td>6773±757.5*</td>
</tr>
<tr>
<td>MIF</td>
<td>100.0±4.4</td>
<td>261.2±27.2*</td>
<td>95.7±12.9</td>
<td>1051±263.7*</td>
</tr>
<tr>
<td>FXR-/+</td>
<td>95.1±6.9</td>
<td>226.7±5.8*</td>
<td>88.6±9.0</td>
<td>1216.8±108.5*</td>
</tr>
<tr>
<td>IL-10</td>
<td>100.0±12.2</td>
<td>178.8±19.0</td>
<td>90.7±6.8</td>
<td>852.0±184.6*</td>
</tr>
<tr>
<td>FXR-/+</td>
<td>88.6±4.9</td>
<td>179.5±5.5</td>
<td>175.3±19.4</td>
<td>794.1±186.0*</td>
</tr>
<tr>
<td>MIF</td>
<td>100.0±7.3</td>
<td>75.8±6.0</td>
<td>138.2±7.6</td>
<td>1051.7±7.7</td>
</tr>
<tr>
<td>FXR-/+</td>
<td>107.4±8.3</td>
<td>85.2±3.3</td>
<td>144.7±26.2</td>
<td>118.7±14.3</td>
</tr>
<tr>
<td>TGF-β</td>
<td>100.0±8.3</td>
<td>185.8±38.4*</td>
<td>98.5±10.5</td>
<td>90.8±8.2</td>
</tr>
<tr>
<td>FXR-/+</td>
<td>97.1±5.6</td>
<td>148.5±8.7</td>
<td>75.1±19</td>
<td>104.9±14.1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>100.0±11.2</td>
<td>109.4±32.1</td>
<td>90.4±9.2</td>
<td>142±10.2*</td>
</tr>
<tr>
<td>FXR-/+</td>
<td>91.0±6.2</td>
<td>73.5±1.1</td>
<td>63.7±9.6</td>
<td>248±64.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE, expressed as percentage of naive farnesoid X receptor wild-type (FXR-/#) mice. CBDL, common bile duct ligation; CA, cholic acid; IL-1RA, IL-1 receptor antagonist; MIF, macrophage inhibitory factor. *p < 0.05 vs. naive FXR-/# mice.

reduce HNF-4α promoter activity and expression, resulting in reduced HNF-1α transactivation (25, 51, 52). Nuclear protein levels, as well as DNA binding activities, of HNF-1α, HNF-4α, and RXRα-RARα remained unchanged in CA-fed FXR-/# mice in the present study, suggesting that reduced activities of these factors cannot be responsible for Ntcp repression in wild-type mice. Unexpectedly, DNA binding of HNF-1α, HNF-4α, and RXRα-RARα was markedly reduced in CA-fed FXR -/# mice, despite no change in Ntcp mRNA levels. Reduced DNA binding might be due to increased hepatic bile acid retention and toxicity in FXR -/# mice, reflected in markedly elevated serum transaminases and the appearance of single-cell necroses as described previously (54), thus representing a nonspecific finding. However, the exact mechanism remains to be determined. These findings, together with reduced protein levels of HNF-1α, RXRα, and RARα after CBDL in FXR -/# mice, despite no change in Ntcp expression, imply that other factors may play a dominant role in regulation of mouse Ntcp. As such, HNF-3β, which has recently been identified as a negative regulator of murine Ntcp promoter activity (24, 38), was downregulated in CBDL and LPS treatment. HNF-3β repression may thus represent an attempt to counteract downregulation of Ntcp and could explain increased Ntcp levels in CA-fed FXR -/# mice. However, complete absence of HNF-3β in CBDL FXR-/# mice did not restore Ntcp expression. This may be due to different expression patterns of yet unidentified Ntcp regulators in CBDL, LPS-treated, and CA-fed animals as observed for HNF-1α, HNF-4α, RXRα, and RARα. Ntcp repression due to loss of essential transactivators in CBDL and LPS-injected mice might not be overcome by reduced levels of the repressor HNF-3β. However, the exact mechanism remains to be determined. Further hints to so far unidentified functionally important Ntcp transactivators are also derived from cytokine-inactivation studies in endotoxin-treated rats, where Ntcp gene expression cannot be explained solely by the observed HNF-1α and RXRα-RARα binding activities (15). Consistent with our results, regulation of the murine Ntcp promoter by HNF-1α and RXRα-RARα was recently questioned by Jung and co-workers (24), who did not identify respective response elements in a minimal mouse Ntcp promoter. Markedly reduced levels of HNF-1α, RXRα, and RARα in CBDL FXR -/# mice and reduced DNA binding in CA-fed FXR -/# mice with preserved Ntcp expression strongly argue that these factors may not play the predominant role in Ntcp regulation in cholestasis or that they may be overruled by other yet unidentified factors.

In addition to bile acid-activated FXR, redundant pathways could also mediate Ntcp repression, as shown for bile acidsynthesizing enzymes (28, 50). Such redundant mechanisms also include induction of proinflammatory cytokines by bile acids. Hydrophobic bile acids have been shown to be capable of inducing IL-1β and TNF-α expression in mice (35). In contrast, we show that CA has no influence on hepatic mRNA levels of proinflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, and IFN-γ) in vivo. These apparent discrepancies could be related to the atherogenic (2% cholesterol-enriched) CA-supplemented diet used by Miyake at al. (35), which was recently demonstrated to induce severe nonsuppurative cholangitis, whereas an exclusively CA-enriched diet (also used in our study) did not induce such an inflammatory response in mouse liver (49). Taken together, induction of proinflammatory cyto-
cytokines and associated regulatory pathways [e.g., activation of c-Jun NH2-terminal kinase pathway leading to induction of SHP (22) or reduced nuclear activity of RXRα-RARα and HNF-1α] is not relevant to Ntcp repression in CA-fed mice.

To compare the effects of bile acids with the effects of cytokines, we also investigated expression of nuclear receptors and hepatocyte-enriched factors regulating Ntcp expression in LPS-challenged mice. LPS administration does not reflect the circumstance associated with CBDL, nor does it cause cholestasis, at this early time point in mice. We therefore have chosen an early time point after LPS administration, when serum bile acid levels were still within the normal range (56), to study sole cytokine effects. LPS administration leads to induction of proinflammatory cytokines and repression of Ntcp expression. LPS repressed Ntcp expression in an FXR/SHP-independent manner, but the nuclear protein levels of the previously postulated Ntcp transactivators HNF-1α, RXRs, and RARs were dramatically reduced. Reductions in nuclear RXRα protein by LPS and IL-1β were recently attributed to translocation of RXRα from the nucleus to the cytoplasm (18, 19, 53). This was observed only within the 1st h after treatment, which might explain the lack of intracellular RXRα redistribution in our study, where a later time point was investigated. In summary, these findings clearly indicate that FXR/SHP-independent mechanisms are involved in endotoxemia. However, in light of the findings in CBDL and CA-fed FXR−/− mice outlined above, mechanisms other than the known transactivators must be involved.

Taken together, our findings clearly demonstrate involvement of distinct transcriptional pathways in regulation of Ntcp expression during cholestasis. Accumulating bile acids may lead to Ntcp repression via activation of FXR without induction of proinflammatory cytokines. As expected, inflammatory cytokines do not require FXR/SHP and lead to Ntcp repression via other mechanisms. In obstructive cholestasis, accumulating bile acids, leading to activation of FXR, lead to repression of Ntcp; however, the exact mechanisms remain to be determined.

REFERENCES

This work was supported by Austrian Science Foundation Grant P15502 (to M. Trauner), Deutsche Forschungsgemeinschaft Grants SFB542TPC1 and DI 729/31 (to A. Geier), and Grant DI 729/3-1.


2. Beigneux AP, Moser AH, Shigenaga JK, Grunfeld C, and Feingold KR. Reduction in cytochrome P-450 enzyme expression is associated with repression of CAR (constitutive androstane receptor) and PXR (pregnane X receptor) in mouse liver during the acute phase response. Biochem Biophys Res Commun 293: 145–149, 2002.


GRANTS

This work was supported by Austrian Science Foundation Grant P15502 (to M. Trauner), Deutsche Forschungsgemeinschaft Grants SFB542TPC1 and DI 729/31 (to A. Geier), and Grant DI 729/3-1.

AJP-Gastrointest Liver Physiol • VOL 289 • NOVEMBER 2005 • www.ajpgi.org
REGULATION OF \( \text{Ntcp} \) EXPRESSION IN CHOLESTASIS


36. Pleyani M, Panozzo MP, Basso D, De Paoli M, Biasin R, and Infan- 

37. Plebani M, Panozzo MP, Basso D, De Paoli M, Biasin R, and Infan- 

38. Rausa FM, Tan Y, Zhou H, Yoo KW, Stolz DB, Watkins SC, Frank- 


41. Shenieder BL, Fox VL, Schwarz KB, Watson CL, Ananthanarayanan M, Thevananther S, Christie DM, Hardikar W, Setchell KD, Mieli- 


44. Trauner M, Arrese M, Soroka CJ, Ananthanarayanan M, Koeppel TA, Schlosser SF, Suchy FJ, Kepler D, and Boyer JL. The rat canalicular conjugate export pump (Mrp2) is down-regulated in intrahe- 


51. Yang Y, Zhang M, Eggersen G, and Jhang JY. On the mechanism of bile acid inhibition of rat sterol 12α hydroxylase gene (CYP8B1) trans- 

52. Yang Y, Zhang M, Eggersen G, and Jhang JY. On the mechanism of bile acid inhibition of rat sterol 12α hydroxylase gene (CYP8B1) trans- 

53. Yang Y, Zhang M, Eggersen G, and Jhang JY. On the mechanism of bile acid inhibition of rat sterol 12α hydroxylase gene (CYP8B1) trans- 

54. Yang Y, Zhang M, Eggersen G, and Jhang JY. On the mechanism of bile acid inhibition of rat sterol 12α hydroxylase gene (CYP8B1) trans- 

55. Yang Y, Zhang M, Eggersen G, and Jhang JY. On the mechanism of bile acid inhibition of rat sterol 12α hydroxylase gene (CYP8B1) trans- 

56. Yang Y, Zhang M, Eggersen G, and Jhang JY. On the mechanism of bile acid inhibition of rat sterol 12α hydroxylase gene (CYP8B1) trans- 

57. Yang Y, Zhang M, Eggersen G, and Jhang JY. On the mechanism of bile acid inhibition of rat sterol 12α hydroxylase gene (CYP8B1) trans-