Disrupted coordinate regulation of farnesoid X receptor target genes in a patient with cerebrotendinous xanthomatosis

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Abstract   Cerebrotendinous xanthomatosis (CTX), sterol 27-hydroxylase (CYP27A1) deficiency, is associated with markedly reduced chenodeoxycholic acid (CDCA), the most powerful activating ligand for farnesoid X receptor (FXR). We investigated the effects of reduced CDCA on FXR target genes in humans. Liver specimens from an untreated CTX patient and 10 control subjects were studied. In the patient, hepatic CDCA concentration was markedly reduced but the bile alcohol level exceeded CDCA levels in control subjects (73.5 vs. 37.8 $\pm$ 6.2 nmol/g liver). Cholesterol 7α-hydroxylase (CYP7A1) and Na$^+$/taurocholate-cotransporting polypeptide (NTCP) were upregulated 84- and 8-fold, respectively. However, small heterodimer partner (SHP) and bile salt export pump were normally expressed. Marked CYP7A1 induction with normal SHP expression was not explained by the regulation of liver X receptor α (LXRα) or pregnane X receptor. However, another nuclear receptor, hepatocyte nuclear factor 4α (HNF4α), was induced 2.9-fold in CTX, which was associated with enhanced mRNA levels of HNF4α target genes, CYP7A1, 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase, CYP27A1, and NTCP. In conclusion, the coordinate regulation of FXR target genes was lost in CTX. The mechanism of the disruption may be explained by a normally stimulated FXR pathway attributable to markedly increased bile alcohols with activation of HNF4α caused by reduced bile acids in CTX liver.—Honda, A., G. Salen, Y. Matsuzaki, A. K. Batta, G. Xu, T. Hirayama, G. S. Tint, M. Doy, and S. Shefer. **Disrupted coordinate regulation of farnesoid X receptor target genes in a patient with cerebrotendinous xanthomatosis.** *J. Lipid Res.* 2005. **46: 287–296.**

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Bile acids are necessary for the preservation of cholesterol homeostasis in the human body. They are natural detergents that play important roles in biliary cholesterol transport and in the digestion and absorption of dietary cholesterol. In addition, bile acids themselves are degradation products of cholesterol. Most of the secreted bile acids are recycled by the enterohepatic circulation, but some are eliminated into feces as a result of incomplete intestinal absorption. To replace this loss, the liver synthesizes new bile acids from cholesterol (1).

Recent studies have shown that bile acids are physiological ligands for the farnesoid X receptor (FXR; NR1H4), an orphan nuclear receptor, and chenodeoxycholic acid (CDCA) activates the receptor function most powerfully (2–4). Activated FXR induces the expression of small heterodimer partner (SHP; NR0B2), and the increased SHP protein forms an inactivating heterodimeric complex with α-fetoprotein transcription factor (FTF; NR5A2), turning off the transcription of cholesterol 7α-hydroxylase (CYP7A1) (5–8), the rate-limiting enzyme in the classic bile acid bio-

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synthetic pathway. Not only biosynthesis but also transport of bile acids is regulated by FXR. In the liver, FXR down-regulates the transcription of Na^+/taurocholate-cotransporting polypeptide (NTCP; SLC10A1) (9), which is responsible for bile acid uptake into the hepatocyte, and upregulates the expression of the bile salt export pump (BSEP; ABCB11) (9, 10), which is responsible for bile acid efflux into the bile. Thus, FXR is a pivotal nuclear transcription factor that regulates bile acid metabolism in the liver.

Cerebrotendinous xanthomatosis (CTX) is a recessively inherited disorder caused by mutations in the sterol 27-hydroxylase (CYP27A1) gene located on human chromosome 2 (11–13). Patients are characterized clinically by tendon and brain xanthomas, premature atherosclerosis, and nervous system dysfunction, including mental retardation, dementia, cerebellar ataxia, epileptic seizures, and peripheral neuropathy (14). Biochemically, the pool size of CDCA is markedly reduced, whereas that of cholic acid (CA) is almost normal (15), and large amounts of 25-hydroxylated C_{27} bile alcohols are excreted into bile and urine (16, 17). Hydroxylation at the C-27 position by CYP27A1 is an essential reaction for the biosynthesis of CDCA, whereas CA is synthesized not only by the CYP27A1 pathway but also alternatively by the 5β-cholestane-3α,7α,12α-triol 25-hydroxylase (CYP3A) side chain hydroxylation pathway via 25-hydroxylated C_{27} bile alcohols as intermediates (15, 18). CYP7A1 activities are upregulated (18, 19), which preserves CA biosynthesis but stimulates the production of bile alcohols in this disease. Treatment with CDCA improves neurologic function (20) and reduces the increased bile alcohol concentrations in blood, bile, and urine (17, 21) by inhibition of CYP7A1 activity. These results lead us to expect that FXR is deactivated and SHP expression is downregulated in the liver of CTX subjects as a result of depleted CDCA.

Deactivation of FXR has been demonstrated in *Cyp27a1* knockout mice. In these animals, the expression of SHP was reduced to only 35% of that in $Cyp27a1^{+/+}$ controls, whereas the mRNA levels for CYP7A1 were markedly increased (22). However, $Cyp27a1^{-/-}$ mice showed neither typical CTX-related pathological abnormalities (23) nor hepatic accumulation of bile alcohols compared with CTX patients (24). In addition, CDCA is only a minor component of the bile acid pool in mice (25), and characteristic changes of biliary bile acids in $Cyp27a1^{-/-}$ mice showed markedly reduced concentrations of CA and muricholic acid rather than CDCA (23). Thus, the $Cyp27a1^{-/-}$ mouse is not a good model for studying the regulation of nuclear receptor functions in human CTX.

The current study was undertaken to investigate the regulation of bile acid metabolism through nuclear receptors in CTX. This was the only opportunity to make such an evaluation in CTX liver. Therefore, although our results may not show characteristic features of all CTX patients, disrupted coordinate regulation of FXR target genes presumably attributable to the activation of hepatocyte nuclear factor 4α (HNF4α; NR2A1) was observed in this patient.

MATERIALS AND METHODS

Chemicals

22*R*-Hydroxycholesterol, 24*S*-hydroxycholesterol, 24*S*,25-epoxy-cholesterol, 25-hydroxycholesterol, and authentic bile acids were obtained from Steraloids (Newport, RI). 25(*R*),26-Hydroxycholesterol (27-hydroxycholesterol) was synthesized from diosgenin (26), and the pure compound was obtained by preparative TLC. 5β-Cholestane-3α,7α,12α,25,27-pentol was a gift from Drs. T. Hoshita and K. Kihira (Pharmaceutical Institute, Hiroshima University, Hiroshima, Japan). 5β-Cholestane-3α,7α,12α,25-tetrol was synthesized from CA by the method of Dayal et al. (27). 5β-Cholestane-3α,7α,12α,23*R*,25-pentol was isolated from bile and feces of patients with CTX (28). 5β-Cholestane-3α,7α,12α,24*R*, 25-pentol and 5β-cholestane-3α,7α,12α,24*S*,25-pentol were prepared from 5β-cholestane-3α,7α,12α,25-tetrol by the method of Hoshita (29). [25,26,26,26,27,27,27-$^2H_7$]cholesterol, [2,2,4,4-$^2H_4$] CA, [2,2,4,4-$^2H_4$]deoxycholic acid (DCA), and [2,2,4,4-$^2H_4$]litho-cholic acid (LCA) were obtained from MSD Isotopes (Montreal, Canada), and [$^2H_7$]27-hydroxycholesterol was prepared by the method of Shoda, Axelson, and Sjövall (30). [11,11,12,12-$^2H_4$] CDCA and [11,11,12,12-$^2H_4$]ursodeoxycholic acid (UDCA) were supplied by the research laboratories of Nippon Kayaku Co. and Tokyo Tanabe Co. (Tokyo, Japan), respectively. β-Glucuronidase from *Helix pomatia* (type H-1) and choloylglycine hydrolase from *Clostridium perfringens* were purchased from Sigma Chemical Co. (St. Louis, MO).

Patients

Two CTX patients were studied. Patient 1 (CTX1) was a 45 year old male with dementia, spinal cord paresis, and cerebellar ataxia. Xanthomas were located in both Achilles tendons. Serum cholestanol concentration was 6.4 mg/dl (normal level, 0.2 ± 0.2 mg/dl). The result of mutation analysis of this patient (patient 1100-3) has been described in a previous report (31). A liver biopsy was obtained for diagnostic histology, and the extra tissue was available for biochemical analyses. Patient 2 (CTX2) was a 42-year-old woman with severe neurologic dysfunction (spinal cord paresis and cerebellar ataxia), bilateral cataracts, and tendon and cerebral xanthomas. She had been treated with CDCA for 3 years until she died of pneumonia. A liver specimen was obtained at postmortem (performed within 1 h of death). Control liver specimens were from 10 healthy adults who died unexpectedly and whose livers became available because no suitable recipient for liver transplantation could be found (University of Minnesota Hospital National Institutes of Health Contract 1-DK-62274). All liver specimens were immediately frozen and stored at −70°C until used. The research protocol was approved by the Human Studies Committees at the University of Medicine and Dentistry of New Jersey-New Jersey Medical School (Newark, NJ) and the VA Medical Center (East Orange, NJ).

Hepatic sterol concentrations

An aliquot of the liver specimen was weighed and homogenized with a loose-fitting Teflon pestle in 24 volumes of distilled water. Concentrations of cholesterol in 12.5 μl of the whole liver homogenate were determined by gas chromatography-mass spectrometry with selected ion monitoring (GC-SIM) as described previously (24). Hepatic oxysterol concentrations were determined as follows: [$^2H_7$]27-hydroxycholesterol (32 ng) as internal recovery standard and 5 μg of butylated hydroxytoluene were added to 100 μl of the whole homogenate, and saponification was carried out in 1 ml of 1 N ethanolic KOH at 37°C for 1 h. After addition of 0.4 ml of distilled water, sterols were extracted twice with 2 ml of *n*-hexane. Oxysterols were purified by a Bond

Elut SI cartridge according to the method of Dzeletovic et al. (32). Derivatization into trimethylsilyl (TMS) ether and GC-SIM analysis were carried out according to previously described methods for cholesterol determination (24) except that the column oven was programmed to change from 100°C to 270°C at 30°C/min after a 1 min delay from the start time and the multiple ion detector was focused on *m/z* 173.1360 for 22*R*-hydroxycholesterol, *m/z* 343.3000 for 24*S*,25-epoxycholesterol, *m/z* 413.3239 for 24*S*-hydroxycholesterol, *m/z* 456.3787 for 25-hydroxycholesterol and 27-hydroxycholesterol, and *m/z* 463.4226 for [^{2}H$_7$]27-hydroxycholesterol. Hepatic concentrations of 25-hydroxylated bile alcohols were quantified after treatment with β-glucuronidase (16, 17). Briefly, 50 μl of the whole homogenate was incubated for 3 h at 55°C in 1.5 ml of 0.5 M acetate buffer (pH 5.0) containing 5,000 units of β-glucuronidase (33). At the end of the incubation, the pH of the mixture was adjusted to 10 with 8.9 M KOH. After the addition of cholestane-3β,5α,6β-triol (200 ng) as an internal recovery standard, sterols were extracted twice with 4.5 ml of ethyl acetate and the solvent was evaporated to dryness. The residue was dissolved in 500 μl of chloroform-acetone (35:25, v/v) and applied to a Bond Elut SI (500 mg) cartridge that was prewashed with 1 ml of the same solvent. After washing out cholesterol with 4 ml of the same solvent, cholestane-3β,5α,6β-triol and bile alcohols were eluted with 3 ml of chloroform-acetone-methanol (35:25:20, v/v/v). TMS ether derivatization and quantification by GC-SIM were performed as described previously (24).

Hepatic bile acid concentrations

Bile acid concentrations and profiles in the liver were determined by our previously described method (34) with some modifications. In brief, deuterium-labeled bile acids as internal recovery standards were added to 125 μl of the whole homogenate and incubated in 5% KOH at 80°C for 20 min, and bile acids were extracted by a Bond Elut C$_{18}$ cartridge and subjected to enzymatic hydrolysis with choloylglycine hydrolase. The resulting deconjugated bile acids were converted into the ethyl ester dimethylethylsilyl ether derivatives and quantified by GC-SIM. The column oven was programmed to change from 100°C to 280°C at 30°C/min after a 1 min delay from the start time, and the multiple ion detector was focused on *m/z* 459.3294 for CDCA, *m/z* 461.3451 for LCA, *m/z* 563.3952 for DCA and UDCA, *m/z* 665.4453 for CA, *m/z* 463.3546 for [^{2}H$_4$]CDCA, *m/z* 465.3702 for [^{2}H$_4$]LCA, *m/z* 567.4203 for [^{2}H$_4$]DCA and [^{2}H$_4$]UDCA, and *m/z* 669.4705 for [^{2}H$_4$]CA.

RNA measurements

mRNA was extracted from frozen tissue by a MagNA Pure LC mRNA Isolation Kit II (Roche Diagnostics, Mannheim, Germany). Reverse transcription was performed on 80 ng of mRNA using a First Strand cDNA Synthesis Kit for RT-PCR (Roche). Real-time quantitative PCR assay was performed in triplicate using aliquots of the cDNA (∼1.6 ng each) with the FastStart DNA Master SYBR Green I and the LightCycler Instrument (Roche). The sequences of the oligonucleotide primer pairs used to amplify the mRNAs are listed in **Table 1**. CYP3A4 and β-actin mRNAs were quantified by use of ready-to-use amplification primer mix for the LightCycler Instrument. PCR amplification began with a 10 min preincubation step at 95°C followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 62°C or 58°C (CYP7A1) for 10 s, and elongation at 72°C for 6 s, 10 s [sterol regulatory element binding protein 1 (SREBP1), BSEP, NTCP, multidrug-resistant protein (MDR1), and HNF4α], or 14 s [organic anion transport protein 2 (OATP2)]. The relative concentration of PCR product derived from the target gene was calculated using software of the LightCycler system. A standard curve for each run was constructed by plotting the crossover point against the log concentration. The concentration of target molecules in each sample was then calculated automatically by reference to this curve ($r = -1.00$). Results were expressed relative to the number of β-actin transcripts used as an internal control. Amplification products were checked by electrophoresis on 3% agarose gels, and the specificity of each PCR product was assessed by melting curve analysis.

Statistics

Data are reported here as means ± SEM. In all statistical tests, significance was accepted at the level of $P < 0.05$.

TABLE 1.   Primer sequences used in mRNA quantification by real-time RT-PCR

mRNA	GenBank Accession Number	Forward	Reverse	Amplicon Length
				bp
FXR	NM_005123	5′-TGGGGAACTGAAAATGACTC-3′	5′-ACAGGCAAAGTGTTGAGGAT-3′	181
LXRα	NM_005693	5′-GACCGACTGATGTTCCCACG-3′	5′-CCATCCGGCCAAGAAAACAG-3′	187
PXR	AF061056	5′-GGGAGGATGGCAGTGTCT-3′	5′-GGTCCTCGATGGGCAAGT-3′	165
SREBP1	NM_004176	5′-GCCCAGGTGACTCAGCTATT-3′	5′-GTCAGAGAGGCCCACCACTT-3′	245
SHP	NM_021969	5′-CCATCCTCTTCAACCCCGA-3′	5′-CGGAATGGACTTGAGGGTG-3′	170
FTF	U93553	5′-GATGGATACCAACACGGTCAGA-3′	5′-TCCCCGCTGTATAAGCCT-3′	119
HNF4α	NM_178850	5′-TCGCAGATGTGTGTGAGTC-3′	5′-AGTGCCGAGGGACAATGTA-3′	213
PGC-1α	NM_013261	5′-GCTGCTCTTGAAAATGGATAC-3′	5′-AAGCAGGGTCAAAGTCATC-3′	136
HMGCR	NM_000859	5′-CTGGGGAATTGTCACTTATG-3′	5′-GCTATTCAGGCTGTCTTCTT-3′	130
CYP7A1	XM_044651	5′-GAATGCTGGTCAAAAAGTC-3′	5′-TGAAATCCTCCTTAGCTGT-3′	152
CYP7B1	AF127090	5′-CATCTTTCCTCCAGTCCTAC-3′	5′-GGCATTAGGTAACACTTCAG-3′	141
CYP8B1	NM_004391	5′-TCGCTTCCTCAACCCTAATG-3′	5′-TCAGGGTCCACCAACTCTAA-3′	189
CYP27A1	BC051851	5′-CACAAACTCCCCGGATCAT-3′	5′-AGGCTCAGAGAAGGCAGT-3′	121
BSEP	AF091582	5′-CCACTTCTGCCTTAGACACA-3′	5′-GATTCTTGCATTGGGTCAAC-3′	248
NTCP	NM_003049	5′-GGATGCCAAAATGTCCAACT-3′	5′-TAGGTGCCATTTCCCAGA-3′	242
MDR1	NM_000927	5′-CATCGTTTGTCTACAGTTCGT-3′	5′-GACATTTCCAAGGCATCAA-3′	203
OATP2	NM_006446	5′-AGGGTCTACTTGGGCTTG-3′	5′-GTGAAAGGACCAGGAACT-3′	305

BSEP, bile salt export pump; CYP7A1, cholesterol 7α-hydroxylase; CYP7B1, oxysterol 7α-hydroxylase; CYP8B1, 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase; CYP27A1, sterol 27-hydroxylase; FTF, α-fetoprotein transcription factor; FXR, farnesoid X receptor; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; HNF4α, hepatocyte nuclear factor 4α; LXRα, liver X receptor α; MDR1, multidrug-resistant protein 1; NTCP, Na$^+$/taurocholate-cotransporting polypeptide; OATP2, organic anion transport protein 2; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; PXR, pregnane X receptor; SHP, small heterodimer partner; SREBP1, sterol regulatory element binding protein 1. Real-time quantitative PCR was performed as described in Materials and Methods.

TABLE 2. Hepatic bile acid concentrations in controls and CTX patients

Bile Acids	CTX1 (Untreated)	CTX2 (Treated)	Controls (n = 10)
		nmol/g liver	
Lithocholic acid (LCA)	0.7	1.1	2.3 ± 1.3[a] (0.0–13.7)
Deoxycholic acid (DCA)	0.3	2.3	6.0 ± 3.1[a] (0.4–29.2)
Chenodeoxycholic acid (CDCA)	0.9	5.3	37.8 ± 6.2[a] (15.0–69.0)
Ursodeoxycholic acid (UDCA)	0.4	1.4	3.4 ± 1.2[a] (0.4–10.4)
Cholic acid (CA)	11.5	42.0	29.9 ± 5.2[a] (11.7–59.5)
Total	13.8	52.1	79.3 ± 11.9[a] (32.1–134.5)

CTX, cerebrotendinous xanthomatosis. Means ± SEM and (range of results) are given.
[a] Ninety-five percent confidence intervals for control means of LCA, DCA, CDCA, UDCA, CA, and total bile acid concentrations are 0.0–5.2, 0.0–7.0, 23.8–51.8, 0.7–6.1, 18.1–41.7, and 52.4–106.2 nmol/g liver, respectively.

RESULTS

Hepatic bile acid composition and concentrations in untreated and CDCA-treated CTX patients were compared with those in controls (**Table 2**). In untreated patient CTX1, hepatic CDCA concentration was markedly reduced (2% of control mean) and CA concentration was also low compared with those in controls (38% of control mean). Therefore, hepatic total bile acid concentration in this patient was only 17% of the control mean. In contrast, the liver from CDCA-treated patient CTX2 contained a significant amount of CDCA (5.9-fold of CTX1) with normal CA level, so that hepatic total bile acid concentration was within the normal range.

Data in **Table 3** show hepatic concentrations of 25-hydroxylated C_{27} bile alcohols. In untreated CTX1 liver, all bile alcohols (i.e., 5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α,7α,12α,23R,25-pentol, 5β-cholestane-3α,7α,12α,24R,25-pentol, 5β-cholestane-3α,7α,12α,24S,25-pentol, and 5β-cholestane-3α,7α,12α,25,27-pentol) were markedly increased and total bile alcohol concentration was increased more than 100-fold compared with that of the control mean. This total bile alcohol level exceeded CDCA levels in any control subjects determined in this study and was similar to total bile acid concentration of the control mean (Table 2). In CDCA-treated patient CTX2, hepatic total bile alcohol concentration was much lower than that in untreated patient CTX1 but still increased more than 10-fold compared with that of the control mean.

Hepatic gene expression levels of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), CYP7A1, oxysterol

7α-hydroxylase (CYP7B1), 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase (CYP8B1), and CYP27A1 were analyzed by real-time quantitative PCR (**Fig. 1**). HMGCR mRNA level in patient CTX1 was similar to those in controls. CYP7A1 mRNA level was markedly increased (84-fold) in the patient, which was associated with markedly increased CYP7A1 activity (22-fold) (18). In contrast, CYP7B1 mRNA level in the patient was reduced to 0.4-fold. CYP8B1 mRNA in the patient was increased 3.8-fold, but the result was not consistent with CYP8B1 activity, which was not significantly increased compared with controls (18). Although this patient could not make active CYP27A1 protein and the enzyme activity was virtually absent (18), transcription of mRNA measured using our primer set designed between exons 7 and 8 was upregulated 2.6-fold.

Hepatic expression levels of FXR and its target genes are depicted in **Fig. 2**. In these genes, SHP and BSEP were almost equally expressed between CTX and controls. FXR mRNA level tended to be low in patient CTX1 but was still within the 95% confidence interval for the control mean. In contrast, NTCP was significantly upregulated (8.0-fold) in patient CTX1 than in controls.

To evaluate the status of another nuclear receptor, liver X receptor α (LXRα; NR1H3), hepatic cholesterol and oxysterol concentrations were determined (**Table 4**). Hepatic cholesterol concentrations were increased in both untreated patient CTX1 (+39%) and CDCA-treated patient CTX2 (+13%) compared with controls. In patient CTX1, hepatic 27-hydroxycholesterol was virtually absent but 22R-hydroxycholesterol, 24S-hydroxycholesterol, 25-hydroxycholesterol, and 24S,25-epoxycholesterol concen-

TABLE 3. Hepatic bile alcohol concentrations in controls and CTX patients

Bile Alcohols	CTX1 (Untreated)	CTX2 (Treated)	Controls (n = 4)
		nmol/g liver	
5β-Cholestane-3α,7α,12α,25-tetrol	41.3	7.5	0.4 ± 0.1[a] (0.2–0.6)
5β-Cholestane-3α,7α,12α,23R,25-pentol	21.9	1.9	0.1 ± 0.0[a] (0.0–0.1)
5β-Cholestane-3α,7α,12α,24R,25-pentol	6.1	0.3	0.1 ± 0.0[a] (0.0–0.2)
5β-Cholestane-3α,7α,12α,24S,25-pentol	3.9	0.1	0.1 ± 0.0[a] (0.0–0.1)
5β-Cholestane-3α,7α,12α,25,27-pentol	0.3	0.1	0.1 ± 0.0[a] (0.0–0.1)
Total	73.5	10.0	0.7 ± 0.2[a] (0.4–1.0)

Means ± SEM and (range of results) are given.
[a] Ninety-five percent confidence intervals for control means of 5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α,7α,12α,23R,25-pentol, 5β-cholestane-3α,7α,12α,24R,25-pentol, 5β-cholestane-3α,7α,12α,24S,25-pentol, 5β-cholestane-3α,7α,12α,25,27-pentol, and total bile alcohol concentrations are 0.1–0.7, 0.0–0.2, 0.0–0.3, 0.0–0.1, 0.0–0.1, and 0.2–1.2 nmol/g liver, respectively.
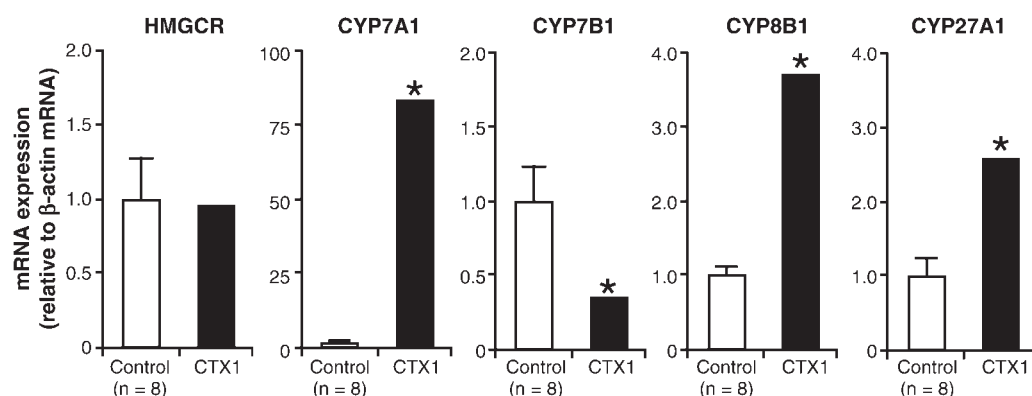
Fig. 1. Relative amounts of mRNA for enzymes involved in cholesterol and bile acid biosynthesis in the livers of controls and an untreated cerebrotendinous xanthomatosis (CTX) patient (CTX1). Quantitative real-time PCR was performed as described in Materials and Methods with the primers listed in Table 1. All data were standardized for β-actin mRNA. Expression in controls was set to 1.0. Data are presented as means ± SEM. Ninety-five percent confidence intervals for control means of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), cholesterol 7α-hydroxylase (CYP7A1), oxysterol 7α-hydroxylase (CYP7B1), 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase (CYP8B1), and sterol 27-hydroxylase (CYP27A1) mRNA levels are 0.3–1.7, 0.2–1.8, 0.4–1.6, 0.7–1.3, and 0.3–1.7, respectively. Asterisks indicate that the values for patient CTX1 are out of the 95% confidence interval for control means.

trations did not differ significantly from those in controls. In contrast, CDCA-treated patient CTX2 showed slightly increased concentrations of oxysterols except for 27-hydroxy-cholesterol. Hepatic gene expression levels of LXRα and SREBP1, a target gene for LXRα, in patient CTX1 and controls are compared in **Fig. 3**. Consistent with hepatic oxysterol concentrations, LXRα and SREBP1 expression levels did not change significantly in untreated patient CTX1.

The expression levels of pregnane X receptor (PXR; NR1I2) and its target genes, CYP3A4, MDR1 (ABCB1), and OATP2 (SLC21A5), were studied. As shown in **Fig. 4**, the mRNA level of PXR was increased 9.1-fold in untreated patient CTX1 compared with controls. However, expression levels of the target genes in patient CTX1 were not changed compared with those in control subjects.

Figure 5 represents mRNA levels of other nuclear receptors, FTF and HNF4α, and a coactivator, peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), that are also known to regulate CYP7A1 gene transcription. Expression levels of both FTF and PGC-1α were not significantly different between CTX1 and controls, but HNF4α in CTX1 was induced 2.9-fold compared with control subjects.

DISCUSSION

It seems likely that FXR plays the major role in the feedback regulation of bile acid biosynthesis (9), and the previous study on $Cyp27a1^{-/-}$ mice clearly showed that mark-
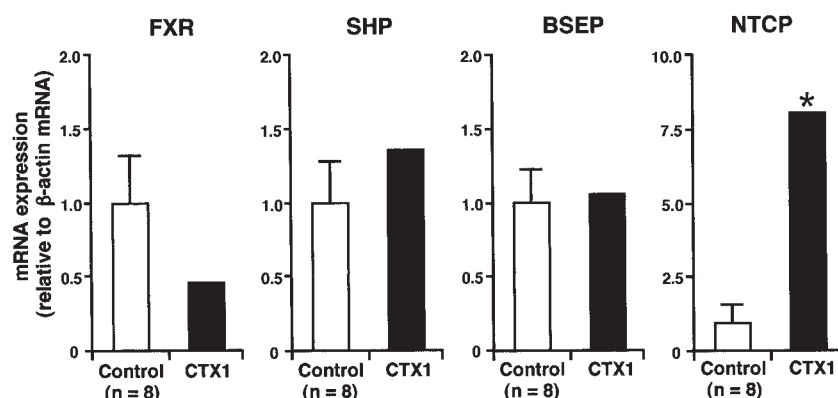

Fig. 2. Relative amounts of mRNA for farnesoid X receptor (FXR) and related proteins in the livers of controls and untreated patient CTX1. Quantitative real-time PCR was performed as described in Materials and Methods with the primers listed in Table 1. All data were standardized for β-actin mRNA. Expression in controls was set to 1.0. Data are presented as means ± SEM. Ninety-five percent confidence intervals for control means of FXR, small heterodimer partner (SHP), bile salt export pump (BSEP), and Na$^+$/taurocholate-cotransporting polypeptide (NTCP) mRNA levels are 0.3–1.7, 0.3–1.7, 0.3–1.7, and 0.0–2.8, respectively. An asterisk indicates that the value for patient CTX1 is above the 95% confidence interval for the control mean.

TABLE 4. Hepatic cholesterol and oxysterol concentrations in controls and CTX patients

Cholesterol or Oxysterols	CTX1 (Untreated)	CTX2 (Treated)	Controls (n = 8)
		$\mu mol/g\ liver$	
Cholesterol	17.5	14.3	12.6 ± 0.5^a (10.0–14.1)
		$nmol/g\ liver$	
Oxysterols			
22R-Hydroxycholesterol	0.08	0.40	0.14 ± 0.02^b (0.07–0.24)
24S-Hydroxycholesterol	0.12	0.55	0.29 ± 0.10^b (0.10–0.95)
24S,25-Epoxycholesterol	$<0.1^c$	0.44	$<0.1^c$
25-Hydroxycholesterol	0.39	1.36	0.36 ± 0.06^b (0.19–0.66)
27-Hydroxycholesterol	$<0.05^c$	$<0.05^c$	0.96 ± 0.10^b (0.67–1.45)
Total	0.59	2.75	1.74 ± 0.12^b (1.30–2.19)

Means ± SEM and (range of results) are given.

[a] Ninety-five percent confidence interval for control mean is 11.4–13.8 μmol/g liver.

[b] Ninety-five percent confidence intervals for control means of 22R-hydroxycholesterol, 24S-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, and total oxysterol concentrations are 0.08–0.20, 0.05–0.53, 0.22–0.50, 0.72–1.19, and 1.46–2.02 nmol/g liver, respectively.

[c] Limit of detection.

edly upregulated CYP7A1 was associated with significantly reduced SHP expression as a result of deactivated FXR (22). In CTX, however, CYP7A1 expression and activity were markedly upregulated in spite of normal SHP expression, which means that unlike in $Cyp27a1^{-/-}$ mice, upregulation of CYP7A1 in CTX is not simply explained by deactivation of the FXR/SHP/FTF cascade attributable to a reduced CDCA pool.

Normally stimulated SHP and BSEP in CTX liver suggests that FXR is not deactivated in this disease. In contrast to $Cyp27a1^{-/-}$ mice, three types of compounds (i.e., cholestanol, bile acid intermediates, and C_{27} bile alcohols) were abnormally accumulated in CTX liver (24). Although cholestanol and bile acid intermediates, including 7α-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one, and 5β-cholestane-3α,7α,12α-triol, are not good ligands for mouse FXR (35, 36), at least 5β-cholestane-3α,7α,12α,25-tetrol, one of the C_{27} bile alcohols accumulated in CTX, was very recently shown to be a potent ligand for FXR (37). Total C_{27} bile alcohol level in CTX liver was ~74 μM, as high as the total bile acid concentration in controls (~79 μM). Therefore, it is strongly suggested that accumulated bile alcohols stimulate the FXR pathway in CTX liver.

If the FXR pathway is normally activated in CTX, we must prove another pathway that causes the marked upregulation of both CYP7A1 and NTCP independent of FXR. Recent study in Fxr knockout mice revealed that the mice showed 80% reduction in hepatic SHP mRNA, but CYP7A1 mRNA levels were increased only 1.6-fold compared with those in wild-type mice (9). Two other groups produced Shp knockout mice and reported that the increase of CYP7A1 mRNA in the knockout mice was 1.5- to 2.5-fold (38, 39). More important, treatment with cholestyramine, a polyanionic polymer that binds intestinal bile acids and reduces their pool size, further enhanced CYP7A1 expression in the Shp null mice (38). Thus, although the FXR/SHP/FTF cascade is one of the important mechanisms for feedback regulation of bile acid biosynthesis, there is no room for doubt that additional FXR-independent mechanisms also regulate bile acid biosynthesis.

At least three FXR-independent pathways have been proposed to explain the regulation of CYP7A1 expression. First, $CYP7A1$ gene transcription is regulated by LXRα, a nuclear receptor for oxysterols (40, 41). In rodent liver, LXRα positively regulates $CYP7A1$ gene transcription (42), whereas activation of LXRα directly induces SHP mRNA and downregulates CYP7A1 expression in human hepatocytes (43). In agreement with the latter finding, the LXRα response element is not conserved in the human $CYP7A1$ promoter (44), and dietary cholesterol does not induce human $CYP7A1$ in transgenic mice (45). Thus, there are fundamental differences in the regulation of CYP7A1 by LXRα between rodents and humans, and dissociating SHP repression from CYP7A1 induction in human liver is not explained by the LXRα pathway.
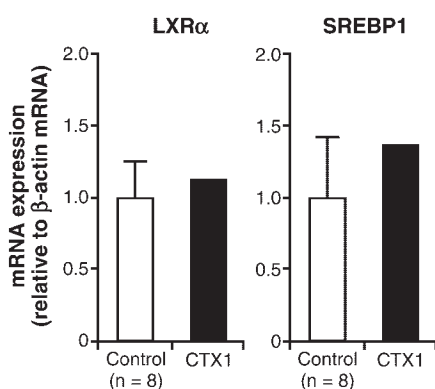
In addition, the activation status of LXRα was not differ-

Fig. 3. Relative amounts of mRNA for liver X receptor α (LXRα) and sterol regulatory element binding protein 1 (SREBP1) in the livers of controls and untreated patient CTX1. Quantitative real-time PCR was performed as described in Materials and Methods with the primers listed in Table 1. All data were standardized for β-actin mRNA. Expression in controls was set to 1.0. Data are presented as means ± SEM. Ninety-five percent confidence intervals for control means of LXRα and SREBP1 mRNA levels are 0.5–1.5 and 0.0–2.1, respectively.
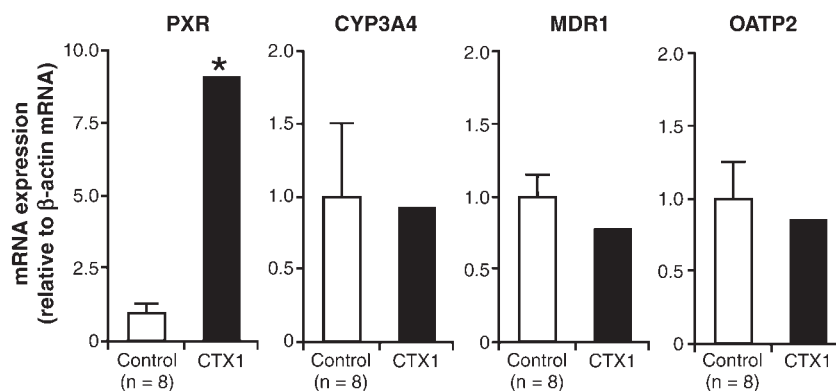
Fig. 4. Relative amounts of mRNA for pregnane X receptor (PXR), 5β-cholestane-3α,7α,12α-triol 25-hydroxylase (CYP3A4), multidrug-resistant protein 1 (MDR1), and organic anion transport protein 2 (OATP2) in the livers of controls and untreated patient CTX1. Quantitative real-time PCR was performed as described in Materials and Methods with the primers listed in Table 1. All data were standardized for β-actin mRNA. Expression in controls was set to 1.0. Data are presented as means ± SEM. Ninety-five percent confidence intervals for control means of PXR, CYP3A4, MDR1, and OATP2 mRNA levels are 0.4–1.6, 0.0–2.2, 0.6–1.4, and 0.4–1.6, respectively. An asterisk indicates that the value for patient CTX1 is above the 95% confidence interval for the control mean.

ent between CTX and control livers. Although cholesterol concentration was increased in CTX liver, oxysterol levels were not increased, which seems to be attributable to markedly upregulated CYP7A1 activity because not only cholesterol but also many oxysterols, including 24S-, 25-, and 27-hydroxycholesterols, can be substrates for CYP7A1 (46, 47). This hypothesis was supported by the observation that hepatic levels of oxysterols as well as cholesterol were increased in CTX after CDCA treatment compared with controls (Table 4). We could not measure hepatic CYP7A1 expression in CDCA-treated patient CTX2, but
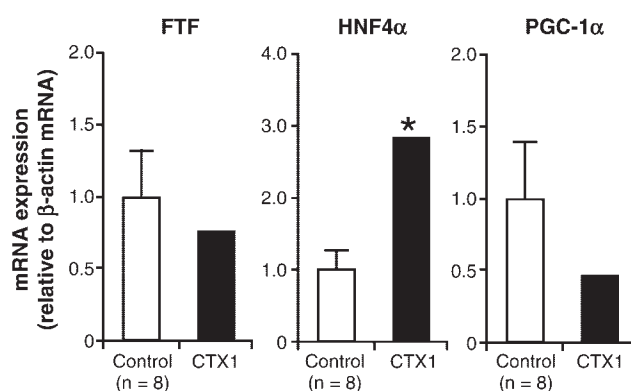
the expression must have been decreased as a result of activation of the FXR pathway. Consistent with hepatic oxysterol concentrations, expression of SREBP1, a target gene for LXRα, was not changed in untreated patient CTX1 (Fig. 3). It should be noted that although we quantified total SREBP1 instead of SREBP1c, 1c transcript predominates over 1a transcript by a 6:1 ratio in human liver (48). A recent report showed that the upregulation of SREBP1c by LXRα was inhibited by SHP in rodents (49). In fact, $Cyp27a1^{-/-}$ mice show hypertriglyceridemia, probably as a result of the activation of LXRα with reduced SHP expression (22). However, SREBP1 expression in our CTX patient does not seem to have been influenced by SHP, because the expression of SHP was normal and hypertriglyceridemia is not a characteristic feature of CTX (50).
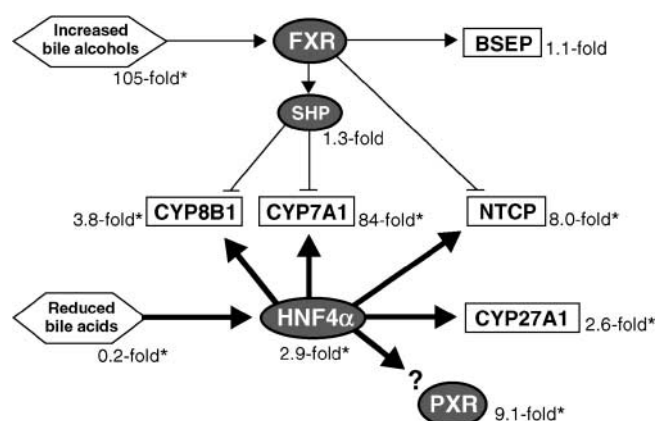

Fig. 5. Relative amounts of mRNA for α-fetoprotein transcription factor (FTF), hepatocyte nuclear factor 4α (HNF4α), and peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) in the livers of controls and untreated patient CTX1. Quantitative real-time PCR was performed as described in Materials and Methods with the primers listed in Table 1. All data were standardized for β-actin mRNA. Expression in controls was set to 1.0. Data are presented as means ± SEM. Ninety-five percent confidence intervals for control means of FTF, HNF4α, and PGC-1α mRNA levels are 0.2–1.8, 0.3–1.7, and 0.0–2.0, respectively. An asterisk indicates that the value for patient CTX1 is above the 95% confidence interval for the control mean.

Fig. 6. A simplified model describing the regulation of bile acid metabolism by FXR and HNF4α in CTX liver. Arrows indicate positive regulation, and brakes indicate negative regulation. Fold changes of gene expression and bile alcohol as well as bile acid concentrations in patient CTX1 compared with those in controls are indicated. Asterisks indicate that the values for patient CTX1 are out of the 95% confidence intervals for control means.

Second, LCA and other toxic bile acids are ligands for xenobiotic receptor PXR (51) and repress CYP7A1 expression (52). The PXR-dependent mechanism was recently unveiled by Li and Chiang (53). HNF4α interacts with several coactivators, including PGC-1α, and the complex activates *CYP7A1* gene transcription in the absence of ligands (54). Ligands for PXR activate PXR to promote its interaction with HNF4α, which disrupts the interaction between HNF4α and PGC-1α and results in the suppression of CYP7A1 expression. Because hepatic bile acid concentrations are reduced in CTX, PXR may be deactivated in this disease. Inversely, PXR may be activated by abnormally accumulated sterols in CTX liver. Our results demonstrated that the PXR pathway was not significantly influenced in CTX, because the expressions of CYP3A4, MDR1, and OATP2, which are target genes for PXR, were not changed compared with those in controls (Fig. 4). In addition, previous reports have shown that some bile acid intermediates accumulated in CTX liver are good ligands for mouse PXR but not for human PXR (35, 36).

The third FXR-independent mechanism for the regulation of CYP7A1 is based on the activation of c-Jun N-terminal kinase (JNK) (55). This JNK/c-Jun signaling cascade is activated by bile acids, particularly taurocholate. Activated c-Jun represses CYP7A1 expression by both SHP-dependent and -independent mechanisms (39). Although we could not evaluate the SHP-independent JNK/c-Jun pathway, this pathway does not seem to contribute to marked CYP7A1 induction in CTX liver, because the effects of JNK inhibitor on the stimulation of CYP7A1 expression were small in hepatocytes from *Shp*$^{-/-}$ mice (39). The downstream target of the SHP-independent JNK/c-Jun pathway in bile acid inhibition of CYP7A1 expression is not certain. However, it is possible that HNF4α is a primary target of JNK phosphorylation and that the activation of the JNK/c-Jun pathway inactivates HNF4α followed by inhibition of *CYP7A1* gene transcription (56).

Because LXRα does not bind to the human *CYP7A1* promoter (44), HNF4α seems to be the only nuclear receptor that is able to stimulate human *CYP7A1* gene transcription (8, 57). Although bile acids inhibit HNF4α activity through activation of the PXR or JNK/c-Jun pathway, reduction of HNF4α mRNA and protein by bile acids has also been reported in rat liver and HepG2 cells (58). Our result of 2.9-fold induced HNF4α mRNA in CTX liver was probably attributable to reduced hepatic bile acid levels in this disease. Upregulated CYP7A1, despite normally expressed SHP in the CTX liver, may be explained by the mechanism that positive regulation of CYP7A1 by HNF4α dominates over the negative effect of the FXR/SHP/FTF pathway (**Fig. 6**). Like *CYP7A1, CYP8B1* (58), *CYP27A1* (59), and *NTCP* (but not *BSEP*) (60) are known to be target genes for HNF4α. In addition, the HNF4α binding site has been characterized in the PXR promoter and found to be required for the expression of PXR in fetal hepatocytes (61). Because PXR was normally expressed in the *Hnf4α* null adult mice, another factor(s) is also involved in the baseline expression of PXR in adult mice. Although it is still controversial whether markedly increased PXR

expression in our CTX patient can be explained by the induction of HNF4α, the fact that other HNF4α target genes (i.e., *CYP7A1, CYP8B1, CYP27A1,* and *NTCP*) were all upregulated in CTX liver lends support to our idea that HNF4α is activated in CTX.

In summary, CTX liver exhibited a dramatic reduction in CDCA and markedly increased bile alcohol concentrations. SHP and BSEP were normally expressed, reflecting activation of the FXR pathway by accumulating bile alcohols, whereas CYP7A1 and NTCP, which are negatively controlled by activated FXR, were markedly upregulated. The disrupted coordinate regulation of FXR target genes in CTX liver can be explained by normal stimulation of the FXR pathway by increased bile alcohols with induction of HNF4α attributable to reduced bile acids. However, because the data shown in this report are derived from a single untreated patient, we cannot conclude that our observations are characteristic features of all CTX patients.

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