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

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The main hepatocellular 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 (RXRα)-retinoic acid receptor (RARα) 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-RARα 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 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).

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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).

**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, 5 mM KCl, 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-thiols packed nuclei 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 1:20 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 previously described (16). Nuclear extracts (5–10 μg) were incubated on ice for 30 min with a 2 × 106 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′-GGTTAATAATTACCA3′ for HNF-1α (33), 5′-AGAGGGAGAAGGTAGTGTA3′ for HNF-4α (37), and 5′-GGGAGTGAGGCTACGAG-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 electrophoresis through a non-denaturing 6% polyacrylamide gel and quantified by phosphor imaging (Bio-Rad, Munich, Germany).

**Western blot analysis.** Similar amounts of nuclear protein (10 μg) were loaded onto 10% SDS-polyacrylamide gels and subjected to electrophoresis (12, 30). After electrophoresis onto nitrocellulose membranes (Bio-Rad, Richmond, CA), the blots were incubated with polyclonal antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA) against HNF-1α (dilution 1:500; clone H-205), HNF-3β (dilution 1:500; clone M-20), HNF-4α (dilution 1:1,000; clone C-1B), RXRα (dilution 1:500; clone D-20), and RARα (dilution 1:1,000; clone C-20), and immune complexes were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; fragments as described elsewhere (12). Equal protein loading was confirmed by reprobing membranes for β-actin as described previously (12).

Immunofluorescence microscopy for demonstration of RXRα localization. Cryosections of liver tissue were fixed with 4% buffered formaldehyde solution for 5 min and then with methanol for 5 min (−20°C) and acetone for another 3 min (−20°C). Immunofluorescence staining for RXRα was performed with an RXRα antibody (dilution 1:20; Santa Cruz Biotechnology) using a protocol described previously (12).

Statistical analysis. In each group, three to four animals were studied. Data are reported as arithmetic means ± SE. Differences between experimental groups were analyzed by ANOVA with Bonferroni’s post test using the Sigmastat statistic program (Jandel Scientific, San Rafael, CA). P < 0.05 was considered significant.

**RESULTS**

**CBDL-mediated Ntcp repression strictly depends on FXR.** We previously demonstrated that induction of SHP is paralleled by reduction of Ntcp mRNA levels in CBDL mice, but the relative contribution of this pathway to Ntcp repression remained open to question (56). Moreover, the postulated role of SHP in mediating repression of key enzymes involved in bile acid synthesis has been questioned recently (28, 28, 50). To determine the consequences of the absence of FXR/SHP on Ntcp regulation in CBDL, mRNA levels of Ntcp and HNF-1α were studied in CBDL FXR−/− and FXR+/+ mice (Fig. 1). Baseline Ntcp steady-state mRNA levels were comparable in both genotypes as reported previously (42). In CBDL FXR−/− mice, Ntcp steady-state mRNA levels were reduced to 56 ± 7% and 58 ± 8% of those in sham-operated animals after 3 and 7 days, respectively (P < 0.05 for both time points; Fig. 1). Concomitant with reduction of Ntcp levels, SHP mRNA was induced 3 days after CBDL (222 ± 7%, P < 0.05) and returned to baseline 7 days after CBDL (106 ± 8%), when Ntcp remained repressed (Fig. 1). In contrast, Ntcp expression in CBDL FXR−/− mice remained unchanged after 3 and 7 days (88 ± 3% and 101 ± 10%, respectively, P = not significant), and the already reduced baseline expression of SHP (62 ± 21% of controls, P < 0.05) further declined to 7 ± 5% and 10 ± 6% at 3 and 7 days after CBDL, respectively (P < 0.05 for both; Fig. 1). RNA loading was controlled by staining membranes for 28S rRNA, because expression of GAPDH tended to be higher in CBDL (Fig. 1) and CA-fed FXR−/− mice (Fig. 2A). These data therefore underline the predominant role of an intact FXR pathway in the downregulation of Ntcp in CBDL mice.

CA- but not LPS-mediated downregulation of Ntcp depends on FXR and SHP. Because bile acids and proinflammatory cytokines are elevated in obstructive cholestasis, we attempted to compare effects of bile acids with effects of cytokines by feeding mice a diet supplemented with CA (representing a
fold change relative to sham-operated FXR
rRNA to control RNA loading. Densitometry data below blots are expressed as 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. 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 levels to \(\approx 27\%\) of controls \((P < 0.05)\) in FXR\(^{+/+}\) mice (Fig. 2A), whereas CA significantly increased Ntcp expression in FXR\(^{-/-}\) mice (Fig. 2A). CA feeding increased SHP mRNA levels to \(\approx 181\%\) of controls \((P < 0.05)\) in FXR\(^{+/+}\) mice but had no effect on SHP expression in FXR\(^{-/-}\) mice (Fig. 2A). LPS decreased Ntcp steady-state mRNA to \(\approx 23\%\) of FXR\(^{+/+}\) mice and \(\approx 27\%\) in FXR\(^{-/-}\) mice, respectively \((P < 0.05)\) for both, without inducing SHP expression in both LPS-challenged genotypes (Fig. 2B). LPS even reduced SHP expression as reported previously (29). These findings clearly demonstrate that an intact FXR/SHP pathway is required for Ntcp repression by bile acids and that repression of Ntcp by LPS-induced proinflammatory cytokines is independent of FXR/SHP.

CBDL and LPS, but not CA, reduce nuclear protein levels of HNF-1\(\alpha\), HNF-4\(\alpha\), RXR\(\alpha\), and RAR\(\alpha\). Reduced expression and nuclear binding activities of HNF-1\(\alpha\), HNF-4\(\alpha\), RXR\(\alpha\), and RAR\(\alpha\) have previously been reported after LPS challenge and may explain, at least in part, the FXR independence of cytokine-mediated Ntcp repression (1, 2, 15, 43, 48). However, similar alterations of these liver-enriched factors and nuclear receptors might also be caused by bile acids (25, 51, 52) or bile acid-induced inflammatory cytokines (35). We therefore analyzed protein levels of these liver-enriched factors and nuclear receptors in CBDL FXR\(^{+/+}\) and FXR\(^{-/-}\) mice fed the CA-supplemented diet and treated with LPS.

No differences in baseline HNF-1\(\alpha\), HNF-3\(\beta\), HNF-4\(\alpha\), RXR\(\alpha\), and RAR\(\alpha\) levels were observed between naïve FXR\(^{+/+}\) and FXR\(^{-/-}\) mice (Figs. 3–5). CBDL for 7 days (Fig. 3) and LPS (Fig. 4) markedly reduced nuclear levels of HNF-1\(\alpha\), HNF-3\(\beta\), HNF-4\(\alpha\), RXR\(\alpha\), and RAR\(\alpha\) in both genotypes. The specificity of the changes is confirmed by unchanged nuclear β-actin levels (3). Downregulation of Ntcp expression in LPS-treated animals may thus be attributed to reduced levels...
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−/− animals 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

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.

Fig. 3. Effects of CBDL on nuclear protein levels of hepatocyte nuclear factor (HNF)-1α, HNF-3β, HNF-4α, retinoic X receptor-α (RXRα), and retinoic acid receptor-α (RARα) in FXR+/+ and FXR−/− mice. Nuclear protein was isolated from FXR+/+ and FXR−/− mice subjected to sham operation (control) and at 7 days after CBDL 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 sham-operated mice. Values are averages from 3 animals in each group. CBDL led to reduction of nuclear levels of HNF-1α, HNF-3β, HNF-4α, RXRα, and RARα in FXR+/+ and FXR−/− mice. *P < 0.05 vs. control. nd, Not detectable.

Fig. 4. Effects of LPS 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 injected with LPS (S. typhi murium, 15 mg/kg body wt) and vehicle (saline) 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 sham-injected FXR+/+ mice. Values are averages from 3 animals in each group. LPS injection led to a pronounced decline of nuclear protein levels of HNF-1α, HNF-3β, HNF-4α, RXRα, and RARα in FXR+/+ and FXR−/− mice. *P < 0.05 vs. control.
mRNA levels were comparable between CBDL FXR observed 7 days after CBDL, when FXR from FXR mice. Densitometry data below blots are expressed as fold change relative to control diet-fed FXR, and RAR/RAR/H9252 against HNF-3. 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, RARα, 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. 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+/+.

**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+/+.**
regulation of Ntcp expression in cholestasis

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

<table>
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<tr>
<th>Control</th>
<th>CBDL</th>
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<tr>
<td>IFN-γ</td>
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Values are means ± SE, expressed as percentage of naïve farnesoid X receptor wild-type (FXR+/-) mice. CBDL, common bile duct ligation; CA, cholic acid; IL-1α/β, IL-1 receptor antagonist; MIF, macrophage inhibitory factor. *p < 0.05 vs. naïve FXR+/-.

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 in FXR−/− mice but do not appear to be involved in Ntcp repression, because a comparable induction of proinflammatory cytokines was not associated with downregulation of Ntcp in FXR−/− mice. Moreover, we demonstrated recently that administration of specific antagonists of TNF-α and IL-1β and depletion of Kupffer cells did not restore Ntcp expression in CBDL mice and rats (17). Taken together, downregulation of Ntcp after CBDL is clearly dependent on FXR, although the exact SHP-independent mechanisms remain to be elucidated.

CA-fed mice represent a commonly used model to study the hepatocellular effects of the major primary bile acid retained during cholestasis (12, 42, 46, 56). CA induced SHP and downregulated Ntcp expression in FXR+/−, but not FXR−/−, mice, which is consistent with the hypothesis that FXR/SHP is required for bile acid-mediated Ntcp repression (8, 42). SHP has been demonstrated not only to reduce RXRα-RARα transactivation of the Ntcp promoter (8) but also possibly to exert its negative effects on Ntcp by repressing HNF-1α transactivation by HNF-4α (25, 31). Furthermore, bile acids may directly 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 acids-synthesizing 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-
kines 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, RXRα, and RARα 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 endotoxia. 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.

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