Bile acid synthesis in the Smith-Lemli-Opitz syndrome: effects of dehydrocholesterols on cholesterol 7α-hydroxylase and 27-hydroxylase activities in rat liver

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Abstract The Smith-Lemli-Opitz syndrome (SLOS) is a congenital birth defect syndrome caused by a deficiency of 3β-hydroxysterol Δ^7-reductase, the final enzyme in the cholesterol biosynthetic pathway. The patients have reduced plasma and tissue cholesterol concentrations with the accumulation of 7-dehydrocholesterol and 8-dehydrocholesterol. Bile acid synthesis is reduced and unnatural cholenoic and cholestenoic acids have been identified in some SLOS patients. To explore the mechanism of the abnormal bile acid production, the activities of key enzymes in classic and alternative bile acid biosynthetic pathways (microsomal cholesterol 7α-hydroxylase and mitochondrial sterol 27-hydroxylase) were measured in liver biopsy specimens from two mildly affected SLOS patients. The effects of 7- and 8-dehydrocholesterols on these two enzyme activities were studied by using liver from SLOS model rats that were treated with the Δ^7-reductase inhibitor (BM15.766) for 4 months and were comparable with more severe SLOS phenotype in plasma and hepatic sterol compositions. In the SLOS patients, cholesterol 7α-hydroxylase and sterol 27-hydroxylase were not defective. In BM15.766-treated rats, both enzyme activities were lower than those in control rats and they were competitively inhibited by 7- and 8-dehydrocholesterols. Rat microsomal cholesterol 7α-hydroxylase did not transform 7-dehydrocholesterol or 8-dehydrocholesterol into 7α-hydroxylated sterols. In contrast, rat mitochondrial sterol 27-hydroxylase catalyzed 27-hydroxylation of 7- and 8-dehydrocholesterols, which were partially converted to 3β-hydroxycholestadienoic acids. Addition of microsomes to the mitochondrial 27-hydroxylase assay mixture reduced 27-hydroxydehydrocholesterol concentrations, which suggested that 27-hydroxydehydrocholesterols were further metabolized by microsomal enzymes. These results suggest that reduced normal bile acid production is characteristic of severe SLOS phenotype and is caused not only by depletion of hepatic cholesterol but also by competitive inhibition of cholesterol 7α-hydroxylase and sterol 27-hydroxylase activities by accumulated 7- and 8-dehydrocholesterols. Unnatural bile acids are synthesized mainly by the alternative pathway via mitochondrial sterol 27-hydroxylase in SLOS.—Honda, A., G. Salen, S. Shefer, A. K. Batta, M. Honda, G. Xu, G. S. Tint, Y. Matsuzaki, J. Shoda, and N. Tanaka. **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.* **1999.** 40: 1520–1528.

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The Smith-Lemli-Opitz syndrome (SLOS) (1) is an autosomal recessive inherited birth defect. The syndrome is caused by deficient activity of 3β-hydroxysteroid Δ^7-reductase (2, 3), the final enzyme in the cholesterol biosynthetic pathway, and mutations in the Δ^7-reductase gene have been identified in a number of SLOS patients (4–6). Patients are characterized clinically by mental retardation, failure to thrive, gastrointestinal malfunction, and multiple congenital anomalies including microcephaly, facial dysmorphism, limb anomalies, genital malformations, endocrine hypofunction, cataracts, and heart and kidney dysfunction (1, 7–10). Biochemically, the unusually low activity of 3β-hydroxysteroid Δ^7-reductase causes plasma and tissue cholesterol levels to be abnormally reduced and leads to the accumulation of the cholesterol precursor, 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol), and its isomer, 8-dehydrocholesterol (cholesta-5,8-dien-3β-ol) (11–14).

According to current theory, cholesterol is the immediate precursor of bile acids exclusively synthesized in the liver that facilitate intestinal absorption of dietary lipids and fat-soluble vitamins. It is suggested that reduced cholesterol synthesis causes decreased formation of bile acids, which contributes to gastrointestinal malfunction and failure to thrive in SLOS. We previously reported (12) that fecal

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bile acids were virtually absent in a 6-month-old girl who was affected with a severe SLOS phenotype which, traditionally, has been designated SLOS type II (8). However, normal bile acids (chenodeoxycholic and cholic acids) were excreted in bile and feces several weeks after the subject was treated with a high cholesterol diet (15). Natowicz and Evans (16) analyzed urinary bile acids from SLOS patients by fast atom bombardment (FAB) mass spectrometry and reported a deficiency of normal bile acids (cholanoic acids) and the presence of abnormal species postulated to be mono-, di-, and tri-hydroxylated cholenoic and cholestenoic acid sulfates. We also reported extremely reduced normal bile acid excretion and the presence of a dihydroxycholenoic acid in SLOS patient's urine by gas chromatography–mass spectrometry (GC–MS) (17). However, the mechanisms of deficient normal bile acid production and the formation of unnatural bile acids have not been elucidated.

Normal bile acid formation occurs via two major pathways starting with either 7α-hydroxylation or 27-hydroxylation of cholesterol. 7α-Hydroxylation is catalyzed by microsomal cholesterol 7α-hydroxylase which is the rate-controlling enzyme in the classical pathway of bile acid biosynthesis (18, 19). On the other hand, the alternative pathway is initiated by mitochondrial sterol 27-hydroxylase (cholesterol 27-hydroxylase) (20, 21). In this report, we measured both enzyme activities in liver biopsy specimens from two less clinically and biochemically affected (type I) SLOS patients. In addition, the effects of 7- and 8-dehydrocholesterols on the enzyme activities were studied by using liver from SLOS model rats which were treated with the Δ7-reductase inhibitor (BM15.766) and were comparable with type II SLOS phenotype in plasma and hepatic sterol compositions. The results indicate that not only lack of cholesterol but also accumulation of 7- and 8-dehydrocholesterols play an important role for the abnormal bile acid biosynthesis in SLOS.

MATERIALS AND METHODS

Chemicals

Cholesterol and 7-dehydrocholesterol were purchased from Sigma Chemical Co. (St. Louis, MO) and Aldrich Chemical Company, Inc. (Milwaukee, WI), respectively, and each was purified three times by recrystallization. 8-Dehydrocholesterol was synthesized according to the method of Wilson et al. (22) and purified by HPLC. 27-Hydroxycholesterol was synthesized from diosgenin (23) and the pure compound was obtained by preparative TLC. 27-Hydroxy-7-dehydrocholesterol (cholesta-5,7-dien-3β, 27-diol) was synthesized via 7α-bromination of 27-hydroxycholesterol diacetate with 1,3-dibromo-5,5-dimethylhydantoin followed by dehydrobromination with triethylphosphite/o-xylene and alkaline saponification (23). The pure compound was obtained by HPLC. The purities of these compounds were checked by gas–liquid chromatography (GLC), and each gave only a single peak. [²H7] 7α-hydroxycholesterol, [²H₇]27-hydroxycholesterol, and [²H₇]3β-hydroxy-5-cholestenoic acid were prepared as described previously (24, 25). [³H]7-dehydrocholesterol was synthesized by the method of Batta et al. (26). BM 15.766, 4-(2-[1-(4-chlorocinnamyl)piperazin-4-yl]ethyl)-benzoic acid, was a gift from Boehringer Mannheim GmbH (Mannheim, Germany).

Patients

The Institutional Review Board approved the use of fibroblasts from SLOS patients. Two SLOS patients were studied. Patient A was a 4-month-old boy with the typical type I phenotype. His plasma cholesterol level was abnormally low and 7- and 8-dehydrocholesterol concentrations were elevated nearly 1000 times compared to mean healthy control levels (27, 28) (**Table 1**). Patient B was a 1-month-old girl with the type I phenotype but who had an unexpectedly high concentration of plasma cholesterol. However, her plasma 7- and 8-dehydrocholesterol levels were more than 500 times increased over control levels (Table 1). In both patients, deficient 3β-hydroxysteroid Δ7-reductase activity was confirmed (Table 1) in cultured skin fibroblasts using the conversion of ergosterol to brassicasterol assay (29). Liver biopsies were obtained by the caring pediatrician from each patient for diagnosis and were stored at −70°C until used. Control liver specimens were from 11 healthy children who died unexpectedly and whose livers became available because no suitable recipient for liver transplantation could be found (National Institutes of Health contract No. 1-DK62274).

Animals

Male Sprague-Dawley rats (50–70 g) were purchased from Charles River (Wilmington, MA) and kept under regular light–dark cycles (7 am–7 pm). The rats were fed rat chow powder plus BM15.766 (30 mg/kg/day) by gavage for 4 months, with the BM15.766 suspended in water (10 mg/ml) by sonication. At the completion of the experiment, the animals were killed and livers were obtained for assay of hepatic enzyme activities. The animal protocol was approved by Subcommittee on Animal Studies at VA Medical Center (East Orange, NJ) and Institutional Animal Care and Use Committee at University of Medicine and Dentistry of New Jersey–New Jersey Medical School (Newark, NJ).

TABLE 1. Plasma concentrations of cholesterol (CHOL), 7-dehydrocholesterol (7DHC), and 8-dehydrocholesterol (8DHC) and conversion of ergosterol to brassicasterol in cultured fibroblasts from patients with the Smith-Lemli-Opitz syndrome

	Plasma			Conversion from Ergosterol to Brassicasterol
Subject	CHOL	7DHC	8DHC	
	mg/dl			*%*
Patient A	95	16	7.8	1.5
Patient B	190	5.0	10	2.6
Control				
Mean ± SEM (n)	143 ± 2(147)[a]	<0.01 (35)[b]	<0.01 (35)[b]	24 ± 2 (10)[c]

[a] Data from 2-month-old infants by Kallio et al. (27).
[b] Data from healthy adult subjects by Axelson et al. (28).
[c] Data from control subjects by Honda et al. (29).

Plasma and hepatic sterol analysis

Cholesterol, 7-dehydrocholesterol, and 8-dehydrocholesterol were extracted with n-hexane from plasma or liver (microsomes or mitochondria) after saponification in 1 N ethanolic NaOH for 1 h at 70°C. Trimethylsilyl (TMS) ether derivatives were prepared by addition of Sil Prep (Alltech Associates, Deerfield, IL) and quantitated by capillary-column GLC as described previously (13).

Assay of cholesterol 7α-hydroxylase activity

Hepatic microsomes and mitochondria were prepared by differential ultracentrifugation, and the protein was determined by the method of Lowry et al. (30). The activity of cholesterol 7α-hydroxylase was measured by the stable-isotope dilution mass spectrometry according to Honda et al. (24) with some modifications. The reaction mixture (final volume 0.5 ml) consisted of 100 mm potassium phosphate buffer (pH 7.4) containing 0.1 mm EDTA, 5 mm DTT, 0.1 mm BM15.766, and 0.1–0.5 mg of microsomal protein, and various amounts of cholesterol, 7-dehydrocholesterol or 8-dehydrocholesterol dissolved in 30 μl of 2.5% (w/v) Triton X-100 solution were added to the mixture. The incubation was started by addition of NADPH (final 1.2 mm), and continued for 15 min at 37°C. The reaction was stopped with 0.5 ml of 1 N ethanolic KOH, 5 μg of BHT (butylated hydroxytoluene), and 8 ng of [²H₇]7α-hydroxycholesterol as an internal recovery standard. After saponification at 37°C for 1 h, sterols were extracted three times with 1 ml of n-hexane, and the extracts were evaporated to dryness under nitrogen. The residue was dissolved in 0.3 ml of n-hexane–2-propanol 97:3 (v/v), and applied to a Bond Elut SI cartridge (500 mg). After washing with 1 ml of n-hexane followed by 4 ml of n-hexane–2-propanol 97:3 (v/v), 7α-hydroxycholesterol was eluted with 3 ml of n-hexane–2-propanol 80:20 (v/v) and converted into dimethylethylsilyl (DMES) ether derivative. GC–MS with selected-ion monitoring (SIM) was performed using a JMS-SX102 instrument equipped with a data-processing system JMA DA-6000 (JEOL, Tokyo, Japan). An Ultra Performance capillary column (25 m × 0.32 mm ID) coated with methylsilicone (Hewlett-Packard, Palo Alto, CA) was used with a flow-rate of helium carrier gas of 1.0 ml/min. The column oven was programmed to change from 100 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* 470.3944 for 7α-hydroxycholesterol and *m/z* 477.4383 for [²H₇]7α-hydroxycholesterol. The assays were carried out in duplicate.

Assay of cholesterol 27-hydroxylase activity

The activity of cholesterol 27-hydroxylase was measured by GC–MS method which is similar to cholesterol 7α-hydroxylase assay described above. The composition of the standard reaction mixture (final volume 0.5 ml) was 100 mm potassium phosphate buffer (pH 7.4) containing 0.1 mm EDTA, 5 mm DTT, 0.1 mm BM15.766, 0.1–0.5 mg of mitochondrial protein, and 12 μl of a 33% aqueous solution of 2-hydroxypropyl-β-cyclodextrin (Pharmatec Inc., Alachua, FL). Various amounts of cholesterol, 7-dehydrocholesterol, or 8-dehydrocholesterol were added in the β-cyclodextrin solution. The reaction was initiated by adding a mixture of NADPH (final 1.2 mm), isocitrate (final 5 mm) and 0.2 IU of isocitrate dehydrogenase, and continued for 30 min at 37°C. The reaction was stopped by adding 0.5 ml of 1 N ethanolic KOH, 5 μg of BHT, and 16 ng of [²H₇]27-hydroxycholesterol as an internal recovery standard. After saponification at 37°C for 1 h, sterols were extracted three times with 1 ml of n-hexane, purified by Bond Elut SI cartridge, and derivatized to DMES ether by the same procedure as 7α-hydroxylase assay described above. The column oven 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* 545.4210 for 27-hydroxycho-

lesterol, *m/z* 552.4649 for [²H₇]27-hydroxycholesterol, and *m/z* 572.4445 for 27-hydroxydehydrocholesterols. The assays were carried out in duplicate.

Determination of 27-hydroxycholesterol, 27-hydroxydehydrocholesterols, 3β-hydroxy-5-cholestenoic acid, and 3β-hydroxycholestadienoic acids in incubation mixture

To study further metabolism of 27-hydroxycholesterol and 27-hydroxydehydrocholesterols, 60 nmol of cholesterol or dehydrocholesterol in 12 μl of a 33% aqueous solution of β-cyclodextrin was incubated in 0.5 ml of 100 mm potassium phosphate buffer (pH 7.4) containing 0.1 mm EDTA, 5 mm DTT, 0.1 mm BM15.766, and 0.1 mg of mitochondrial protein with intact or boiled 0.3 mg of microsomal protein. The reaction was initiated by adding a mixture of NADPH (final 1.2 mm), isocitrate (final 5 mm) and 0.2 IU of isocitrate dehydrogenase, and continued for 2 or 5 h at 37°C. The reaction was stopped by adding 0.5 ml of 1 N ethanolic KOH, 5 μg of BHT, and [²H₇]27-hydroxycholesterol (64 ng) and [²H₇]3β-hydroxy-5-cholestenoic acid (13 ng) as an internal recovery standard. After saponification, sterols were extracted with n-hexane, purified by Bond Elut SI cartridge, derivatized to DMES ether, and measured by GC-SIM by the same procedure as above. The resulting aqueous phase of n-hexane extraction was diluted with 5 ml of 0.5 m sodium phosphate buffer (pH 7.0) and applied to a Bond Elut C18 cartridge (500 mg) (31). After washing with 6 ml of water, 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-cholestadienoic acids were eluted with 5 ml of ethanol. Ethylation and dimethylethylsilylation were performed as described previously (31). The column oven 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* 501.3763 for 3β-hydroxy-5-cholestenoic acid, *m/z* 508.4203 for [²H₇]3β-hydroxy-5-cholestenoic acid, and *m/z* 528.3998 for 3β-hydroxycholestadienoic acids.

Conversion of [³H]7-dehydrocholesterol into 7-hydroxylated sterols

[³H]7-dehydrocholesterol (150 nmol, 100,000 dpm) dissolved in 30 μl of 2.5% (w/v) Triton X-100 solution was incubated in 0.5 ml of 100 mm potassium phosphate buffer (pH 7.4) containing 0.1 mm EDTA, 5 mm DTT, 0.1 mm BM15.766, and 0.5 mg of microsomal protein. In some experiments, 1.0 nmol (100,000 dpm) of [¹⁴C]cholesterol was also added to the incubation mixture. The incubation was started by adding NADPH (final 1.2 mm), and continued for 20 min at 37°C. The reaction was stopped and sterols were extracted by adding 2.5 ml of dichloromethane–ethanol 5:1 (v/v). The extracts were applied to a TLC plate, developed in diethyl ether, and the pertinent spots were visualized by spraying with 3.5% phosphomolybdic acid in isopropanol (32). The observed R_f values were: cholesterol, 0.88; 7-dehydrocholesterol, 0.85; 7β-hydroxycholesterol, 0.54; 7α-hydroxycholesterol, 0.40. The radioactivity of the pertinent spots was determined by liquid scintillation spectroscopy.

RESULTS

In **Table 2**, hepatic concentrations of cholesterol and dehydrocholesterols in patients and controls are listed. In the typical type I patient, A, hepatic microsomal and mitochondrial cholesterol concentrations were 54% and 42% of the control means, respectively, which was below the 95% confidence limits. In contrast, an atypical type I patient, B, showed high microsomal and normal mito-

TABLE 2. Concentrations of cholesterol (CHOL), 7-dehydrocholesterol (7DHC), and 8-dehydrocholesterol (8DHC) in hepatic microsomes and mitochondria from patients with the Smith-Lemli-Opitz syndrome

	Liver (Microsomes)			Liver (Mitochondria)		
Subject	CHOL	7DHC	8DHC	CHOL	7DHC	8DHC
	$\mu g/mg$ protein			$\mu g/mg$ protein		
Patient A	9.7	4.0	6.6	13	8.1	8.7
Patient B	23	0.5	0.8	26	0.7	2.2
Control						
Mean ± SEM (n = 10)	18 ± 1[a]	<0.05[b]	<0.05[b]	31 ± 4[c]	<0.05[b]	<0.05[b]

[a] 95% confidence interval for control mean is 14.6 to 20.6 $\mu g/mg$ protein.

[b] Trace amounts of dehydrocholesterols.

[c] 95% confidence interval for control mean is 22.8 to 39.9 $\mu g/mg$ protein.

chondrial cholesterol concentrations compared with control means. In both patients, microsomal and mitochondrial 7- and 8-dehydrocholesterol concentrations were abnormally elevated.

Hepatic microsomal cholesterol 7α-hydroxylase and mitochondrial cholesterol 27-hydroxylase activities measured by using endogenous cholesterol as a substrate are shown in **Table 3**. Cholesterol 7α-hydroxylase activity in patient A was 245% of the control mean, which was above the 95% confidence limit, while the enzyme activity was not up-regulated in the atypical patient B. Cholesterol 27-hydroxylase activities were within the 95% confidence interval in both patients.

Cholesterol 7α-hydroxylase and 27-hydroxylase activities in BM15.766-treated rats used in the following experiments were compared with those in untreated rats (**Fig. 1**). In BM15.766-treated rats, cholesterol 7α-hydroxylase activity measured without addition of exogenous cholesterol was low compared with that in controls, while the activity markedly increased when assays were carried out at saturation level of cholesterol. In contrast, both 27-hydroxylase activities measured with and without addition of exogenous cholesterol were low in BM15.766-treated rats compared with controls.

The effects of 7- and 8-dehydrocholesterols on cholesterol 7α-hydroxylase and cholesterol 27-hydroxylase activities are

TABLE 3. Activities of microsomal cholesterol 7α-hydroxylase and mitochondrial cholesterol 27-hydroxylase in patients with the Smith-Lemli-Opitz syndrome

Subject	Cholesterol 7α-Hydroxylase	Cholesterol 27-Hydroxylase
	pmol/min/mg protein	
Patient A	2.7	10
Patient B	0.8	16
Control		
Mean ± SEM (n = 11)	1.1 ± 0.2[a]	12 ± 2[b]

[a] 95% confidence interval for control mean is 0.7 to 1.4 pmol/min/mg protein.

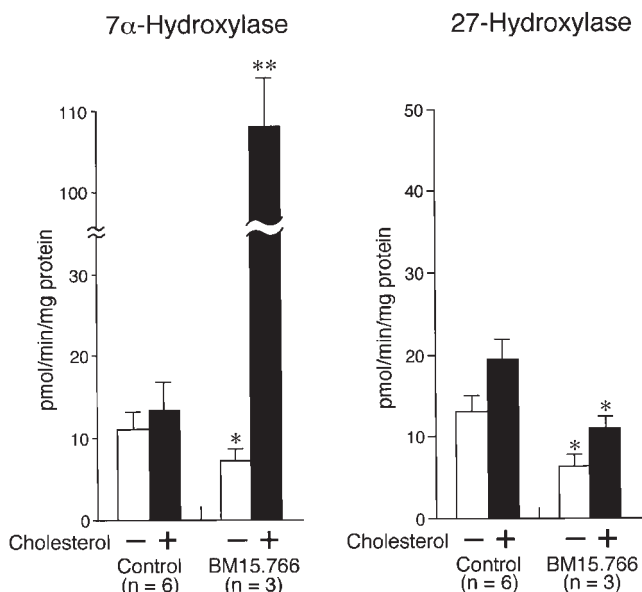[b] 95% confidence interval for control mean is 7.5 to 17 pmol/min/mg protein.

Fig. 1. Effects of BM15.766 treatment on hepatic microsomal cholesterol 7α-hydroxylase and mitochondrial cholesterol 27-hydroxylase activities in rats. The assays were carried out with (+) and without (−) exogenous cholesterol (200 μm). * $P < 0.05$, significantly different from control; ** $P < 0.001$, significantly different; from control.

demonstrated in **Fig. 2**. In this experiment, incubations were carried out with increasing amounts of 7-dehydrocholesterol or 8-dehydrocholesterol added to microsomes or mitochondria from BM15.766-treated rat liver. Microsomal concentrations of cholesterol, 7-dehydrocholesterol, and 8-dehydrocholesterol in the BM15.766-treated rat were 2.4, 14.3, and 2.8 μg/mg protein, respectively, and mitochondrial concentrations of these sterols were 1.5, 4.1, and 1.1 μg/mg protein, respectively. Cholesterol concentrations derived from microsomes and mitochondria in the incubation mixture were 6.1 and 4.0 μm, respectively. When either dehydrocholesterol was added to the incubation mixture, both cholesterol 7α-hydroxylase and cholesterol 27-hydroxylase activities were suppressed in a dose-dependent manner but the suppression due to 8-dehydrocholesterol tended to be greater than that caused by 7-dehydrocholesterol at the same concentration. For the determination of the type of inhibition, enzyme activities were measured with 120 μm of 7-dehydrocholesterol or 8-dehydrocholesterol and increasing concentrations of cholesterol substrate (**Fig. 3**). The Lineweaver-Burk double reciprocal plots showed straight lines that intersected at the same point on the ordinates, indicating competitive inhibition of cholesterol 7α-hydroxylase and cholesterol 27-hydroxylase by 7- and 8-dehydrocholesterols.

To test the possibility that 7-dehydrocholesterol directly converts to 7α-hydroxycholesterol without changing to cholesterol, [³H]7-dehydrocholesterol and [¹⁴C]cholesterol were incubated with BM15.766-treated rat liver microsomes. The final concentrations of 7-dehydrocholesterol and cholesterol in the incubation mixture were 300 and 8.1 μm, respectively. The mixture also contained 0.1 mm BM15.766 which completely inhibited Δ^7-reductase activ-
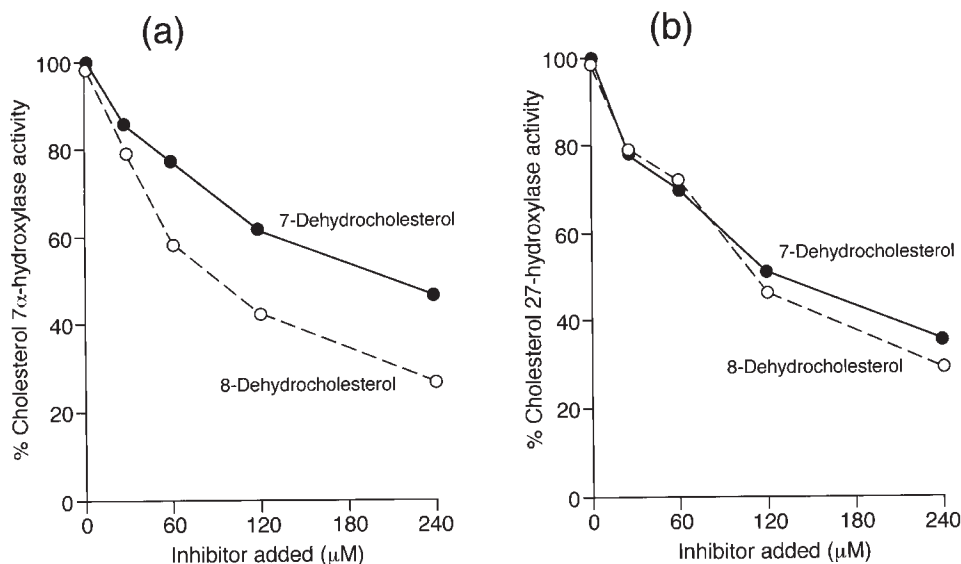
Fig. 2. Effects of varying concentrations of 7-dehydrocholesterol and 8-dehydrocholesterol on microsomal cholesterol 7α-hydroxylase activity (a) and mitochondrial cholesterol 27-hydroxylase activity (b) in BM15.766-treated rat liver. 100% activities of cholesterol 7α-hydroxylase and cholesterol 27-hydroxylase are 7.3 and 6.3 pmol/min per mg protein, respectively. Each point represents the average of duplicate experiments.

ity (33). After incubation, no [^{3}H]7α-hydroxycholesterol, [^{3}H]7β-hydroxycholesterol, or [^{14}C]7β-hydroxycholesterol was detected, while 0.44 ± 0.02% (n = 4) of [^{14}C]cholesterol was converted to [^{14}C]7α-hydroxycholesterol.

The formation of 7α-hydroxylated 8-dehydrocholesterol was also tested by using BM15.766-treated rat liver microsomes. The microsomes were incubated in 240 μm of 8-dehydrocholesterol and 7α-hydroxylated 8-dehydrocholesterol was sought by GC/MS. A reference compound for 7α-hydroxy-8-dehydrocholesterol was not available, but the change of retention time on 8-desaturation (**Table 4**) and mass spectrum for this sterol was expected. There was no significant peak after 7α-hydroxycholesterol in

total ion chromatograms, and SIM showed no peaks at m/z 572 (M$^+$), m/z 468 (M – (CH$_3$)$_2$C$_2$H$_5$SiOH), or m/z 453 (M – (CH$_3$)$_2$C$_2$H$_5$SiOH – CH$_3$).

The transformations of 7- and 8-dehydrocholesterols to 27-hydroxylated dehydrocholesterols and 3β-hydroxycholestadienoic acids were quantitated in BM15.766-treated rat liver mitochondrial fraction. Relative retention times and major fragments in mass spectra of the studied compounds are summarized in Table 4. Difference of relative retention time (ΔRRT) showed the expected characteristic changes on introduction of double bond at C-7 or C-8. The position of double bond at C-7 or C-8 did not cause significant difference of major fragments in mass spectra. Incubation of
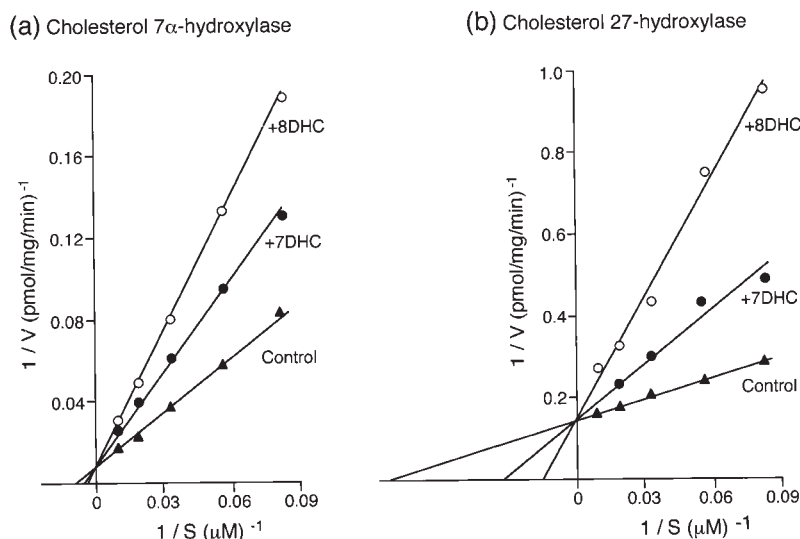

Fig. 3. Lineweaver-Burk plots of microsomal cholesterol 7α-hydroxylase activity (a) and mitochondrial cholesterol 27-hydroxylase activity (b) in BM15.766-treated rat liver. S, cholesterol substrate concentration; V, enzyme activity. Each point represents the average of duplicate experiments.

TABLE 4. GC/MS characteristics of DMES derivatives of C_{27} steroids and C_{27} acid ethyl esters

			Ions (m/z) and Their Relative Intensities (%)[c]			
Compound	RRT[a]	ΔRRT[b]	M[+]	[M−29][+]	[M−104][+]	[M−119][+]
3β-Hydroxysterols						
Δ^5 (cholesterol)	1.00	—	472 (12)	443 (93)	368 (52)	—
$\Delta^{5,7}$ (7-dehydrocholesterol)	1.04	0.04[d]	470 (23)	—	366 (34)	351 (100)
$\Delta^{5,8}$ (8-dehydrocholesterol)	1.01	0.01[e]	470 (21)	—	366 (35)	351 (100)
3β,27-Dihydroxysterols						
Δ^5 (27-hydroxycholesterol)	1.72	0.72[f]	574 (12)	545 (89)	470 (38)	—
$\Delta^{5,7}$ (27-hydroxy-7-dehydrocholesterol)	1.80	0.08[d]	572 (19)	—	468 (38)	453 (100)
$\Delta^{5,8}$ (27-hydroxy-8-dehydrocholesterol)[g]	1.74	0.02[e]	572 (20)	—	468 (37)	453 (100)
3β-Hydroxy-C_{27}-acids						
Δ^5 (3β-5-cholestenoic acid)	1.58	0.58[h]	530 (15)	501 (100)	426 (68)	—
$\Delta^{5,7}$ (3β-5,7-cholestadienoic acid)[g]	1.65	0.07[d]	528 (19)	—	424 (44)	409 (100)
$\Delta^{5,8}$ (3β-5,8-cholestadienoic acid)[g]	1.59	0.01[e]	528 (24)	—	424 (29)	409 (100)

[a] Retention times are expressed relative to that of cholesterol. Column oven was programmed to change from 100 to 270°C at 30°C/min after a 1-min delay from the start time. Retention time of cholesterol was 11.00 min.
[b] Change of RRT on desaturation, hydroxylation or oxidation.
[c] M−29 = M−C_2H_5; M−104 = M−$(CH_3)_2C_2H_5SiOH$; M−119 = M−$(CH_3)_2C_2H_5SiOH$−CH_3.
[d] Insertion of a double bond at C-7.
[e] Insertion of a double bond at C-8.
[f] Introduction of the 27-hydroxyl group.
[g] Data from incubation mixture for 27-hydroxylase assay.
[h] Oxidation of C-27 methyl to the carboxyl group.

cholesterol, 7-dehydrocholesterol, or 8-dehydrocholesterol with mitochondria yielded corresponding 27-hydroxylated sterols in a time-dependent manner (**Table 5**). In addition, small but significant amounts of corresponding 3β-hydroxy-C_{27}-acids were detected in the mixture. Addition of microsomes to the above incubation mixture containing mitochondria caused less accumulation of corresponding 27-hydroxylated sterols in the mixture.

DISCUSSION

There is no doubt that the hepatic cholesterol pool is important for bile acid biosynthesis because cholesterol is the immediate precursor of bile acids. Markedly decreased hepatic concentration of cholesterol as seen in severe SLOS phenotype appears to be responsible for abnormally reduced bile acid production. The idea is supported by our previous observation that virtually no bile acids were detected in the feces of a SLOS patient, but the primary bile acids, cholic and chenodeoxycholic acids, were found in bile and feces of the patient after high cholesterol was introduced in the diet (12, 15). In mildly affected patients A and B, however, cholesterol was still the most abundant sterol in the liver and cholesterol 7α-hydroxylase and 27-hydroxylase activities were not decreased (Table 3).

In rats, the effects of 4 months treatment with BM15.766 on plasma and hepatic sterol concentrations were previously reported by Xu et al. (34). In this model, plasma and

TABLE 5. Formation of 3β,27-dihydroxysterols and 3β-hydroxy-C_{27}-acids after incubation of cholesterol (CHOL), 7-dehydrocholesterol (7DHC), or 8-dehydrocholesterol (8DHC) with BM15.766-treated rat liver mitochondria

Substrate	Incubation Time	Microsome	3β-27-Dihydroxysterols			3β-Hydroxy-C_{27}-Acids		
			Δ^5	$\Delta^{5,7}$	$\Delta^{5,8}$	Δ^5	$\Delta^{5,7}$	$\Delta^{5,8}$
	h		*pmol*			*pmol*		
CHOL	2	−	671	<1	<1	4	<1	<1
	2	+	422	<1	<1	4	<1	<1
	5	−	1399	<1	<1	15	<1	<1
	5	+	437	<1	<1	24	<1	<1
7DHC	2	−	45	457	<1	<1	13	<1
	2	+	48	296	<1	<1	11	<1
	5	−	85	723	<1	<1	9	<1
	5	+	42	261	<1	2	65	<1
8DHC	2	−	39	<1	585	<1	<1	51
	2	+	35	<1	463	<1	<1	50
	5	−	70	<1	1176	<1	<1	67
	5	+	37	<1	418	2	<1	301

hepatic 7-dehydrocholesterol concentrations were higher than cholesterol levels, which was comparable to severely affected (type II) SLOS phenotype. Hepatic microsomal cholesterol 7α-hydroxylase and mitochondrial 27-hydroxylase activities measured by using endogenous cholesterol as a substrate were low compared with those in controls. However, cholesterol 7α-hydroxylase activities were markedly up-regulated when assays were carried out with addition of exogenous cholesterol, which suggests that protein mass of cholesterol 7α-hydroxylase was markedly increased in BM15.766-treated rat liver. It is known that decreased liver cholesterol substrate supply is associated with inhibited cholesterol 7α-hydroxylase activity while reduced hepatic bile acid flux up-regulates this enzyme (35–37). Our results suggest that the markedly increased protein mass

of cholesterol 7α-hydroxylase somewhat compensates for reduced bile acid biosynthesis caused by the decreased hepatic cholesterol pool in the BM15.766-treated rats.

The experiments using rats suggest that not only depleted hepatic cholesterol but also accumulated 7- and 8-dehydrocholesterols appear to inhibit normal bile acid production in severely affected type II SLOS. Plasma concentrations of cholesterol and dehydrocholesterols in one of the most profoundly affected type II SLOS patients were 7.0 and 87 mg/dl, respectively (2). If hepatic sterol concentrations change in proportion to plasma sterol levels, hepatic dehydrocholesterol concentration in this patient is more than 12 times higher than cholesterol level, which is equivalent to 50–80 μm of dehydrocholesterol in incubation mixture (Fig. 2). The double reciprocal plots
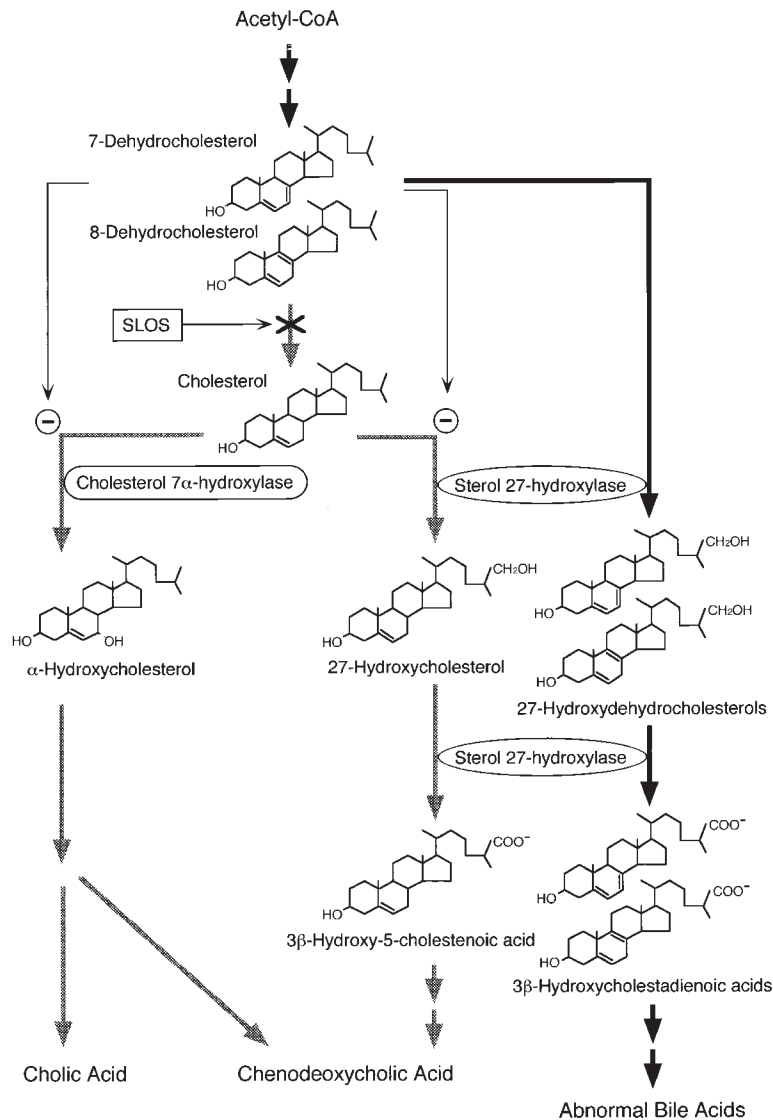

Fig. 4. A simplified scheme of the mechanism of abnormal bile acid biosynthesis in the Smith-Lemli-Opitz syndrome. Both 7-dehydrocholesterol and 8-dehydrocholesterol competitively inhibit microsomal cholesterol 7α-hydroxylase and mitochondrial cholesterol 27-hydroxylase activities. Cholesterol 7α-hydroxylase does not catalyze 7α-hydroxylation of 7-dehydrocholesterol or 8-dehydrocholesterol. In contrast, cholesterol (sterol) 27-hydroxylase catalyzes 27-hydroxylation of 7-dehydrocholesterol and 8-dehydrocholesterol, and these 27-hydroxylated dehydrocholesterols appear to convert to unusual bile acids.

clearly demonstrated that both dehydrocholesterols were competitive inhibitors of cholesterol 7α-hydroxylase and cholesterol 27-hydroxylase (Fig. 3). The apparent K_m values of cholesterol 7α-hydroxylase measured in the presence of 120 μm 7-dehydrocholesterol and 120 μm 8-dehydrocholesterol were 190 μm and 307 μm, respectively, compared with the K_m of 115 μm observed in the absence of any added inhibitor. Calculated K_i values of 7-dehydrocholesterol and 8-dehydrocholesterol were 183 μm and 72 μm, respectively. On the other hand, K_m values of cholesterol 27-hydroxylase determined in the presence of 120 μm 7-dehydrocholesterol and 120 μm 8-dehydrocholesterol and absence of inhibitor were 32 μm, 67 μm, and 12 μm, respectively. K_i values of 7-dehydrocholesterol and 8-dehydrocholesterol were 73 μm and 26 μm, respectively. These results indicate that 8-dehydrocholesterol is a more potent inhibitor of cholesterol 7α-hydroxylase and cholesterol 27-hydroxylase activities than 7-dehydrocholesterol.

Preliminary analyses of urinary bile acids showed the existence of unusual bile acids in SLOS patients (16, 17). Although structural confirmation will have to await the synthesis of appropriate reference standards, mass spectral data were strongly suggestive of cholenoic and cholestenoic acids derivatives. The presence of monounsaturated bile acids (cholenoic acids) suggests the existence of pathways for the formation of Δ^7 and Δ^8 bile acids from 7-dehydrocholesterol and 8-dehydrocholesterol, respectively. To test the possibility, we studied early steps of classic and alternative bile acid biosynthetic pathways. As shown in **Fig. 4**, microsomal cholesterol 7α-hydroxylase did not transform these dehydrocholesterols to hydroxylated derivatives. In contrast, mitochondrial sterol 27-hydroxylase catalyzed 27-hydroxylation of both 7- and 8-dehydrocholesterols, and formed respective 27-hydroxydehydrocholesterols that were partially converted to 3β-hydroxycholestadienoic acids. This second reaction from 27-hydroxydehydrocholesterols to 3β-hydroxycholestadienoic acids is considered to be catalyzed by the same mitochondrial sterol 27-hydroxylase (38). These results show that abnormal monounsaturated bile acids are synthesized mainly by an alternative pathway via mitochondrial sterol 27-hydroxylase. Further metabolism of 27-hydroxydehydrocholesterols and 3β-hydroxycholestadienoic acids remains unclear. However, some of the 27-hydroxydehydrocholesterols appear to be further metabolized by microsomal enzymes, e.g., oxysterol 7α-hydroxylase, because addition of microsomes to the incubation mixture containing dehydrocholesterols and mitochondrial fraction resulted in less accumulation of corresponding 27-hydroxylated dehydrocholesterols (Table 5).

Bile acids (cholanoic acids) are necessary for the development of bile flow and for absorption of lipids (39), and certain unsaturated bile acids (cholenoic acids) might be hepatotoxic (40, 41). Decreased primary bile acids and increased unsaturated bile acids might contribute to gastrointestinal malfunction, hepatic abnormalities, and failure to thrive as seen in SLOS patients. The present study lends support to the idea that a high cholesterol diet is an effective treatment to increase primary bile acid synthesis and decrease abnormal bile acid production in SLOS.

The reasons are *1*) key enzymes in normal bile acid biosynthetic pathways are active; *2*) both dehydrocholesterols are competitive inhibitors so that increased hepatic cholesterol concentrations could attenuate the inhibitory effects of dehydrocholesterols; and *3*) cholesterol could reduce elevated hepatic dehydrocholesterol concentrations by inhibiting the abnormal endogenous cholesterol biosynthetic pathway (42).

In summary, this study demonstrated that 7-dehydrocholesterol and 8-dehydrocholesterol adversely affect bile acid biosynthesis in SLOS. Reduced normal bile acid synthesis in this disease appears to be caused by both depletion of hepatic cholesterol pool and competitive inhibition of key enzymes in classic and alternative bile acid biosynthetic pathways by the dehydrocholesterols. Abnormal bile acids seen in SLOS are mainly synthesized by an alternative pathway via mitochondrial sterol 27-hydroxylase.

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