7-Dehydrocholesterol down-regulates cholesterol biosynthesis in cultured Smith-Lemli-Opitz syndrome skin fibroblasts

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Abstract The Smith-Lemli-Opitz syndrome (SLOS) is a common birth defect–mental retardation syndrome caused by a defect in the enzyme that reduces 7-dehydrocholesterol to cholesterol. Because of this block, patients' plasma cholesterol levels are generally low while 7-dehydrocholesterol concentrations are markedly elevated. In addition, plasma total sterols are abnormally low and correlate negatively with the percent of 7-dehydrocholesterol ($r = -0.65$, $P < 0.0001$) suggesting that 7-dehydrocholesterol might inhibit the activity of HMG-CoA reductase. Cultured skin fibroblasts from SLOS patients grown in fetal bovine serum or for 1 day in delipidated medium contain little 7-dehydrocholesterol (3 ± 1% of total sterols) and HMG-CoA reductase activities are indistinguishable from that measured in control cells. However, raising the 7-dehydrocholesterol concentration to 20 ± 3% of total sterols, equal to the mean proportion in plasma of SLOS patients, by either growing cells for 1 week in delipidated medium or adding 20 μg/ml 7-dehydrocholesterol directly to the cells reduced HMG-CoA reductase activities from 74 ± 7 to 9 ± 2 pmol/min per mg protein, or from 92 ± 22 to 16 ± 4 pmol/min per mg protein, respectively ($P < 0.01$). In contrast, adding 20 μg/ml cholesterol evoked a 2- to 4-fold lesser suppression of activity (39 ± 8 pmol/min per mg protein, $P < 0.05$, vs. 7-dehydrocholesterol). HMG-CoA synthase and LDL binding were inhibited equally by 7-dehydrocholesterol and cholesterol. Ketaconazole prevented the down-regulation of HMG-CoA reductase by 7-dehydrocholesterol, suggesting that an hydroxylated derivative of 7-dehydrocholesterol may be especially important in suppressing cholesterol synthesis. These results demonstrate that 7-dehydrocholesterol, perhaps as an hydroxylated derivative(s), is a very effective feedback inhibitor of HMG-CoA reductase.—**Honda, M., G. S. Tint, A. Honda, L. B. Nguyen, T. S. Chen, and S. Shefer.** 7-Dehydrocholesterol down-regulates cholesterol biosynthesis in cultured Smith-Lemli-Opitz syndrome skin fibroblasts. *J. Lipid Res.* 1998. **39:** 647–657.

Supplementary key words HMG-CoA reductase • HMG-CoA synthase • ketoconazole • LDL receptor • plasma sterols

The Smith-Lemli-Opitz syndrome (SLOS) (1) is a common birth disorder caused by a defect in 7-dehydrocholesterol Δ^7-reductase (2, 3), the last enzyme in the cholesterol biosynthetic pathway. Patients are characterized clinically by severe mental retardation with incomplete myelination of both brain and peripheral nerves, failure to thrive, a characteristic facial dysmorphism, limb abnormalities, endocrine hypofunction, ambiguous genitalia in severely affected males, cataracts, and cardiac, hepatic, lung, digestive system and renal malformations (1, 4–7). The syndrome is inherited in an autosomal recessive manner and the prevalence is estimated to be about 1 in 20,000 births with a carrier frequency between 1 and 2% in North American Caucasian populations.

The unusually low activity of 7-dehydrocholesterol 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) (8, 9). Because cholesterol is essential for the functioning of every mammalian cell and is required for normal embryonic growth (10), it is not surprising that developmental abnormalities could be caused by markedly inhibiting its synthesis or by substituting another sterol.

The cholesterol biosynthetic pathway involves many

Abbreviations: cholesterol, cholest-5-en-3β-ol; 7-dehydrocholesterol, cholesta-5,7-dien-3β-ol; 8-dehydrocholesterol, cholesta-5,8-dien-3β-ol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein; PBS, phosphate-buffered saline; SLOS, Smith-Lemli-Opitz syndrome.

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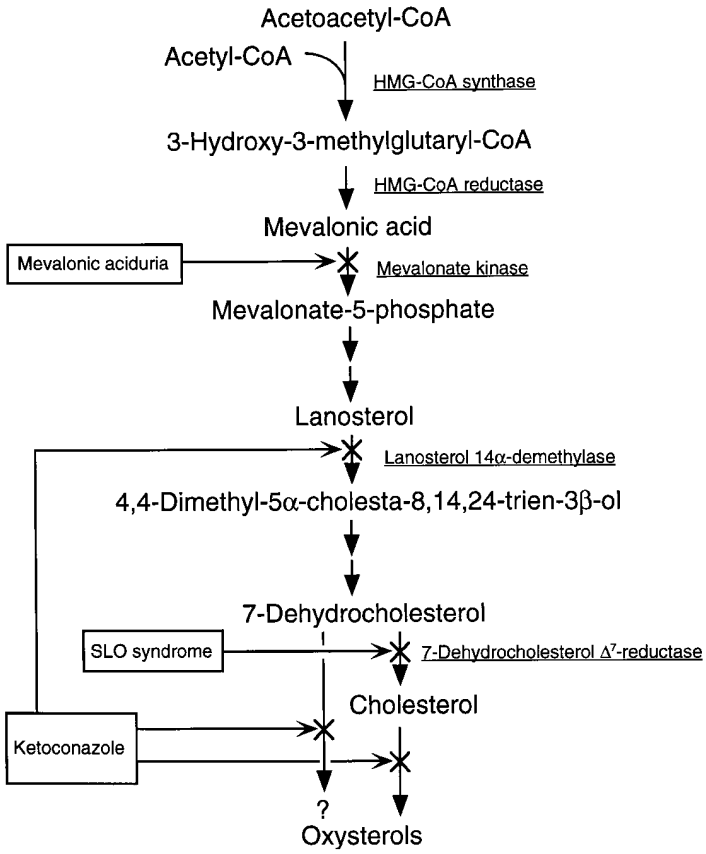

Fig. 1. Cholesterol biosynthetic pathway. The rate-controlling enzyme is HMG-CoA reductase; the defective enzyme in the SLOS is 7-dehydrocholesterol Δ^7-reductase (3β-hydroxysterol Δ^7-reductase).

steps (**Fig. 1**), but the rate-controlling enzyme is 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. One is tempted to predict that, in SLOS patients, HMG-CoA reductase activities should be stimulated because plasma and tissue concentrations of cholesterol are reduced. However, it seems unlikely that up-regulation takes place because the concentration of plasma total sterols, including the unusual sterols, in 90–95% of SLOS patients is abnormally low compared to cholesterol levels in age-matched control subjects (11, 12). Consistent with this latter observation, we recently assayed HMG-CoA reductase activity in cultured skin fibroblasts from several patients with the syndrome (13) and found that they were not increased. These findings suggested to us that cholesterol might not be the only sterol that could down-regulate cholesterogenesis.

The present investigation was undertaken to determine the ability of 7-dehydrocholesterol to inhibit cholesterol biosynthesis. Although both 7-dehydrocholesterol and 8-dehydrocholesterol accumulate in the syndrome, 7-dehydrocholesterol is more abundant and its origin has been clarified. Furthermore, patients' skin fibroblasts make very little 8-dehydrocholesterol (14). We report here the results of a study in which we compare the effects of 7-dehydrocholesterol and cholesterol on HMG-CoA reductase, HMG-CoA synthase, and LDL receptor binding activities in cultured skin fibroblasts. The results provide ample evidence that accumulated 7-dehydrocholesterol could readily inhibit sterol biosynthesis in the Smith-Lemli-Opitz syndrome.

MATERIALS AND METHODS

Chemicals

BM 15.766 and ketoconazole were gifts from Boehringer Mannheim GmbH (Mannheim, Germany) and The Janssen Research Foundation (Beerse, Belgium), respectively. Cholesterol and 7-dehydrocholesterol were purchased from Sigma Chemical Co. (St. Louis, MO) and Aldrich Chemical Company, Inc. (Milwaukee, WI), respectively, and purified three times by recrystallization. Purities were checked by high-performance liquid

chromatography (HPLC), and each of them gave only a single peak. Coprostanol (5β-cholestan-3β-ol) was obtained from Steraloids (Wilton, NH), tetrabutylammonium hydroxide (40% aqueous solution) and phosphoric acid were from Aldrich, and acetyl-CoA and acetoacetyl-CoA were from Sigma. [3-^{14}C]HMG-CoA, [1-^{14}C] acetyl-CoA, and Na^{125}I were purchased from Amersham Co. (Arlington Heights, IL), and [1,2-^{3}H]7-dehydrocholesterol was synthesized as described previously (15, 16).

Plasma sterol analysis

Plasma sterols from 83 patients with the Smith-Lemli-Opitz syndrome (39 females and 44 males, ages 1 day to 34 years) were determined as described previously (11, 12). After addition of coprostanol as an internal recovery standard, plasma was hydrolyzed in 1 N ethanolic NaOH, extracted with n-hexane, converted to trimethylsilyl (TMS) ether derivatives, and analyzed by capillary gas chromatography.

Fibroblast culture

Skin biopsies were obtained from twelve patients. Seven of the patients were among the less clinically and biochemically affected phenotypes (type I), while the other five were among the most profoundly affected phenotypes which, traditionally, have been designated SLOS type II (2, 5). Controls were patients from other metabolic studies without abnormalities in cholesterol metabolism. Fibroblasts were grown and maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (14). All cells were used before the 20th passage. To study the effects of cholesterol or 7-dehydrocholesterol on HMG-CoA reductase, HMG-CoA synthase, and LDL receptor binding activities, the following procedures were used. On day 1, 12.5 cm^2 tissue culture flasks were seeded with 1×10^5 cells/flask. On day 7 when the cells were nearly confluent (approximately 5×10^5 cells/flask), the original growth medium was removed, the attached cells were washed twice with phosphate-buffered saline (PBS), and the medium was replaced with 2 ml of fresh DMEM supplemented with 20% FBS or 2.5 mg/ml of delipidated serum prepared from FBS (17). In some experiments, cholesterol, 7-dehydrocholesterol, and/or ketoconazole were then added in 18 μl of ethanol to the medium, and cells were incubated for 24 h at 37°C in a humidified incubator containing 5% CO_2, 95% air.

Cell sterol analysis

Cell sterols were quantitated as previously described (14). Cells were harvested by trypsinization and washed twice with PBS. The cells plus coprostanol added as an internal recovery standard were hydrolyzed in 1 N ethanolic NaOH, and free neutral sterols were extracted with n-hexane. After conversion into trimethylsilyl ether derivatives, sterols were determined by gas chromatography–mass spectrometry (GC-MS) with selected-ion monitoring (SIM).

Assay of HMG-CoA reductase activity

HMG-CoA reductase activities in fibroblasts were measured according to the method described by Goldstein, Basu, and Brown (18) with minor modifications. Medium from each flask was discarded and the flask was rinsed twice with 2 ml of PBS. Cells were then harvested by use of a cell scraper and washed twice with PBS. Cell-free extracts were prepared by adding 65 μl of phosphate buffer containing 0.25% (v/v) Brij 96 to the cell pellets, and aliquots were used for determination of protein concentration (19). [^{14}C]HMG-CoA was incubated in a final volume of 100 μl of 100 mm potassium phosphate buffer (pH 7.4) containing a NADPH generating system. The reaction was started with the addition of 50 μl of the cell extract and stopped after 30 min at 37°C with the addition of 10 μl of 6 N HCl. After adding unlabeled mevalonolactone (1 mg) as a marker for visualization on thin-layer plates, [^{3}H]mevalonolactone (40,000 dpm) as an internal recovery standard, 200 μl of ethanol, 0.2 g of anhydrous Na_2SO_4, and 100 μl of water, mevalonolactone was extracted twice with 1 ml of diethyl ether, separated by thin-layer chromatography, and measured by dual-label liquid scintillation counting.

Assay of HMG-CoA synthase activity

The harvested cell pellet from a 25 cm^2 flask was homogenized in 0.2 ml of 10 mm Tris-HCl buffer (pH 7.3) containing 0.2 m sucrose, 0.1 mm dithiothreitol, and 0.1 mm EDTA. The cytosolic fractions were prepared by ultracentrifugation (100,000 *g* supernatant) and dialyzed for 24 h at 4°C against a 1000-fold excess of 20 mm potassium phosphate buffer (pH 7.7) containing 0.1 mm EDTA and 0.5 mm dithiothreitol to inhibit HMG-CoA lyase activity (20). The dialyzed cytosol was then concentrated to about 40 μl by Centricon-30 (Amicon, Inc., Beverly, MA), and an aliquot was used for measurement of protein concentration (19). The assay of cytosolic HMG-CoA synthase activity was based on the methods of Scharnagl et al. (21) with minor modifications. The composition of the standard reaction mixture (final volume 100 μl) was 100 mm Tris-HCl buffer (pH 8.0) containing 0.1 mm EDTA, 60 μm acetoacetyl-CoA, 400 μM [^{14}C]acetyl-CoA (28,000 dpm/nmol), and 20–50 μg of dialyzed cytosolic protein. The mixture was preincubated for 2 min at 37°C and the reac-

tion was initiated by the addition of the substrate mixture. After 15 min, the reaction was stopped by adding 200 μl of 200 mm TBAP buffer (pH 5.5) dissolved in methanol–water 3:2 containing 15 nmol of HMG-CoA. The incubated mixture was then centrifuged for 1 min at 10,000 rpm in a Beckman microfuge and was kept on ice until analyzed. An aliquot (100 μl) of the supernatant was injected onto a μ-Bondapak C_{18} column (3.9 × 300 mm, Waters Corp., Milford, MA) equilibrated with 50 mm TBAP buffer (pH 5.5) dissolved in methanol–water 43:57, and the column was eluted with the same solvent at a rate of 1.3 ml/min. The HMG-CoA-containing fraction was collected, evaporated under N_2 at 90°C, and the radioactivity was measured.

Assay of LDL receptor binding activity

Receptor-mediated binding of ^{125}I-labeled LDL in cultured fibroblasts was assayed by previously published methods (18, 22). Cells were washed twice with 2 ml of Tris-buffered saline containing 0.2% (w/v) bovine serum albumin and incubated for 2 h at 37°C with ^{125}I-labeled LDL (10 μg/ml; 290 cpm/ng protein) in the presence and absence of 400 μg/ml of unlabeled LDL, in 1 ml of RPMI Medium 1640 (Life Technologies, Inc.) supplemented with 10% delipidated serum and 2% bovine serum albumin. At the end of the incubation, cells were washed with Tris-buffered saline with and without 0.2% bovine serum albumin, dissolved by incubation in 1 ml of 0.1 N NaOH, and radioactivity in 0.5 ml of the cell suspension was counted. LDL receptor binding activity was calculated as the difference between total binding (without unlabeled LDL) and nonspecific binding (with 40-fold excess unlabeled LDL). This method measures the combination of surface-bound and internalized LDL.

Statistics

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

RESULTS

We measured the concentrations of cholesterol (55 ± 5 mg/dl), 7-dehydrocholesterol (16 ± 1 mg/dl) and 8-dehydrocholesterol (14 ± 1 mg/dl) in plasma from 83 SLOS patients with a mean age of 6.6 ± 0.9 years. Their total plasma sterol concentration, 85 ± 4 mg/dl, was well below the 5th centile for plasma cholesterol levels in age-matched controls which increases from about 70 to 90 to 100 mg/dl at 0, 4, and 19 weeks, respectively, then rises to a value of 150–160 mg/dl for a typical 6-year-old (23–25). On the average, 7-dehydrocholesterol represented 18 ± 2% of total sterols in affected individuals. When we plotted total plasma sterols against the % of 7-dehydrocholesterol in plasma, we discovered a highly significant inverse correlation (**Fig. 2**, r = −0.65, $P < 0.0001$). These results suggested that 7-dehydrocholesterol might be down-regulating cholesterol biosynthesis at the level of HMG-CoA reductase.

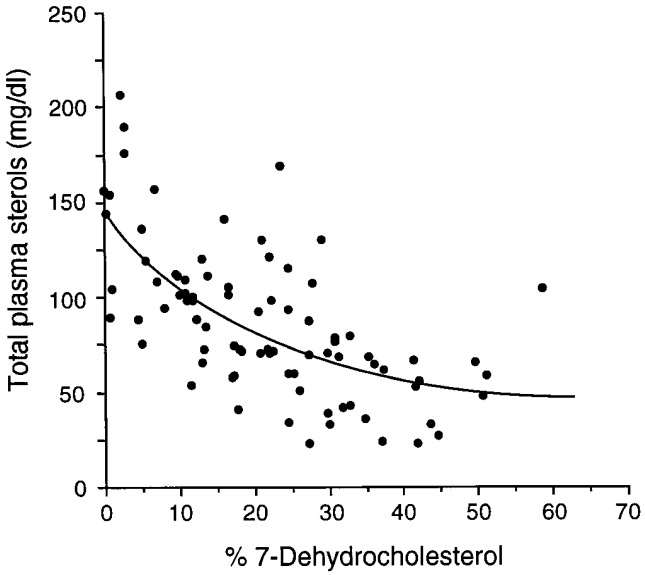

Fig. 2. Relationship between % 7-dehydrocholesterol and total sterol levels in plasma from SLOS patients. Total sterol level was calculated as the sum of cholesterol, 7-dehydrocholesterol, and 8-dehydrocholesterol, while % 7-dehydrocholesterol was calculated as 7-dehydrocholesterol concentration divided by total sterol level ×100%. The regression curve satisfies the equation Y = 101exp (−0.0515X) + 46.4 (r = −0.65, $P < 0.0001$, n = 83).

When cells are grown with exogenous cholesterol then exposed to a delipidated medium for 24 h, 7-dehydrocholesterol is virtually undetectable in control cells and represents only 3.0 ± 0.6% of total cellular sterols in SLOS fibroblasts (**Table 1** and **Fig. 3**). However, after adding 20 μg/ml of 7-dehydrocholesterol to the SLOS fibroblasts, the proportion of this diunsaturated sterol increased to 19% becoming similar to its mean proportion in SLOS plasma. When 7-dehydrocholesterol was added to control cells, 7-dehydrocholesterol increased to only 6.9% of total sterols because these cells readily convert this precursor to cholesterol. Total cellular sterol concentrations in SLOS cells after the addition of 20 μg/ml of either cholesterol or 7-dehydrocholesterol were identical (103 ± 20 vs. 102 ± 20 μg/mg protein, respectively).

TABLE 1. Sterol concentrations in cultured fibroblasts from controls and from patients with the Smith-Lemli-Opitz syndrome before and after incubating them with cholesterol or 7-dehydrocholesterol

Treatment	7-Dehydrocholesterol	Cholesterol
	μg/mg protein (%)[a]	*μg/mg protein*
Controls (n = 4)		
DLS[b]	0.09 ± 0.04 (0.17 ± 0.06)	51 ± 5
DLS + cholesterol[c]	0.12 ± 0.04 (0.18 ± 0.04)	62 ± 9
DLS + 7-dehydrocholesterol[c]	5.7 ± 0.4 (6.9 ± 1.1)[d]	81 ± 9[e]
SLOS (n = 4)		
DLS[b]	2.6 ± 0.8 (3.0 ± 0.6)[e]	86 ± 27
DLS + cholesterol[c]	3.1 ± 1.0 (2.9 ± 0.6)	100 ± 19[g]
DLS + 7-dehydrocholesterol[c]	19 ± 4 (19 ± 1)[f]	83 ± 16[g]

[a] In parentheses, 7-dehydrocholesterol as % of total sterols (7-dehydrocholesterol + cholesterol).
[b] Cells were exposed for 24 h to DMEM containing 2.5 mg/ml of delipidated protein from fetal bovine serum.
[c] Cells were exposed for 24 h to DMEM containing 2.5 mg/ml of delipidated protein from fetal bovine serum plus 20 μg/ml of cholesterol or 7-dehydrocholesterol.
[d] $P < 0.005$, significantly different from controls treated with DLS alone.
[e] $P < 0.05$, significantly different from controls treated with DLS alone.
[f] $P < 0.01$, significantly different from type II patients treated with DLS alone and from controls treated with DLS + 7-dehydrocholesterol.
[g] Total sterol concentrations of cells incubated in DLS + cholesterol and DLS + 7-dehydrocholesterol are 103 ± 20 and 102 ± 20 μg/mg protein, respectively.

The 7-dehydrocholesterol concentration in SLOS cells can also be raised by incubating them in delipidated medium for one week which forces the cells to synthesize their own sterols. Figure 3 compares sterol concentrations in fibroblasts after exposure to delipidated medium for 1 day and 7 days in patients' and control fibroblasts. Prolonged exposure to delipidated medium also reproduced in patients' cells the same relative accumulation of 7-dehydrocholesterol (20 ± 3% of total sterols) noted in the plasma of these individuals. Cholesterol concentrations were not significantly different among the four groups.

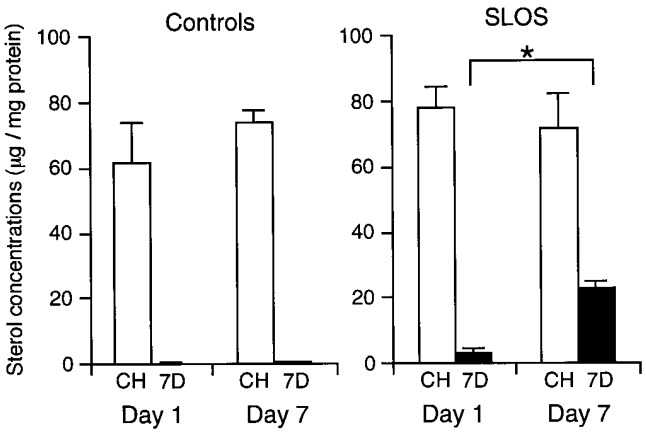

Fig. 3. Effects of delipidated medium on sterol concentrations of fibroblasts from controls and type II SLOS patients. The cells were grown for 7 days as described in Methods. On day 7, the original growth medium was replaced with fresh delipidated medium. After 1 day or 7 days of incubation, the cells were harvested for determination of cell sterols. CH, cholesterol; 7D, 7-dehydrocholesterol. Error bars represent SEM (data from four different subjects). *$P < 0.01$.

HMG-CoA reductase activities in cultured skin fibroblasts from controls and type I and type II (severely affected) patients are summarized in **Table 2**. When the cells were exposed to a cholesterol-rich medium (i.e., with 20% FBS) enzyme activities were suppressed and, after incubation in delipidated medium for 24 h, activities in all cells were up-regulated similarly. It should be noted that in these SLOS cells, 7-dehydrocholesterol concentrations were low. In contrast, the enzyme activities in patients' fibroblasts were markedly decreased after exposure to delipidated medium for 7 days when 7-

TABLE 2. HMG-CoA reductase activities in fibroblasts from controls and from Smith-Lemli-Opitz syndrome type I (less severe) and type II (most severe) patients after growth in delipidated medium

Subjects	HMG-CoA Reductase Activity		
	FBS[a] (n)	DLS 1 Day[b] (n)	DLS 7 Days[c] (n)
	pmol/min/mg protein		
Controls	12 ± 1 (14)	80 ± 12 (14)	42 ± 6 (5)
SLOS Type I	13 ± 3 (7)	77 ± 15 (7)	
SLOS Type II	17 ± 4 (5)	74 ± 7 (5)	9 ± 2[d] (7)

[a] Cells were exposed for 1 day to DMEM containing 20% fetal bovine serum.
[b] Cells were exposed for 1 day to DMEM containing 2.5 mg/ml of delipidated protein from fetal bovine serum.
[c] Cells were exposed for 7 days to DMEM containing 2.5 mg/ml of delipidated protein from fetal bovine serum.
[d] $P < 0.005$, significantly different from controls exposed to DLS for 7 days.

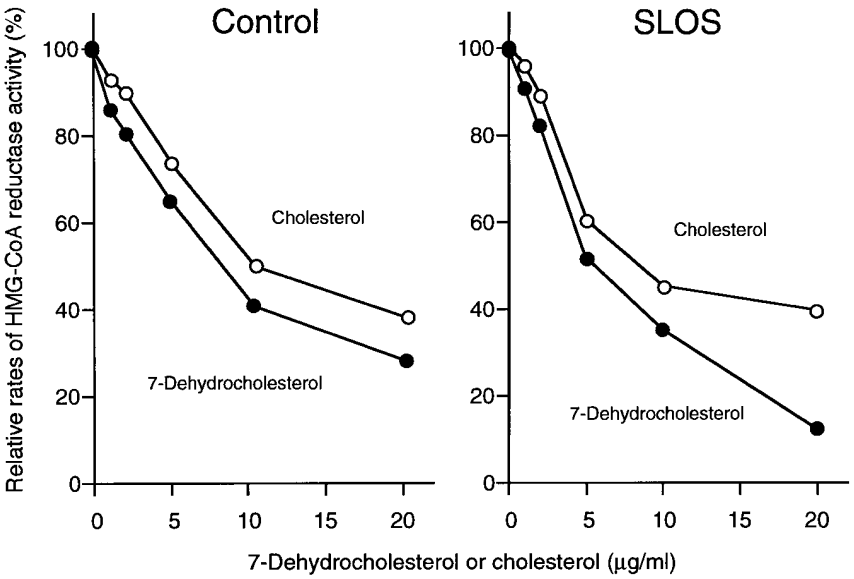

Fig. 4. Effects of varying concentrations of 7-dehydrocholesterol and cholesterol on HMG-CoA reductase activity in fibroblasts from a control and a type II SLOS patient. The cells were grown for 7 days as described in Methods. On day 7, the original growth medium was replaced with fresh delipidated medium containing the indicated concentrations of cholesterol or 7-dehydrocholesterol, incubated for 24 h, then harvested for determination of HMG-CoA reductase activity. Data points represent the mean of duplicate determinations. The absolute activities for control and type II patient without addition of sterols were 103 and 92 pmol/min per mg protein, respectively.

dehydrocholesterol levels increased to 20% of total sterols (Fig. 3). Only a moderate reduction of activity was observed in control fibroblasts grown under the same conditions. In these latter cells 7-dehydrocholesterol was barely detectable (Fig. 3).

Figure 4 illustrates the typical effects of adding increasing amounts of cholesterol and 7-dehydrocholesterol on HMG-CoA reductase activity in cultured fibroblasts from a control and a patient. When either sterol was added to cells grown in cholesterol-deficient medium, HMG-CoA reductase activity was suppressed in a dose-dependent manner but the suppression due to 7-dehydrocholesterol was consistently greater than that caused by cholesterol at the same concentration. In **Table 3**, the inhibition of HMG-CoA reductase activity by these two sterols was compared using fibroblasts from four controls and four patients. The enzyme activity was decreased to 34% of baseline activity in control and 42 ± 9% in patients' cells when 20 μg/ml of cholesterol was added to the delipidated medium. Using the same concentration of added 7-dehydrocholesterol, the enzyme activity was reduced even further, to 28 ± 3% of baseline activity in control cells and to 17 ± 4% in patients' cells. The suppression by 7-dehydrocholesterol in patients' cells was significantly greater ($P < 0.05$) than that achieved by equimolar cholesterol. Note especially (Table 1) that adding either cholesterol or 7-dehydrocholesterol to SLOS fibroblasts raised total cellular sterol concentration equally.

Adding 7-dehydrocholesterol to the medium significantly decreased LDL receptor binding activities to 67% of the baseline value in control cells and to 68% in patients' cells (Table 3). The same amount of 7-dehydrocholesterol also tended to inhibit HMG-CoA synthase activities to 77% of pretreatment levels ($P = 0.053$) in control cells and to 66% ($P = 0.066$) in patients' cells (Table 3). The suppression of LDL receptor binding and HMG-CoA synthase activities by 7-dehydrocholesterol was similar to that achieved with the same concentration of added cholesterol.

To confirm that the suppression of HMG-CoA reductase activity in patients' fibroblasts was caused by the added 7-dehydrocholesterol itself and that these cells could not make cholesterol from the added 7-dehydrocholesterol, cells were exposed to delipidated medium with 20 μg/ml of ^{3}H-labeled 7-dehydrocholesterol for 24 h. Control cells and patients' cells took up 6.5 ± 2.6% (n = 4) and 5.9 ± 1.4% (n = 4) of added ^{3}H-labeled 7-dehydrocholesterol, respectively. No conversion to cholesterol was observed in patients' cells, while 23 ± 8% of 7-dehydrocholesterol taken up by cells was converted to cholesterol in control fibroblasts.

A variety of oxysterols are known to be very potent inhibitors of HMG-CoA reductase activity (26, 27). To

TABLE 3. Effects of cholesterol and 7-dehydrocholesterol on HMG-CoA reductase activity, HMG-CoA synthase activity, and LDL receptor binding in fibroblasts from controls and from patients with Smith-Lemli-Opitz syndrome type II

Treatment	Relative HMG-CoA Reductase Activity	Relative HMG-CoA Synthase Activity	Relative LDL Receptor Binding
		%	
Controls (n = 4)			
DLS[a]	100[c]	100[g]	100[h]
DLS + cholesterol[b]	34 ± 7	87 ± 7	52 ± 7[d]
DLS + 7-dehydrocholesterol[b]	28 ± 3[d]	77 ± 10	67 ± 9[d]
SLOS (n = 4)			
DLS[a]	100[c]	100[g]	100[h]
DLS + cholesterol[b]	42 ± 9[e]	72 ± 22	81 ± 12
DLS + 7-dehydrocholesterol[b]	17 ± 4[e,f]	66 ± 15	68 ± 11[i]

[a]Cells were exposed for 24 h to DMEM containing 2.5 mg/ml of delipidated protein from fetal bovine serum.

[b]Cells were exposed for 24 h to DMEM containing 2.5 mg/ml of delipidated protein from fetal bovine serum plus 20 μg/ml of cholesterol or 7-dehydrocholesterol.

[c]Absolute activities for controls and type II patients were 103 ± 19 and 92 ± 22 pmol/min per mg protein, respectively.

[d]$P < 0.01$, significantly different from controls treated with DLS alone.

[e]$P < 0.01$, significantly different from type II patients treated with DLS alone.

[f]$P < 0.05$, significantly different from type II patients treated with DLS + cholesterol.

[g]Absolute activities for controls and type II patients were 227 ± 49 and 137 ± 39 pmol/min per mg protein, respectively.

[h]The absolute activities for controls and type II patients were 104 ± 14 and 74 ± 17 ng/mg protein, respectively.

[i]$P < 0.05$, significantly different from type II patients treated with DLS alone.

determine whether an hydroxylated derivative of 7-dehydrocholesterol might not be the compound responsible for the observed down-regulation of sterol biosynthesis, we repeated the studies after adding the cytochrome P-450 hydroxylase inhibitor ketoconazole. The results are illustrated in **Fig. 5**. Ketoconazole itself did not inhibit HMG-CoA reductase activity but prevented 7-dehydrocholesterol from affecting HMG-CoA reductase activity in both control and patients' cells.

DISCUSSION

HMG-CoA reductase is one of the most highly regulated enzymes in nature and is controlled through a multivalent feedback mechanism (28). Cholesterol is thought to repress transcription of HMG-CoA mRNA while non-sterol isoprenoids appear to suppress the enzyme post-transcriptionally (29, 30).

Our results provide strong evidence that 7-dehydrocholesterol (or one or more of its oxygenated derivatives) is an effective feedback inhibitor of HMG-CoA reductase and that its effect is greater than that of cholesterol. We could not properly test the inhibition of sterol synthesis by 7-dehydrocholesterol in normal cells because much of any added 7-dehydrocholesterol would be rapidly converted to cholesterol. Moreover, even if we were to have used a 7-dehydrocholesterol Δ^7-reductase inhibitor, we could never have excluded the possibility that the drug itself might have inhibited HMG-CoA reductase. To avoid these problems, we studied the most severely affected mutant SLOS cells, those that convert no measurable 7-dehydrocholesterol to cholesterol (2).

The concentration and proportion of 7-dehydrocholesterol in SLOS fibroblasts grown in conventional cholesterol-rich medium (10% FBS) are much less than that found in the plasma of these patients (14) because cholesterol in the medium markedly suppresses cholesterol biosynthesis. Because there appear to be no defects in the early steps of cholesterol biosynthesis in the SLOS, when 7-dehydrocholesterol levels are low, fibroblasts from these patients appear normal. Thus, incubating control and SLOS fibroblasts in delipidated medium for 1 day evokes an equal up-regulation of HMG-CoA reductase activity (Table 2). However, raising the 7-dehydrocholesterol concentration until it equals the average proportion in the plasma of SLOS patients, by growing cells for 1 week in delipidated medium or by adding 7-dehydrocholesterol directly to the cells, markedly reduced HMG-CoA reductase activities. When we added either cholesterol or 7-dehydrocholesterol to SLOS cells, total sterol concentrations were identical (Table 1). However, when 7-dehydrocholesterol was added, 7-dehydrocholesterol levels increased to a level equal to the average proportion in the plasma of SLOS patients and HMG-CoA reductase activity was suppressed 2.5-fold more than when equimolar choles-

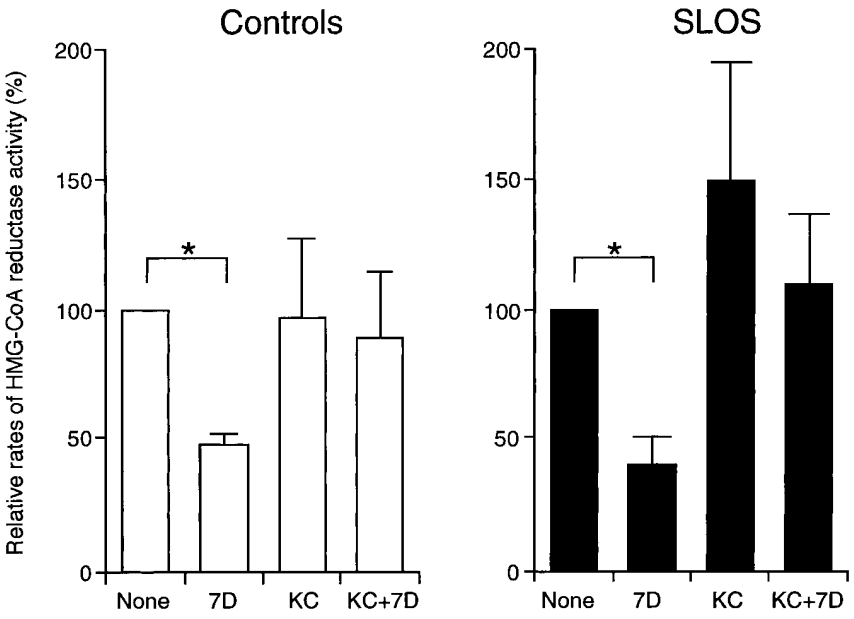

Fig. 5. Effects of ketoconazole on the inhibition of HMG-CoA reductase activity by 7-dehydrocholesterol in fibroblasts from controls and type II SLOS patients. The cells were grown for 7 days as described in Methods. On day 7, cells were fed fresh delipidated medium with or without ketoconazole (30 μm), and 7-dehydrocholesterol (10 μg/ml) was then added to the medium. After 24 h of incubation, the cells were harvested for determination of HMG-CoA reductase activity. None, without ketoconazole or 7-dehydrocholesterol; 7D, 7-dehydrocholesterol alone; KC, ketoconazole alone; KC+7D, ketoconazole plus 7-dehydrocholesterol. Error bars represent SEM (data from four different subjects). *$P < 0.005$.

terol was added (Table 3). We conclude, therefore, that it must have been the 7-dehydrocholesterol that provided the additional inhibition. The reason we observe no significant difference in the suppressive effects of cholesterol and 7-dehydrocholesterol when these two sterols are added to control fibroblasts (Table 3) is that normal cells can readily convert 7-dehydrocholesterol to cholesterol.

The regulation of HMG-CoA reductase in the SLOS is different from that observed in mevalonic aciduria, an inherited disorder (31, 32) caused by abnormally reduced activity of mevalonate kinase, the enzyme that follows HMG-CoA reductase in the cholesterol biosynthetic pathway. In mevalonic aciduria, mevalonic acid accumulates but plasma cholesterol concentrations are frequently normal or only moderately decreased (31, 33, 34). HMG-CoA reductase activities in fibroblasts from these patients when grown in cholesterol-containing medium are nearly 6-fold higher than activities noted in control cells (34). Although levels of mevalonic acid are greatly elevated in these cells, there does not appear to be an increase in the concentration of cholesterol or of any steroidal precursors of cholesterol.

Furthermore, in a Chinese hamster ovary mutant cell line (35) with a defect in the enzyme that demethylates lanosterol, HMG-CoA reductase activity was found to be down-regulated by the accumulated lanosterols (36). Similar observations of potent inhibition of HMG-CoA reductase have also been made for a series of non-metabolizable derivatives of lanosterol (37). The above reports combined with our own data suggest that it is very likely that many sterols, including cholesterol precursors, are able to reduce the function of HMG-CoA reductase.

Many hydroxylated sterol derivatives (oxysterols) are also efficient suppressors of HMG-CoA reductase activity (26, 27), and it has been suggested that one of the oxygenated derivatives of cholesterol, rather than cholesterol itself, is the actual inhibitor of cellular cholesterol biosynthesis (38–41). If this hypothesis is correct, then the suppression of HMG-CoA reductase by 7-dehydrocholesterol might be mediated by the formation of oxygenated 7-dehydrocholesterol. To test the possibility, we studied the effects of ketoconazole on the inhibition. Ketoconazole is an inhibitor of lanosterol 14α-demethylase and other cytochrome P450-linked reactions (42, 43) that are also involved in the formation of oxysterols from endogenously or exogenously supplied cholesterol. It is probably for this reason that ketoconazole abolishes inhibition of HMG-CoA reductase activity by LDL cholesterol (39). In the present study, ketoconazole also prevented the inhibi-

tion of HMG-CoA reductase activity by 7-dehydrocholesterol (Fig. 5), suggesting that down-regulation of HMG-CoA reductase by 7-dehydrocholesterol may require that 7-dehydrocholesterol be hydroxylated. Vitamin D_3 and its hydroxylated derivatives also inhibit HMG-CoA reductase activity (44) and ultraviolet light easily converts 7-dehydrocholesterol into vitamin D_3, but we did not detect vitamin D_3 in cells treated with 7-dehydrocholesterol or in the media from our experiments.

The effects of 7-dehydrocholesterol on HMG-CoA synthase activity and LDL receptor function are intriguing. The promoter regions of the HMG-CoA reductase, HMG-CoA synthase, and LDL receptor genes contain the sterol regulatory element 1 sequence (30) so that synthesis of these two enzymes and the receptor are coordinately regulated via transcription by sterol regulatory element-binding protein 1 (SREBP1) (45, 46). Our data demonstrated that adding 7-dehydrocholesterol down-regulated LDL receptor binding, HMG-CoA synthase, and HMG-CoA reductase activities but that the degree of inhibition of HMG-CoA reductase by 7-dehydrocholesterol was much greater than that of the other two. Thus, it may be that the particular effect of 7-dehydrocholesterol on HMG-CoA reductase activity may not be completely explicable by a transcriptional mechanism alone.

7-Dehydrocholesterol that accumulates in plasma and tissue may well exacerbate the cholesterol deficiency in patients with the SLOS by suppressing sterol synthesis at its source. On the other hand, elevated levels of 7-dehydrocholesterol may prevent a much greater accumulation of this abnormal and potentially toxic intermediate. Whether either effect might be better or worse for the patient is unknown. In normal subjects, the effect of 7-dehydrocholesterol on cholesterol biosynthesis is negligible because plasma (47) and tissue (8) concentrations are extremely low. In patients, however, when the proportion of 7-dehydrocholesterol in plasma is highest, the total sterol concentration is lowest (Fig. 2). This observation again suggests that 7-dehydrocholesterol is an effective suppressor of sterol synthesis in vivo at the level of HMG-CoA reductase. Recently, Steiner et al. (48) reported in a preliminary study that sterol synthesis in SLOS patients as measured by sterol balance was not stimulated as might be expected. This result also lends support to the idea that in SLOS patients up-regulation of HMG-CoA reductase activity may be difficult to attain.

In summary, our studies demonstrate that 7-dehydrocholesterol, perhaps as an hydroxylated derivative(s), is an effective feedback inhibitor of HMG-CoA reductase and may serve to unnaturally limit sterol synthesis in the SLOS.

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