Differences in hepatic levels of intermediates in bile acid biosynthesis between Cyp27$^{-/-}$ mice and CTX

Akira Honda,* Gerald Salen,[1,†,§] Yasushi Matsuzaki,* Ashok K. Batta,† Guorong Xu,[†,§] Eran Leitersdorf, G. Stephen Tint,[†,§] Sandra K. Erickson,[††] Naomi Tanaka,* and Sarah Shefer[†]**

Department of Gastroenterology,* University of Tsukuba, Tsukuba City 305-8575, Japan; Gastrointestinal Division, Department of Medicine, and Liver Center,† University of Medicine and Dentistry of New Jersey-New Jersey Medical School, Newark, NJ 07103; Veterans Affairs Medical Center,§ East Orange, NJ 07018; Department of Medicine,** Center for Research, Prevention, and Treatment of Atherosclerosis, Hadassah University Hospital, 91120 Jerusalem, Israel; and Department of Medicine,†† University of California and Veterans Affairs Medical Center, San Francisco, CA 94121

Abstract **Cerebrotendinous xanthomatosis (CTX) is a rare, recessively inherited lipid storage disease characterized by a markedly reduced production of chenodeoxycholic acid and an increased formation of 25-hydroxylated bile alcohols and cholestanol. Patients with this disease are known to have mutations in the sterol 27-hydroxylase (*Cyp27*) gene. However, one study showed that mice with a disrupted *Cyp27* gene did not have any CTX-related clinical or biochemical abnormalities. To explore the reason, hepatic cholesterol, cholestanol, and 12 intermediates in bile acid biosynthetic pathways were quantified in 10 Cyp27$^{-/-}$ and 7 Cyp27$^{+/+}$ mice, two CTX patients (untreated and treated with chenodeoxycholic acid), and four human control subjects by high resolution gas chromatography-mass spectrometry. Mitochondrial 27-hydroxycholesterol and 5β-cholestane-3α,7α,12α,27-tetrol were virtually absent in both Cyp27$^{-/-}$ mice and CTX patients. In Cyp27$^{-/-}$ mice, microsomal concentrations of intermediates in the early bile acid biosynthetic pathway (7α-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one, 7α,12α-dihydroxy-4-cholesten-3-one, and 5β-cholestane-3α,7α,12α-triol), 25-hydroxylated bile alcohols (5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α,7α,12α,23*R*,25-pentol, and 5β-cholestane-3α,7α,12α,24*R*,25-pentol), and cholestanol were all significantly elevated compared with those in Cyp27$^{+/+}$ mice, although the levels were lower than those in untreated CTX patients. The intermediate levels in early bile acid biosynthesis were more elevated in male (16–86% of CTX) than in female Cyp27$^{-/-}$ mice (7–30% of CTX). In contrast, 25-hydroxylated bile alcohol concentrations were not significantly different between male and female Cyp27$^{-/-}$ mice and were considerably lower (less than 14%) than those in CTX patients. These results suggest that *1*) in Cyp27$^{-/-}$ mice, especially in females, classic bile acid biosynthesis via 7α-hydroxycholesterol is not stimulated as much as in CTX patients; and *2*) formed 25-hydroxylated bile alcohols are more efficiently metabolized in Cyp27$^{-/-}$ mice than in CTX patients.**—Honda, A., G. Salen, Y. Matsuzaki, A. K. Batta, G. Xu, E. Leitersdorf, G. S. Tint, S. K. Erickson, N. Tanaka, and S. Shefer. **Differences in hepatic levels of intermediates in bile acid biosynthesis between Cyp27$^{-/-}$ mice and CTX. *J. Lipid Res.* 2001. 42: 291–300.**

Supplementary key words cholesterol • cholestanol • bile alcohols

Bile acid biosynthesis from cholesterol is initiated either by a series of ring modifications (classic pathway) or by side-chain hydroxylation (alternative pathway) (1). The first and rate-limiting step of the classic pathway is catalyzed by microsomal cholesterol 7α-hydroxylase, whereas the key enzyme of the alternative pathway is mitochondrial cholesterol (sterol) 27-hydroxylase. In the classic pathway, the ring modifications yield 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol. The side chain of the diol is then hydroxylated by mitochondrial 5β-cholestane-3α,7α-diol 27-hydroxylase (2–4) and finally converted into chenodeoxycholic acid (CDCA). On the other hand, the side chain of 5β-cholestane-3α,7α,12α-triol is hydroxylated by either mitochondrial 5β-cholestane-3α,7α,12α-triol 27-hydroxylase (3–5) or microsomal 5β-cholestane-3α,7α,12α-triol 25-hydroxylase (3, 6, 7), and formed tetrols are finally converted into cholic acid (CA). Hydroxylations of the C-27 position of cholesterol, 5β-cholestane-3α,7α-diol, and 5β-cholestane-3α,7α,12α-triol are catalyzed by the same enzyme, sterol 27-hydroxylase (Cyp27) (4). Therefore, when sterol 27-hydroxylase is defective, the classic pathway via 25-hydroxylated intermediates appears to be the major route for side-chain cleavage and bile acid biosynthesis (**Fig. 1**).

Abbreviations: BHT, butylated hydroxytoluene; CA, cholic acid; CDCA, chenodeoxycholic acid; CTX, cerebrotendinous xanthomatosis; DMES, dimethylethylsilyl; GC, gas chromatography; MS, mass spectrometry; SIM, selected-ion monitoring; TMS, trimethylsilyl.

[1] To whom correspondence should be addressed at the GI Laboratory (15A), VA Medical Center, 385 Tremont Ave., East Orange, NJ 07018-1095.
e-mail: Salenge@UMDNJ.edu

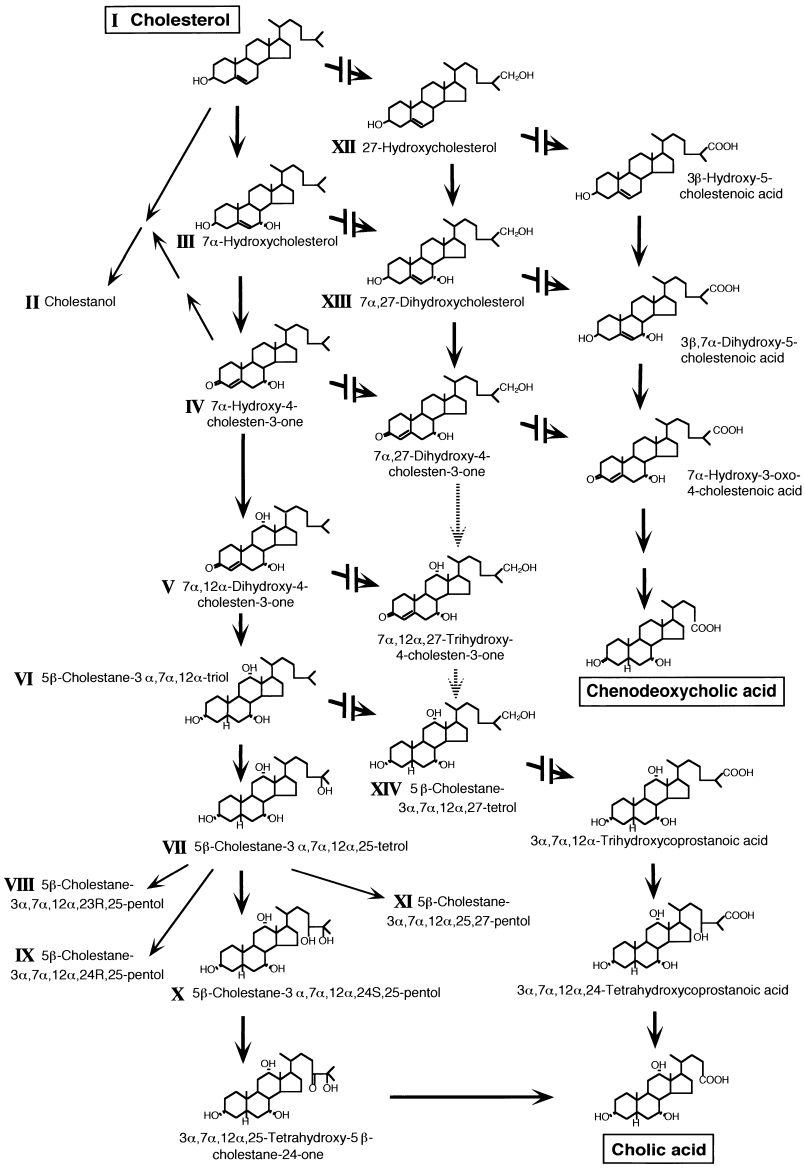

Fig. 1. Cholic and chenodeoxycholic acids biosynthesis from cholesterol in CTX and Cyp27$^{-/-}$ mice. Reactions catalyzed by sterol 27-hydroxylase are indicated by the interrupted lines. Solid arrows represent known enzymatic steps whereas broken arrows represent putative reactions that have yet to be confirmed. Roman numerals correspond to those in Tables 1–5.

Cerebrotendinous xanthomatosis (CTX) is a rare, recessively inherited disorder caused by a mutation in the *Cyp27* gene located on human chromosome 2 (8–10). Patients are characterized clinically by tendon and brain xanthomas, juvenile cataracts, osteoporosis, premature atherosclerosis, and nervous system dysfunction including mental retardation, dementia, cerebellar ataxia, epileptic seizures, and peripheral neuropathy (11). Biochemically, the pool size of CDCA is markedly reduced while that of CA is almost normal (3), and large amounts of 25-hydroxylated C-27 bile alcohols are excreted into bile and urine (12, 13). Increased hepatic concentrations of 7α-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one, 7α,12α-dihydroxy-4-cholesten-3-one, 5β-cholestane-3α,7α-diol, and 5β-cholestane-3α,7α,12α-triol, intermediates in early bile acid biosynthesis, were also reported in CTX liver (14). Another major biochemical feature is increased plasma and tissue concentrations of cholestanol, the 5α-saturated derivative of cholesterol, with particularly large deposits in brain, xanthomas, and bile (15, 16).

Mice with a disrupted *Cyp27* gene (knockout model) were reported by Rosen et al. (17). However, the Cyp27$^{-/-}$ mice showed no CTX-related clinical phenotype, and there was no accumulation of cholestanol in liver and circulation. In bile and feces, only trace amounts of 25-hydroxylated bile alcohols were detected, and CDCA and muricholic acids as well as CA concentrations were reduced compared with Cyp27$^{+/+}$ mice. In mice, muricholic acids are more abundant than CDCA but they are thought to be metabolites of CDCA (18). The only finding simulating CTX was increased plasma and tissue 7α-hydroxycholesterol concentrations. Thus, virtually all the pathological abnormalities observed in CTX were not reproduced in Cyp27$^{-/-}$ mice. The reasons are unclear, but it was speculated that the mice could not compensate for the lack of the mitochondrial sterol 27-hydroxylase through the alternative microsomal 25-hydroxylation pathway (17).

We have developed new assays for several enzyme reactions in 25- and 27-hydroxylation side-chain cleavage path-

ways for bile acid biosynthesis based on high resolution gas chromatography-mass spectrometry (GC-MS) (19). By using the new assay methods, the enzyme activities were compared between mice and humans. In mice, enzyme activities involved in both mitochondrial 27-hydroxylation and microsomal 25-hydroxylation pathways were generally two to four times higher than those in humans. Thus, our results do not support the idea that the microsomal 25-hydroxylation pathway in mice was not as active as that in humans.

The present study was undertaken to explore the reason why Cyp27$^{-/-}$ mice do not reproduce the biochemical features of CTX. To clarify the similarities and differences between CTX subjects and Cyp27$^{-/-}$ mice, we measured hepatic cholesterol, cholestanol, and 12 intermediates in the bile acid biosynthetic pathways by using high resolution GC-MS. The results showed that the concentrations of many intermediates in Cyp27$^{-/-}$ mice were significantly increased compared with Cyp27$^{+/+}$ mice, but were not as large as those in a CTX patient. In particular, 25-hydroxylated bile alcohol concentrations in Cyp27$^{-/-}$ mice were considerably lower than found in an untreated CTX patient.

MATERIALS AND METHODS

Chemicals

7α-Hydroxycholesterol was obtained from Steraloids (Wilton, NH). 27-Hydroxycholesterol was synthesized from diosgenin (20) and the pure compound was obtained by preparative thin-layer chromatography. 7α,27-Dihydroxycholesterol was synthesized from 27-hydroxycholesterol by the method of Shoda, Axelson, and Sjövall (21). 7α-Hydroxy-4-cholesten-3-one, 7α,12α-dihydroxy-4-cholesten-3-one, and 5β-cholestane-3α,7α,12α,25,27-pentol were gifts from T. Hoshita and K. Kihira (Pharmaceutical Institute, Hiroshima University, Hiroshima, Japan). 5β-Cholestane-3α,7α,12α-triol was prepared by electrolytic coupling of cholic acid with isovaleric acid according to Bergström and Krabisch (22). 5β-Cholestane-3α,7α,12α,25-tetrol was synthesized from CA by the method of Dayal et al. (23). 5β-Cholestane-3α,7α,12α,27-tetrol was prepared by lithium aluminum hydride reduction of the methyl ester of 3α,7α,12α-trihydroxy-5β-cholestan-27-oic acid, which was isolated from the bile of *Alligator mississippiensis*. 5β-Cholestane-3α,7α,12α,23*R*,25-pentol was isolated from bile and feces of patients with CTX (24). 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 (25). Sitosterol (24-ethyl-5-cholesten-3β-ol) and cholestane-3β,5α,6β-triol were purchased from Sigma (St. Louis, MO) and purified by recrystallization. [25,26,26,26,27,27,27-$^2H_7$]cholesterol was obtained from MSD Isotopes (Montreal, Canada). [$^2H_7$]7α-hydroxycholesterol (26), [$^2H_7$]27-hydroxycholesterol (21), [$^2H_7$]7α,27-dihydroxycholesterol (21), and [$^2H_7$]7α-hydroxy-4-cholesten-3-one (27) were prepared by previously described methods. The purities of these compounds were checked by gas-liquid chromatography, and each gave only a single peak.

Patients

Two CTX patients were studied. Patient 1 (CTX 1) was a 40-year-old male with dementia, spinal cord paresis, and cerebellar ataxia. Xanthomas were located in both Achilles tendons. Serum cholestanol concentration was 4.8 mg/dl (normal level, 0.2 ± 0.2 mg/dl). A liver biopsy was obtained for diagnostic histology and the extra tissue was available for biochemical analyses. Patient 2 (CTX 2) 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 four healthy adults who died unexpectedly and whose livers became available because no suitable recipient for liver transplantation could be found (University of Minnesota Hospital NIH 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 University of Medicine and Dentistry of New Jersey-New Jersey Medical School (Newark, NJ) and VA Medical Center (East Orange, NJ).

Animals

Cyp27$^{-/-}$ mice were produced by Rosen et al. (17) at the Hadassah-Hebrew University (Jerusalem, Israel) animal facility in a specific pathogen-free unit. Six male and four female Cyp27$^{-/-}$ mice, and three male and four female Cyp27$^{+/+}$ mice, were fed a normal chow diet. The animals, 3 months of age, were killed at noon and livers were obtained for hepatic sterol analyses. The liver specimens were immediately frozen and stored at −70°C until use. The animal protocol was approved by the Subcommittee on Animal Studies at VA Medical Center (East Orange, NJ) and by the Institutional Animal Care and Use Committee at University of Medicine and Dentistry of New Jersey-New Jersey Medical School.

Preparation of liver microsomes and mitochondria

The liver specimen was weighed, minced, and homogenized with a loose-fitting Teflon pestle in 4 volumes of 3 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 700 *g* for 10 min, and the supernatant was centrifuged at 7,000 *g* for 20 min. The 7,000 *g* pellet (mitochondrial fraction) was washed twice with homogenizing medium, and suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 5 mM dithiothreitol, 50 mM KCl, and 20% glycerol (v/v) (storage buffer). The 7,000 *g* supernatant was then centrifuged at 105,000 *g* for 90 min, and the pellet (microsomal fraction) was suspended in storage buffer. Protein concentrations of the mitochondrial and microsomal fractions were determined by the Lowry method (28).

Determination of cholesterol and cholestanol concentrations

[$^2H_7$]cholesterol (160 ng) was added to 20–100 μg of microsomal protein and saponification was carried out in 1 ml of 1 N ethanolic KOH at 37°C for 1 h. After addition of 0.5 ml of distilled water, sterols were extracted twice with 2 ml of *n*-hexane, and the extract was evaporated to dryness under nitrogen. Trimethylsilyl (TMS) ether derivatives were prepared by addition of 100 μl of TMSI-H (GL Sciences, Tokyo, Japan) and incubation at 55°C for 15 min. High resolution GC-MS with selected-ion monitoring (SIM) was performed with a JMS-SX102 instrument equipped with a JMA DA-6000 data-processing system (JEOL, Tokyo, Japan). The accelerating voltage was 10 kV, the ionization energy was 70 eV, the trap current was 300 μA, and the mass spectral resolution was about 10,000. An Ultra Performance capillary column (25 m × 0.32 mm i.d.) coated with methylsilicone (Hewlett-Packard, Palo Alto, CA) was used at a flow rate of he-

lium carrier gas of 1.0 ml/min. The column oven was programmed to change from 100°C to 245°C at 30°C/min, after a 1-min delay from the start time. The multiple ion detector was focused on *m/z* 458.3943 for cholesterol, *m/z* 465.4383 for [$^2H_7$]cholesterol, and *m/z* 445.3865 for cholestanol. [$^2H_7$]cholesterol was used as an internal recovery standard for both cholesterol and cholestanol.

Determination of 7α-hydroxycholesterol concentration

[$^2H_7$]7α-hydroxycholesterol (8 ng) and 5 μg of butylated hydroxytoluene (BHT) were added to 20–100 μg of microsomal protein and saponification was carried out in 1 ml of 1 N ethanolic KOH at 37°C for 1 h. After addition of 0.5 ml of distilled water, sterols were extracted twice with 2 ml of *n*-hexane. Purification by Bond Elut SI cartridge, derivatization into dimethylethylsilyl (DMES) ether, and high resolution GC-SIM analysis were carried out according to our previously described methods (29). The recovery of 7α-hydroxycholesterol from the Bond Elut SI cartridge was 93.5 ± 2.4% (n = 4), and the detection limit was 0.02 pmol/mg protein.

Determination of 27-hydroxycholesterol concentration

[$^2H_7$]27-hydroxycholesterol (16 ng) and 5 μg of BHT were added to 400 μg of mitochondrial protein and saponification was carried out in 1 ml of 1 N ethanolic KOH at 37°C for 1 h. After addition of 0.5 ml of distilled water and extraction twice with 2 ml of *n*-hexane, 27-hydroxycholesterol was purified by Bond Elut SI cartridge, derivatized into DMES ether, and quantified by high resolution GC-SIM (29). The recovery of 27-hydroxycholesterol from the Bond Elut SI cartridge was 91.2 ± 2.7% (n = 4), and the detection limit was 0.2 pmol/mg protein.

Determination of 7α,27-dihydroxycholesterol concentration

[$^2H_7$]7α,27-dihydroxycholesterol (2.6 ng) and 5 μg of BHT were added to 100–400 μg of microsomal protein and saponification was carried out in 0.3 ml of 1 N ethanolic KOH at 37°C for 1 h. After addition of 0.3 ml of distilled water, sterols were extracted with 3 ml of chloroform–methanol 2:1 (v/v) (30), and the solvent was evaporated to dryness. The residue was dissolved in 100 μl of *n*-hexane–isopropanol 9:1 (v/v), and the resulting solution was applied to a Bond Elut SI (500-mg) cartridge prewashed with 1 ml of *n*-hexane. After washing with 1 ml of *n*-hexane followed by 4 ml of *n*-hexane–isopropanol 97:3 (v/v), 7α,27-dihydroxycholesterol was eluted with 3 ml of *n*-hexane–isopropanol 1:1 (v/v). DMES ether derivatization was carried out by adding 100 μl of pyridine and 20 μl of DMES imidazole to the residue and heating at 55°C for 15 min. High resolution GC-SIM analysis was performed after the excess silylating reagent was removed on a Sephadex LH-20 column. The oven temperature was programmed to change from 100 to 280°C at 30°C/min, after a 1-min delay from the start time. The multiple ion detector was focused on *m/z* 572.4445 for 7α,27-dihydroxycholesterol, and *m/z* 579.4884 for [$^2H_7$]7α,27-dihydroxycholesterol. The recovery of 7α,27-dihydroxycholesterol from the Bond Elut SI cartridge was 90.8 ± 3.1% (n = 4), and the detection limit was 0.03 pmol/mg protein.

Determination of 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one concentrations

[$^2H_7$]7α-hydroxy-4-cholesten-3-one (14 ng), 0.2 ml of distilled water, and 0.2 ml of ethanol were added to 50–400 μg of microsomal protein. Sterols were extracted twice with 2 ml of petroleum ether (31), and the solvent was evaporated to dryness. The following purification and quantification steps were based on our previously described methods (32) with some modifications. The residue was dissolved in 100 μl of *n*-hexane–diethyl ether 3:1 (v/v), and the resulting solution was applied to a Bond Elut SI cartridge prewashed with 1 ml of the solvent mixture described above. After washing with 8 ml of the same mixture, 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one were eluted with 3 ml of *n*-hexane–isopropanol 1:1 (v/v). Methyloxime DMES ether derivatization was carried out by adding 100 μl of 1% *O*-methylhydroxylamine hydrochloride in pyridine (w/v) and heating at 60°C for 1 h followed by addition of 50 μl of DMES imidazole and heating at 60°C for 15 min. High resolution GC-SIM analysis was performed after the excess silylating reagent was removed on a Sephadex LH-20 column. The oven temperature was programmed to change from 100°C to 270°C at 30°C/min, after a 1-min delay from the start time. The multiple ion detector was focused on *m/z* 380.3317 for 7α-hydroxy-4-cholesten-3-one, *m/z* 387.3757 for [$^2H_7$]7α-hydroxy-4-cholesten-3-one, and *m/z* 378.3160 for 7α,12α-dihydroxy-4-cholesten-3-one. [$^2H_7$]7α-hydroxy-4-cholesten-3-one was used as an internal recovery standard for both 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one. The recoveries of 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one from the Bond Elut SI cartridge were 93.0 ± 3.9% (n = 4) and 90.2 ± 2.3% (n = 4), respectively, and the detection limits were 0.1 and 0.5 pmol/mg protein, respectively.

Determination of 5β-cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α,7α,12α,27-tetrol, and pentahydroxy bile alcohol concentrations

After addition of cholestane-3β,5α,6β-triol (200 ng) as an internal recovery standard and 0.5 ml of distilled water to 50–400 μg of microsomal or mitochondrial protein, sterols were extracted twice with 2 ml of ethyl acetate and the solvent was evaporated to dryness. The residue was dissolved in 100 μl of chloroform–acetone 35:25 (v/v), and applied to a Bond Elut SI (500-mg) cartridge that was prewashed with 3 ml of the same solvent. After washing out cholesterol with 5 ml of the same solvent, 5β-cholestane-triol, tetrols, and pentols were eluted with 3 ml of chloroform–acetone–methanol 35:25:20 (v/v/v). TMS ether derivatization and quantification by high resolution GC-SIM was performed as described previously (19). The multiple ion detector was focused on *m/z* 456.3787 for cholestane-3β,5α,6β-triol, *m/z* 253.1956 for 5β-cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α,12α,25-tetrol, and 5β-cholestane-3α,7α,12α,27-tetrol, *m/z* 307.2425 for 5β-cholestane-3α,7α,12α,23*R*,25-pentol, *m/z* 321.2582 for 5β-cholestane-3α,7α,12α, 24*R*,25-pentol and 5β-cholestane-3α,7α,12α,24*S*,25-pentol, and *m/z* 349.2895 for 5β-cholestane-3α,7α,12α,25,27-pentol. The detection limits of 5β-cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α,7α,12α,27-tetrol, 5β-cholestane-3α,7α,12α,23*R*,25-pentol, 5β-cholestane-3α,7α,12α,24*R*,25-pentol, 5β-cholestane-3α,7α,12α,24*S*,25-pentol, and 5β-cholestane-3α,7α,12α,25,27-pentol were 0.05, 0.05, 0.05, 0.1, 0.5, 0.5, and 0.2 pmol/mg protein, respectively.

Statistics

Data are reported here as means ± SEM. The significance of differences between the results in the different groups was evaluated by the Student's two-tailed *t*-test and significance was accepted at the level of $P < 0.05$.

RESULTS

Table 1 shows hepatic microsomal cholesterol and cholestanol concentrations. Cholesterol concentration in

TABLE 1. Hepatic microsomal concentrations of cholesterol and cholestanol in CTX patients and Cyp27$^{-/-}$ mice

	I: Cholesterol	**II:** Cholestanol
	nmol/mg protein	
Human		
CTX 1 (untreated)	84	2.4
CTX 2 (treated)	68	1.0
Control (n = 4)	61 ± 4 (48–67)[a]	0.16 ± 0.01 (0.14–0.19)[a]
Mouse		
Male Cyp27$^{-/-}$ (n = 6)	42 ± 1 (37–45)	0.38 ± 0.02 (0.34–0.43)[b]
Male Cyp27$^{+/+}$ (n = 3)	45 ± 4 (40–52)	0.28 ± 0.03 (0.23–0.34)
Female Cyp27$^{-/-}$ (n = 4)	44 ± 4 (36–54)	0.32 ± 0.03 (0.26–0.40)[c]
Female Cyp27$^{+/+}$ (n = 4)	45 ± 2 (39–49)	0.14 ± 0.01 (0.11–0.16)

Roman numerals correspond to those in Fig. 1. Means ± SEM and (range of results) are given.
[a] Ninety-five percent confidence intervals for control means of cholesterol and cholestanol concentrations are 47 to 74 and 0.13 to 0.19 nmol/mg protein, respectively.
[b] $P < 0.05$ versus Cyp27$^{+/+}$ mice.
[c] $P < 0.005$ versus Cyp27$^{+/+}$ mice.

untreated CTX patient 1 was 138% of the control mean, which was above the 95% confidence limit, whereas the concentration in CTX patient 2, treated with CDCA, was not significantly different from control subjects. Cholesterol levels in Cyp27$^{-/-}$ mice were not significantly different from those in Cyp27$^{+/+}$ mice. Cholestanol concentrations in untreated and treated CTX patients were increased 15-fold and 6.3-fold, respectively, compared with human control subjects. In Cyp27$^{-/-}$ mice, cholestanol concentrations were also significantly elevated 1.4-fold (male) and 2.3-fold (female) compared with Cyp27$^{+/+}$ mice.

Hepatic microsomal concentrations of three intermediates in early steps of the classic bile acid biosynthetic pathway are shown in **Table 2**. Concentrations of 7α-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one, and 7α,12α-dihydroxy-4-cholesten-3-one in untreated CTX patient 1 were elevated 8.7-fold, 318-fold, and 449-fold, respectively, compared with human control subjects. CDCA-treated patient 2 had much lower concentrations of the sterols than did untreated patient 1. These sterol concentrations were increased 14-fold, 101-fold, and more than 282-fold, respectively, in male Cyp27$^{-/-}$ mice, and were elevated 3.1-fold, 20-fold, and more than 126-fold, respectively, in female Cyp27$^{-/-}$ mice compared with Cyp27$^{+/+}$ mice. Absolute concentrations of 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one in male Cyp27$^{-/-}$ mice were close to the levels in the untreated CTX patient. However, the others were much lower than those in the untreated CTX patient.

Figure 2 shows representative chromatograms of TMS ether derivatives of 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α,12α,25-tetrol obtained by analyses of hepatic microsomal fractions from untreated CTX patient 1, a human control subject, and Cyp27$^{-/-}$ and Cyp27$^{+/+}$ mice. We used cholestane-3β,5α,6β-triol as an internal recovery standard for these sterols. The recoveries of cholestane-3β,5α,6β-triol and 5β-cholestane-3α,7α,12α-triol and

TABLE 2. Hepatic microsomal concentrations of 7α-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one, and 7α,12α-dihydroxy-4-cholesten-3-one in CTX patients and Cyp27$^{-/-}$ mice

	III: 7α-Hydroxycholesterol	**IV:** 7α-Hydroxy-4-cholesten-3-one	**V:** 7α,12α-Dihydroxy-4-cholesten-3-one
		pmol/mg protein	
Human			
CTX 1 (untreated)	331	191	898
CTX 2 (treated)	139	2.9	11
Control (n = 4)	38 ± 8 (22–61)[a]	0.6 ± 0.1 (0.4–0.9)[a]	2.0 ± 0.3 (1.3–2.6)[a]
Mouse			
Male Cyp27$^{-/-}$ (n = 6)	285 ± 35 (174–430)[b]	151 ± 12 (115–204)[b]	141 ± 8 (120–165)[b]
Male Cyp27$^{+/+}$ (n = 3)	20 ± 5 (11–28)	1.5 ± 0.5 (0.8–2.4)	<0.5[c]
Female Cyp27$^{-/-}$ (n = 4)	74 ± 4 (68–84)[b]	57 ± 4 (48–66)[b]	63 ± 9 (40–81)[b]
Female Cyp27$^{+/+}$ (n = 4)	24 ± 3 (16–29)	2.8 ± 0.3 (2.0–3.1)	<0.5[c]

Roman numerals correspond to those in Fig. 1. Means ± SEM and (range of results) are given.
[a] Ninety-five percent confidence intervals for control means of 7α-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one, and 7α,12α-dihydroxy-4-cholesten-3-one concentrations are 12 to 64, 0.2 to 1.0, and 0.0 to 5.5 pmol/mg protein, respectively.
[b] $P < 0.005$ versus Cyp27$^{+/+}$ mice.
[c] Limit of detection.

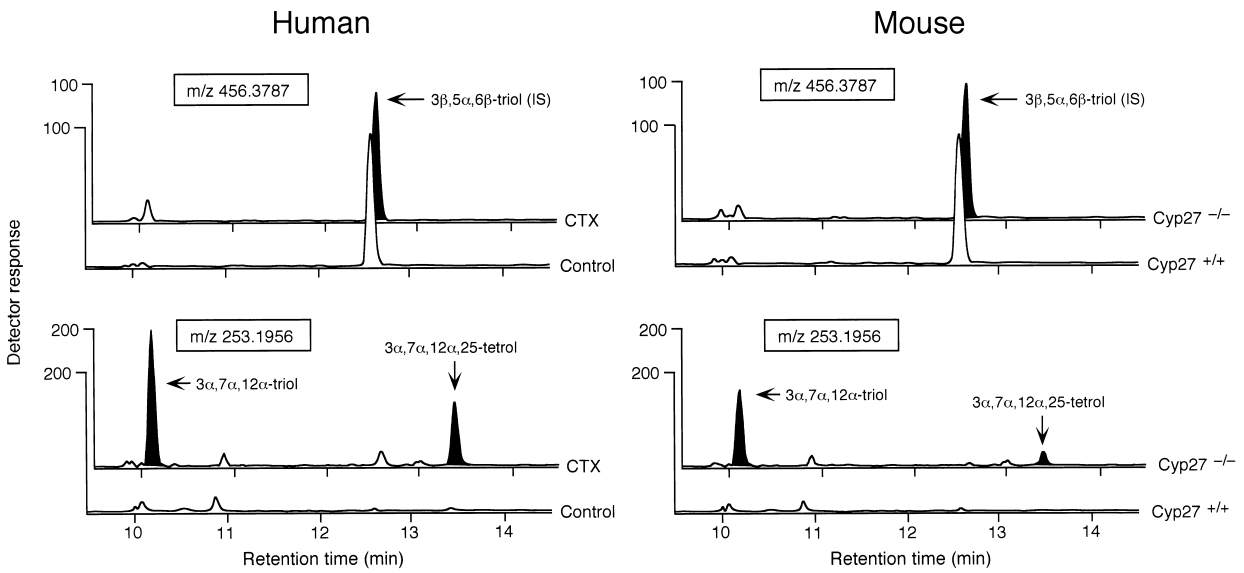

Fig. 2. Representative high resolution GC-SIM chromatograms of hepatic microsomal 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α,12α,25-tetrol in CTX patients, human control subjects, and Cyp27$^{-/-}$ and Cyp27$^{+/+}$ mice. Approximately 100 μg of microsomal protin from a CTX patient, human control subject, a male Cyp27$^{-/-}$ mouse, and a male Cyp27$^{+/+}$ mouse were used in the analyses. IS, internal standard.

the elimination of cholesterol from the Bond Elut SI cartridge were checked by adding 200 ng each of the triols and cholesterol to the column. The sterol amounts were quantified by GC-SIM, using sitosterol as an internal standard. The recoveries of cholestane-3β,5α,6β-triol and 5β-cholestane-3α,7α,12α-triol were 98.5 ± 3.9% (n = 4) and 97.8 ± 3.5% (n = 4), respectively, while more than 99.8% of cholesterol (n = 4) was eliminated by the purification procedure. Microsomal and mitochondrial fractions contained small amounts of endogenous cholestane-3β,5α,6β-triol, and the amounts were less than 2% of the added internal standards.

Table 3 summarizes the microsomal concentrations of 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α,12α,25-tetrol. The triol and tetrol levels in untreated CTX patient 1 were elevated 78-fold and 114-fold, respectively, compared with control subjects. CDCA-treated patient 2 showed much lower concentrations of the sterols, and the levels were 4.3% (triol) and 18% (tetrol) of those in the untreated patient. In Cyp27$^{-/-}$ mice, 5β-cholestane-3α,7α,12α-triol concentrations were elevated 74-fold (male) and 6.1-fold (female), and 5β-cholestane-3α,7α,12α,25-tetrol levels were increased 16-fold (male) and 12-fold (female) compared with those in Cyp27$^{+/+}$ mice. However, absolute concentrations of 5β-cholestane-3α,7α,12α,25-tetrol in both male and female Cyp27$^{-/-}$ mice were lower than that in CDCA-treated patient 2 and were less than 10% of that in untreated CTX patient 1.

Microsomal concentrations of pentahydroxy bile alcohols are shown in **Table 4**. 5β-Cholestane-3α,7α,12α,23*R*,

TABLE 3. Hepatic microsomal concentrations of 5β-cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α,12α,25-tetrol, and 5β-cholestane-3α,7α,12α,26(27)-tetrol in CTX patients and Cyp27$^{-/-}$ mice

	VI: 5β-Cholestane-3α,7α,12α-triol	**VII:** 5β-Cholestane-3α,7α,12α,25-tetrol
	pmol/mg protein	
Human		
CTX 1 (untreated)	391	263
CTX 2 (treated)	17	48
Control (n = 4)	5.0 ± 0.4 (4.1–5.9)[a]	2.3 ± 0.6 (1.4–4.0)[a]
Mouse		
Male Cyp27$^{-/-}$ (n = 6)	176 ± 37 (104–222)[b]	14 ± 2 (12–18)[c]
Male Cyp27$^{+/+}$ (n = 3)	2.4 ± 0.8 (1.4–4.0)	0.9 ± 0.1 (0.6–1.0)
Female Cyp27$^{-/-}$ (n = 4)	35 ± 4 (23–43)[c]	24 ± 2 (20–28)[c]
Female Cyp27$^{+/+}$ (n = 4)	5.7 ± 1.4 (2.0–8.0)	2.0 ± 0.3 (1.3–2.8)

Roman numerals correspond to those in Fig. 1. Means ± SEM and (range of results) are given.

[a] Ninety-five percent confidence intervals for control means of 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α,12α,25-tetrol concentrations are 3.7 to 6.3 and 0.4 to 4.2 pmol/mg protein, respectively.

[b] $P < 0.01$ versus Cyp27$^{+/+}$ mice.

[c] $P < 0.001$ versus Cyp27$^{+/+}$ mice.

TABLE 4. Hepatic microsomal concentrations of 5β-cholestane-3α,7α,12α,23*R*,25-pentol, 5β-cholestane-3α,7α,12α,24*R*,25-pentol, 5β-cholestane-3α,7α,12α,24*S*,25-pentol, and 5β-cholestane-3α,7α,12α,25,27-pentol in CTX patients and Cyp27$^{-/-}$ mice

	VIII: 5β-Cholestane-3α,7α,12α,23*R*,25-pentol	**IX:** 5β-Cholestane-3α,7α,12α,24*R*,25-pentol	**X:** 5β-Cholestane-3α,7α,12α,24*S*,25-pentol	**XI:** 5β-Cholestane-3α,7α,12α,25,27-pentol
		pmol/mg protein		
Human				
CTX 1 (untreated)	514	144	91	6.1
CTX 2 (treated)	44	8.2	2.0	3.2
Control (n = 4)	1.7 ± 0.6 (0.7–3.4)[a]	3.2 ± 0.9 (1.0–5.1)[a]	2.2 ± 0.4 (1.2–3.2)[a]	1.3 ± 0.3 (0.7–2.0)[a]
Mouse				
Male Cyp27$^{-/-}$ (n = 6)	71 ± 10 (57–89)[b]	14 ± 1 (12–17)[b]	<0.5[d]	<0.5[d]
Male Cyp27$^{+/+}$ (n = 3)	<0.1[d]	<0.5[d]	<0.5[d]	<0.5[d]
Female Cyp27$^{-/-}$ (n = 4)	66 ± 6 (49–76)[b]	8.9 ± 2.1 (4.2–13.3)[c]	<0.5[d]	<0.5[d]
Female Cyp27$^{+/+}$ (n = 4)	<0.1[d]	<0.5[d]	<0.5[d]	<0.5[d]

Roman numerals correspond to those in Fig. 1. Means ± SEM and (range of results) are given.

[a] Ninety-five percent confidence intervals for control means of 5β-cholestane-3α,7α,12α,23*R*,25-pentol, 5β-cholestane-3α,7α,12α,24*R*,25-pentol, 5β-cholestane-3α,7α,12α,24*S*,25-pentol, and 5β-cholestane-3α,7α,12α,25,27-pentol concentrations are 0.0 to 3.6, 0.3 to 6.1, 0.9 to 3.5, and 0.3 to 2.3 pmol/mg protein, respectively.

[b] $P < 0.0005$ versus Cyp27$^{+/+}$ mice.

[c] $P < 0.01$ versus Cyp27$^{+/+}$ mice.

[d] Limit of detection.

25-pentol, 5β-cholestane-3α,7α,12α, 24*R*,25-pentol, 5β-cholestane-3α,7α,12α,24*S*,25-pentol, and 5β-cholestane-3α,7α,12α,25,27-pentol levels in untreated CTX patient 1 were elevated 302-fold, 45-fold, 41-fold, and 4.7-fold, respectively, compared with control subjects. In CDCA-treated CTX patient 2, these pentahydroxy bile alcohol levels were 8.6%, 5.7%, 2.2%, and 52% of those in the untreated patient, respectively. In male and female Cyp27$^{-/-}$ mice, 5β-cholestane-3α,7α,12α,23*R*,25-pentol and 5β-cholestane-3α,7α,12α, 24*R*,25-pentol concentrations were significantly increased compared with Cyp27$^{+/+}$ mice, but the levels were similar to those in the CDCA-treated patient.

Table 5 shows hepatic mitochondrial concentrations of 27-hydroxycholesterol and 5β-cholestane-3α,7α,12α,27-tetrol, and microsomal levels of 7α,27-dihydroxycholesterol. Mitochondrial 27-hydroxycholesterol and 5β-cholestane-3α,7α,12α,27-tetrol were virtually absent in treated and untreated CTX patients and Cyp27$^{-/-}$ mice, while significant amounts of these sterols were detected in human control subjects and Cyp27$^{+/+}$ mice. 7α,27-Dihydroxycholesterol was not detected in Cyp27$^{-/-}$ mice, while a significant amount of the sterol was detected in Cyp27$^{+/+}$ mice. Surprisingly, 7α,27-dihydroxycholesterol concentrations were increased 15-fold and 3.9-fold in untreated and CDCA-treated CTX patients, respectively.

DISCUSSION

Hepatic concentrations of intermediates in early bile acid biosynthesis in a CTX patient were previously reported by Oftebro and Björkhem et al. (14, 33). Our data are consistent with their observations that mitochondrial 5β-cholestane-3α,7α,12α,27-tetrol was virtually absent and that microsomal 7α-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one, 7α,12α-dihydroxy-4-cholesten-3-one, and 5β-cholestane-3α,7α,12α-triol concentrations were all ele-

TABLE 5. Hepatic mitochondrial 27-hydroxycholesterol and 5β-cholestane-3α,7α,12α,27-tetrol, and microsomal 7α,27-dihydroxycholesterol concentrations in CTX patients and Cyp27$^{-/-}$ mice

	XII: 27-Hydroxycholesterol	**XIII:** 7α,27-Dihydroxycholesterol	**XIV:** 5β-Cholestane-3α,7α,12α,27-tetrol
		pmol/mg protein	
Human			
CTX1 (untreated)	<0.2[a]	2.16	<0.05[a]
CTX2 (treated)	<0.2[a]	0.54	<0.05[a]
Control (n = 4)	7.5 ± 0.8 (5.4–9.1)[b]	0.14 ± 0.03 (0.07–0.21)[b]	0.15 ± 0.03 (0.10–0.24)[b]
Mouse			
Male Cyp27$^{-/-}$ (n = 6)	<0.2[a,c]	<0.03[a,c]	<0.05[a,d]
Male Cyp27$^{+/+}$ (n = 3)	8.6 ± 3.2 (2.9–13.8)	0.17 ± 0.09 (0.05–0.33)	0.18 ± 0.02 (0.14–0.20)
Female Cyp27$^{-/-}$ (n = 4)	<0.2[a,d]	<0.03[a,c]	<0.05[a,c]
Female Cyp27$^{+/+}$ (n = 4)	9.1 ± 1.1 (7.3–12.2)	0.13 ± 0.03 (0.07–0.18)	0.50 ± 0.13 (0.18–0.75)

Roman numerals correspond to those in Fig. 1. Means ± SEM and (range of results) are given.

[a] Limit of detection.

[b] Ninety-five percent confidence intervals for control means of 27-hydroxycholesterol, 7α,27-dihydroxycholesterol, and 5β-cholestane-3α,7α,12α,27-tetrol concentrations are 5.0 to 10.0, 0.04 to 0.24, and 0.05 to 0.25 pmol/mg protein, respectively.

[c] $P < 0.05$ versus Cyp27$^{+/+}$ mice.

[d] $P < 0.0005$ versus Cyp27$^{+/+}$ mice.

vated in CTX patients. We also showed that mitochondrial 27-hydroxycholesterol was absent in CTX, which supports the contention that a single 27-hydroxylase enzyme (Cyp27) catalyzes C-27 hydroxylation of both cholesterol and 5β-cholestane-3α,7α,12α-triol. To the best of our knowledge, there is no report about hepatic concentrations of 25-hydroxylated bile alcohols in CTX patients. Although most of the bile alcohols are excreted in bile and urine as bile alcohol glucuronides (12, 13), we measured only free forms of bile alcohols in the microsomal fraction because unconjugated bile alcohols appear to reflect synthesis more directly. Actually, microsomal free bile alcohol concentrations were markedly increased in the untreated CTX patient.

It has been well established that treatment with CDCA markedly reduces plasma, urinary, and biliary bile alcohol concentrations and plasma cholestanol levels in CTX patients (13, 34). Reports showed that CDCA was a physiological ligand for the farnesoid X receptor, an orphan nuclear receptor (35–37). When CDCA is bound to the receptor, transcription of cholesterol 7α-hydroxylase gene is repressed. Because CDCA production is markedly reduced in CTX, cholesterol 7α-hydroxylase activity is derepressed (38). Our results showed that CDCA treatment reduced not only hepatic bile alcohol and cholestanol levels but also other intermediates in early steps of bile acid biosynthesis in a CTX patient. The findings support the contention that the major therapeutic effect of CDCA on CTX patients is due to the inhibition of cholesterol 7α-hydroxylase activity.

In female Cyp27$^{-/-}$ mice, absolute microsomal concentrations of intermediates in early bile acid biosynthesis, 25-hydroxylated bile alcohols and cholestanol, were much lower than in the untreated CTX patient. In male Cyp27$^{-/-}$ mice, 25-hydroxylated bile alcohols and cholestanol concentrations were similar to those in female Cyp27$^{-/-}$ mice, but intermediate levels in early bile acid biosynthesis were between those of female Cyp27$^{-/-}$ mice and the untreated CTX patient. The reason for less accumulation of the sterols in Cyp27$^{-/-}$ mice is not clear, but there may be two explanations. First, cholesterol 7α-hydroxylase activity in Cyp27$^{-/-}$ mice, especially in females, might not be upregulated as much as in CTX. Compared with the untreated CTX patient, microsomal concentrations of all intermediates were markedly reduced in the CDCA-treated CTX patient, who had reduced activity of cholesterol 7α-hydroxylase. Although reduced total bile acid synthesis in Cyp27$^{-/-}$ mice has not been proved directly, decreased fecal and biliary bile acid concentrations suggest that the production of bile acids was also low in Cyp27$^{-/-}$ mice (17). Thus, upregulation of cholesterol 7α-hydroxylase activity in Cyp27$^{-/-}$ mice might not be sufficient to compensate for blocked alternative bile acid synthesis via sterol 27-hydroxylase.

Second, 25-hydroxylated bile alcohols seem to be more efficiently metabolized in Cyp27$^{-/-}$ mice than in CTX patients. In female as well as male Cyp27$^{-/-}$ mice, microsomal 25-hydroxylated bile alcohol concentrations were markedly reduced compared with those in the untreated CTX patient. One might expect that the 25-hydroxylation side-chain cleavage pathway in mice was not as active as that in humans. However, our previous results showed that 5β-cholestane-3α,7α,12α-triol 25-hydroxylase and 5β-cholestane-3α,7α,12α,25-tetrol 24*S*-hydroxylase activities in normal mice were 4.2-fold and 2.3-fold higher than those in human control subjects, respectively (19). Thus, the 25-hydroxylation side-chain cleavage pathway appears to be basically more active in mice than in humans. In addition, there are reports that 5β-cholestane-3α,7α,12α-triol 25-hydroxylase activities were not upregulated in CTX patients compared with control subjects (3), and that 5β-cholestane-3α,7α,12α,25-tetrol 24*S*-hydroxylase activity in CTX was lower than in control subjects (39). Microsomal 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol may also be active in Cyp27$^{-/-}$ mice. The reaction has been documented in rat liver, but has yet to be demonstrated in humans and mice. If the microsomal 26-hydroxylation pathway is upregulated in Cyp27$^{-/-}$ mice but not in CTX, it may also explain the lower concentrations of 25-hydroxylated bile alcohols in Cyp27$^{-/-}$ mouse liver.

Comparison of microsomal concentrations of 7α,27-dihydroxycholesterol between Cyp27$^{-/-}$ mice and CTX are intriguing (Table 5). This sterol, as well as 27-hydroxycholesterol, was not detected in Cyp27$^{-/-}$ mice, which is consistent with the lack of sterol 27-hydroxylase. In contrast, 7α,27-dihydroxycholesterol levels were considerably increased in the untreated CTX patient and mildly elevated in the CDCA-treated CTX patient, although 27-hydroxycholesterol concentrations were absent in these patients. The paradoxical elevation of 7α,27-dihydroxycholesterol in CTX patients may be explained by stimulated 27-hydroxylation of 7α-hydroxycholesterol due to increased 7α-hydroxycholesterol concentration. In addition to cholesterol and 5β-cholestane-3α,7α,12α-triol, 7α-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one, 7α,12α-dihydroxy-4-cholesten-3-one, and 5β-cholestane-3α,7α-diol are all good substrates for mitochondrial sterol 27-hydroxylase (40). In CTX patients, sterol 27-hydroxylase activities are markedly reduced (5–50% of normal) but not completely absent (3), whereas the enzyme must be completely devoid in Cyp27$^{-/-}$ mice. Formed 7α,27-dihydroxycholesterol is normally converted into 7α,27-dihydroxy-4-cholesten-3-one by microsomal 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase. A patient with 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase deficiency showed markedly increased serum 7α-hydroxycholesterol concentration and excretion of 3β,7α-dihydroxy- and 3β,7α,12α-trihydroxy-5-cholenoic acids in urine (41). This result suggests that conversion of 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-one and that of 7α,27-dihydroxy cholesterol to 7α,27-dihydroxy-4-cholesten-3-one are both defective in the patient, and both reactions are catalyzed by a single enzyme. In CTX, the reaction from 7α,27-dihydroxycholesterol to 7α,27-dihydroxy-4-cholesten-3-one might be partially blocked because 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase is already oversaturated with another substrate, 7α-hydroxycholesterol.

In summary, hepatic concentrations of cholestanol, all

the intermediates in early steps of classic bile acid biosynthesis, and 25-hydroxylated bile alcohols were significantly increased in Cyp27$^{-/-}$ mice compared with Cyp27$^{+/+}$ mice, but the levels were lower than those in an untreated CTX patient. Our results suggest that in Cyp27$^{-/-}$ mice, cholesterol 7α-hydroxylase activity was not upregulated as much as in CTX patients. In addition, the alternative 25-hydroxylation side-chain cleavage pathway might be much more active in Cyp27$^{-/-}$ mice than in CTX patients.

The authors thank Bibiana Pcolinsky and Eva Paroulek for excellent technical assistance and Barbara Rouse for preparing the manuscript. This work was supported in part by grants from the Department of Veterans Affairs Research Service; by grants HD-31932, HL-17818, and DK-26756 from the National Institutes of Health; and by grant (B)10470130 from the Ministry of Education, Science and Culture of Japan.

Manuscript received 24 February 2000 and in revised form 6 October 2000.

REFERENCES

1. Vlahcevic, Z. R., W. M. Pandak, D. M. Heuman, and P. B. Hylemon. 1992. Function and regulation of hydroxylases involved in the bile acid biosynthesis pathways. *Semin. Liver Dis.* **12:** 403–419.
2. Shefer, S., F. W. Cheng, A. K. Batta, B. Dayal, G. S. Tint, G. Salen, and E. H. Mosbach. 1978. Stereospecific side chain hydroxylations in the biosynthesis of chenodeoxycholic acid. *J. Biol. Chem.* **253:** 6386–6392.
3. Salen, G., S. Shefer, G. S. Tint, G. Nicolau, B. Dayal, and A. K. Batta. 1985. Biosynthesis of bile acids in cerebrotendinous xanthomatosis. *J. Clin. Invest.* **76:** 744–751.
4. Okuda, K., O. Masumoto, and Y. Ohyama. 1988. Purification and characterization of 5β-cholestane-3α,7α,12α-triol 27-hydroxylase from female rat liver mitochondria. *J. Biol. Chem.* **263:** 18138–18142.
5. Taniguchi, S., N. Hoshita, and K. Okuda. 1973. Enzymatic characteristics of CO-sensitive 26-hydroxylase system for 5β-cholestane-3α,7α,12α-triol in rat-liver mitochondria and its intramitochondrial localization. *Eur. J. Biochem.* **40:** 607–617.
6. Björkhem, I., H. Danielsson, and K. Wikvall. 1976. Side chain hydroxylations in biosynthesis of cholic acid. *J. Biol. Chem.* **251:** 3495–3499.
7. Andersson, S., I. Holmberg, and K. Wikvall. 1983. 25-Hydroxylation of C_{27}-steroids and vitamin D_3 by a constitutive cytochrome P-450 from rat liver microsomes. *J. Biol. Chem.* **258:** 6777–6781.
8. Cali, J. J., C-L. Hsieh, U. Francke, and D. W. Russell. 1991. Mutations in the bile acid biosynthetic enzyme sterol 27-hydroxylase underlie cerebrotendinous xanthomatosis. *J. Biol. Chem.* **266:** 7779–7783.
9. Leitersdorf, E., A. Reshef, V. Meiner, R. Levitzki, S. Pressman-Schwartz, E. J. Dann, N. Berkman, J. J. Cali, L. Klapholz, and V. M. Berginer. 1993. Frameshift and splice-junction mutations in the sterol 27-hydroxylase gene cause cerebrotendinous xanthomatosis in Jews of Moroccan origin. *J. Clin. Invest.* **91:** 2488–2496.
10. Kim, K. S., S. Kubota, M. Kuriyama, J. Fujiyama, I. Björkhem, G. Eggertsen, and Y. Seyama. 1994. Identification of new mutations in sterol 27-hydroxylase gene in Japanese patients with cerebrotendinous xanthomatosis (CTX). *J. Lipid Res.* **35:** 1031–1039.
11. Salen, G., S. Shefer, and V. Berginer. 1983. Familial diseases with storage of sterols other than cholesterol: cerebrotendinous xanthomatosis and sitosterolemia with xanthomatosis. *In* The Metabolic Basis of Inherited Diseases. 5th edition. J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, M. S. Brown, and J. L. Goldstein, editors. McGraw-Hill, New York. 713–730.
12. Hoshita, T., M. Yasuhara, M. Une, A. Kibe, E. Itoga, S. Kito, and T. Kuramoto. 1980. Occurrence of bile alcohol glucuronides in bile of patients with cerebrotendinous xanthomatosis. *J. Lipid Res.* **21:** 1015–1021.
13. Batta, A. K., S. Shefer, M. Batta, and G. Salen. 1985. Effect of chenodeoxycholic acid on biliary and urinary bile acids and bile alcohols in cerebrotendinous xanthomatosis: monitoring by high performance liquid chromatography. *J. Lipid Res.* **26:** 690–698.
14. Björkhem, I., H. Oftebro, S. Skrede, and J. I. Pedersen. 1981. Assay of intermediates in bile acid biosynthesis using isotope dilution-mass spectrometry: hepatic levels in the normal state and in cerebrotendinous xanthomatosis. *J. Lipid Res.* **22:** 191–200.
15. Menkes, J. H., J. R. Schimshock, and P. C. Swanson. 1968. Cerebrotendinous xanthomatosis: the storage of cholestanol within the nervous system. *Arch. Neurol.* **19:** 47–53.
16. Salen, G. 1971. Cholestanol deposition in cerebrotendinous xanthomatosis: a possible mechanism. *Ann. Intern. Med.* **75:** 843–851.
17. Rosen, H., A. Reshef, N. Maeda, A. Lippoldt, S. Shpizen, L. Triger, G. Eggertsen, I. Björkhem, and E. Leitersdorf. 1998. Markedly reduced bile acid synthesis but maintained levels of cholesterol and vitamin D metabolites in mice with disrupted sterol 27-hydroxylase gene. *J. Biol. Chem.* **273:** 14805–14812.
18. Mahowald, T. A., J. T. Matschiner, S. L. Hsia, E. A. Doisy, Jr., W. H. Elliot, and E. A. Doisy. 1957. Bile acids. III. Acid I; the principal bile acid in urine of surgically jaundiced rats. *J. Biol. Chem.* **225:** 795–802.
19. Honda, A., G. Salen, S. Shefer, Y. Matsuzaki, G. Xu, A. K. Batta, G. S. Tint, and N. Tanaka. 2000. Regulation of 25- and 27-hydroxylation side chain cleavage pathways for cholic acid biosynthesis in humans, rabbits and mice: assay of enzyme activities by high-resolution gas chromatography-mass spectrometry. *J. Lipid Res.* **41:** 442–451.
20. Kim, H-S., W. K. Wilson, D. H. Needleman, F. D. Pinkerton, D. K. Wilson, F. A. Quiocho, and G. J. Schroepfer, Jr. 1989. Inhibitors of sterol synthesis. Chemical synthesis, structure, and biological activities of (25*R*)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one, a metabolite of 3β-hydroxy-5α-cholest-8(14)-en-15-one. *J. Lipid Res.* **30:** 247–261.
21. Shoda, J., M. Axelson, and J. Sjövall. 1993. Synthesis of potential C_{27}-intermediates in bile acid biosynthesis and their deuterium-labeled analogs. *Steroids.* **58:** 119–125.
22. Bergström, S., and L. Krabisch. 1957. Preparation of some hydroxy coprostanes, 3α,7α- and 3α,12α-dihydroxy coprostane. *Acta. Chem. Scand.* **11:** 1067.
23. Dayal, B., S. Shefer, G. S. Tint, G. Salen, and E. H. Mosbach. 1976. Synthesis of 5β-cholestane-3α,7α,12α,25-tetrol and 5β-cholestane-3α,7α,12α,24ζ,25-pentol. *J. Lipid Res.* **17:** 74–77.
24. Shefer, S., B. Dayal, G. S. Tint, G. Salen, and E. H. Mosbach. 1975. Identification of pentahydroxy bile alcohols in cerebrotendinous xanthomatosis: characterization of 5β-cholestane-3α,7α,12α,24ζ, 25-pentol and 5β-cholestane-3α,7α,12α,23ζ,25-pentol. *J. Lipid Res.* **16:** 280–286.
25. Hoshita, T. 1962. Stero-bile acids and bile sterols. XLVII. Syntheses of 3α,7α,12α,25ζ,26- and 3α,7α,12α,24ζ,25-pentahydroxycoprostanes. *J. Biochem.* **52:** 176–179.
26. Honda, A., J. Shoda, N. Tanaka, Y. Matsuzaki, T. Osuga, N. Shigematsu, M. Tohma, and H. Miyazaki. 1991. Simultaneous assay of the activities of two key enzymes in cholesterol metabolism by gas chromatography-mass spectrometry. *J. Chromatogr.* **565:** 53–66.
27. Shimasue, A. 1974. The action of cholesterol: oxygen oxidoreductase on cholest-5-ene-3β,7α-diol and the enzymatic preparation of the labeled cholest-4-en-7α-ol-3-one. *Hiroshima J. Med. Sci.* **23:** 265–272.
28. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
29. Honda, A., G. Salen, S. Shefer, A. K. Batta, M. Honda, G. Xu, G. S. Tint, Y. Matsuzaki, J. Shoda, and N. Tanaka. 1999. Bile acid synthesis in the Smith-Lemli-Opitz syndrome: effects of dehydrocholesterols on cholesterol 7α-hydroxylase and 27-hydroxylase activities in rat liver. *J. Lipid Res.* **40:** 1520–1528.
30. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.
31. Ogishima, T., and K. Okuda. 1986. An improved method for assay of cholesterol 7α-hydroxylase activity. *Anal. Biochem.* **158:** 228–232.
32. Yoshida, T., A. Honda, N. Tanaka, Y. Matsuzaki, J. Shoda, B-F. He, T. Osuga, and H. Miyazaki. 1994. Determination of 7α-hydroxy-4-cholesten-3-one level in plasma using isotope-dilution mass spectrometry and monitoring its circadian rhythm in human as an index of bile acid biosynthesis. *J. Chromatogr.* **655:** 179–187.
33. Oftebro, H., I. Björkhem, S. Skrede, A. Schreiner, and J. I. Peder-

sen. 1980. Cerebrotendinous xanthomatosis. *J. Clin. Invest.* **65:** 1418–1430.
34. Salen, G., A. K. Batta, G. S. Tint, and S. Shefer. 1994. Comparative effects of lovastatin and chenodeoxycholic acid on plasma cholestanol levels and abnormal bile acid metabolism in cerebrotendinous xanthomatosis. *Metabolism.* **43:** 1018–1022.
35. Wang, H., J. Chen, K. Hollister, L. C. Sowers, and B. M. Forman. 1999. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol. Cell.* **3:** 543–553.
36. Makishima, M., A. Y. Okamoto, J. J. Repa, H. Tu, R. M. Learned, A. Luk, M. V. Hull, K. D. Lustig, D. J. Mangelsdorf, and B. Shan. 1999. Identification of a nuclear receptor for bile acids. *Science.* **284:** 1362–1365.
37. Parks, D. J., S. G. Blanchard, R. K. Bledsoe, G. Chandra, T. G. Consler, S. A. Kliewer, J. B. Stimmel, T. M. Willson, A. M. Zavacki, D. D. Moore, and J. M. Lehmann. 1999. Bile acids: natural ligands for an orphan nuclear receptor. *Science.* **284:** 1365–1368.
38. Nicolau, G., S. Shefer, G. Salen, and E. H. Mosbach. 1974. Determination of hepatic cholesterol 7α-hydroxylase activity in man. *J. Lipid Res.* **15:** 146–151.
39. Salen, G., S. Shefer, F. W. Cheng, B. Dayal, A. K. Batta, and G. S. Tint. 1979. Cholic acid biosynthesis. *J. Clin. Invest.* **63:** 38–44.
40. Björkhem, I., and J. Gustafsson. 1973. ω-Hydroxylation of steroid side-chain in biosynthesis of bile acids. *Eur. J. Biochem.* **36:** 201–212.
41. Buchmann, M. S., E. A. Kvittingen, H. Nazer, T. Gunasekaran, P. T. Clayton, J. Sjövall, and I. Björkhem. 1990. Lack of 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase in fibroblasts from a child with urinary excretion of 3β-hydroxy-Δ^5-bile acids. *J. Clin. Invest.* **86:** 2034–2037.