

# Medication that Reduces Cholesterol by Preventing the body's Production of Cholesterol

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## Abstract

The Smith-Lemli-Opitz syndrome (SLOS) is a multiple malformation/mental retardation syndrome caused by a deficiency of the enzyme 7-dehydrocholesterol  $\Delta^7$ -reductase. This enzyme converts 7-dehydrocholesterol (7-DHC) to cholesterol in the last step in cholesterol biosynthesis. The pathology of this condition may result from two different factors: the deficiency of cholesterol itself and/or the accumulation of precursor sterols such as 7-DHC. Although cholesterol synthesis is defective in cultured SLOS cells, to date there has been no evidence of decreased whole body cholesterol synthesis in SLOS and only incomplete information on the synthesis of 7-DHC and bile acids. In this first report of the sterol balance in SLOS, we measured the synthesis of cholesterol, other sterols, and bile acids in eight SLOS subjects and six normal children. The diets were very low in cholesterol content and precisely controlled. Cholesterol synthesis in SLOS subjects was significantly reduced when compared with control subjects (8.6 vs. 19.6 mg/kg per day, respectively,  $P < 0.002$ ).

**Keywords:** Cholesterol-lowering medication; Enzymes; HMG-CoA reductase; High cholesterol levels; Cardiovascular diseases

## Introduction

Cholesterol precursors 7-DHC, 8-DHC, and 19-nor-cholestatrienol were synthesized in SLOS subjects (7-DHC synthesis was  $1.66 \pm 1.15$  mg/kg per day), but not in control subjects. Total sterol synthesis was also reduced in SLOS subjects (12 vs. 20 mg/kg per day,  $P < 0.022$ ). Bile acid synthesis in SLOS subjects (3.5 mg/kg per day) did not differ significantly from control subjects (4.6 mg/kg per day) and was within the range reported previously in normals. Normal primary and secondary bile acids were identified. This study provides direct evidence that whole body cholesterol synthesis is reduced in patients with SLOS and that the synthesis of 7-DHC and other cholesterol precursors is profoundly increased [1].

It is also the first reported measure of daily bile acid synthesis in SLOS and provides evidence that bile acid supplementation is not likely to be necessary for treatment. Sterol balance in the Smith-Lemli-Opitz syndrome: reduction in whole body cholesterol synthesis and normal bile acid production. The cholesterol metabolites, oxysterols, play central roles in cholesterol feedback control. They modulate the activity of two master transcription factors that control cholesterol homeostatic responses, sterol regulatory element-binding protein-2 (SREBP-2) and liver X receptor (LXR) [2].

## Exogenous oxysterols

Although the role of exogenous oxysterols in regulating these transcription factors has been well established, whether endogenously synthesized oxysterols similarly control both SREBP-2 and LXR remains poorly explored. Here, we carefully validate the role of oxysterols enzymatically synthesized within cells in cholesterol homeostatic responses. We first show that SREBP-2 responds more sensitively to exogenous oxysterols than LXR in Chinese hamster ovary cells and rat primary hepatocytes. We then show that 25-hydroxycholesterol (25-HC), 27-hydroxycholesterol, and 24S-hydroxycholesterol endogenously synthesized by CH25H, CYP27A1, and CYP46A1, respectively, suppress SREBP-2 activity at different degrees by stabilizing Insig (insulin-induced gene) proteins, whereas 7 $\alpha$ -hydroxycholesterol has little impact on SREBP-2. These results demonstrate the role of site-specific hydroxylation of endogenous oxysterols. In contrast, the expression of

CH25H, CYP46A1, CYP27A1, or CYP7A1 fails to induce LXR target gene expression. We also show the 25-HC production-dependent suppression of SREBP-2 using a tetracycline-inducible CH25H expression system. To induce 25-HC production physiologically, murine macrophages are stimulated with a Toll-like receptor 4 ligand, and its effect on SREBP-2 and LXR is examined [3].

## Material and Method

### Control of dietary cholesterol intake SLOS

Control of dietary cholesterol intake SLOS subjects and control subjects 3–6 were admitted to the OHSU General Clinical Research Center (GCRC) for 1-week periods. Instructions were given for an essentially cholesterol-free diet to be fed at home for three or more weeks prior to admission to the GCRC. This was easily accomplished in most cases because many of the infants were receiving exclusively infant formula containing cholesterol concentrations of only 10.5–35.7 mg/1,000 ml. During each admission a very low cholesterol (essentially cholesterol-free) diet was fed. The study diet was fed for at least 3 weeks total to allow for stabilization and steady state conditions. The subjects were studied in the GCRC under metabolic ward conditions. GCRC dietitians and cooks prepared the specialized diets and nurses collected patient samples at baseline and during the study periods. Some SLOS subjects were tube fed because of sucking and swallowing difficulties, but the same principles were applied to the diets of those individuals. The mothers of the children were given diet instruction and were asked to keep intake records. For inpatient studies, food and formula were weighed prior to being served to the subjects and refused food and

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formula were returned to the metabolic kitchen to be reweighed to determine actual intake [4].

## Cell culture

Cell culture CHO-7 and SRD-15 cells were isolated in the laboratories of Drs Joseph Goldstein and Michael Brown (UT Southwestern Medical Center) and Dr Russell DeBose-Boyd (UT Southwestern Medical Center), respectively. CHO-K1 and 25RA cells (51) were kind gifts of Dr Ta-Yuan Chang (Geisel School of Medicine at Dartmouth). All the CHO cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 1:1 mixture supplemented with 7.5% fetal bovine serum (FBS) and penicillin/streptomycin. A2058 (obtained from JCRB Cell Bank) and HEK293T cells were cultured in DMEM with 10% FBS and penicillin/streptomycin. J774.1 cells (RIKEN Cell Bank) were maintained in RPMI1640 medium supplemented with 10% FBS and penicillin/streptomycin. PRHs were isolated from nonfasted male Sprague–Dawley rats at 8 to 10 weeks old (Japan Clea) as described previously (52) with minor modifications. Rats were housed in a 12 h light/12 h dark schedule at  $23 \pm 2^\circ\text{C}$  and fed ad libitum with a standard chow diet (Labo MR Stock; Nosan Corporation) and water [5].

## Plasmid constructs

The coding sequence of human CH25H was amplified from human genomic DNA (because human CH25H does not contain any introns) and cloned into p3×FLAG-CMV-10 expression vector (Sigma) at the BamHI/EcoRI site to generate pFLAG-CH25H. pFLAG-CH25H was used as a template to create the active site mutant CH25H (CH25HH242Q/H243Q) expression plasmid. The 3×FLAG-CH25H sequence was amplified using pFLAG-CH25H as a template and cloned into pTetOne vector (Clontech) at the BamHI/MluI site to generate pFLAG-CH25Htet-on. The coding sequence of human CYP27A1, CYP7A1, and CYP46A1 was amplified by PCR and cloned into p3×FLAG-CMV-14 expression vector (Sigma) at EcoRV/XbaI, HindIII/BamHI, and EcoRI/BamHI sites, respectively. These CYP27A1-FLAG, CYP7A1-FLAG, and CYP46A1-FLAG expression constructs were referred to as pCYP27A1-FLAG, pCYP7A1-FLAG, and pCYP46A1-FLAG, respectively [6].

Complementary DNA (cDNA) for human StAR was cloned into p3×FLAG-CMV-14 expression vector as described previously. Primers used for cloning are listed in Table S2. Transfection and isolation of stable transfectants Cells were seeded into 6-well plates. About 18 to 24 h later, cells were transfected with plasmids using Lipofectamine LTX Reagent according to the manufacturer's protocol. Polyethylenimine Max (Polysciences) was used for plasmid transfection in HEK293T cells. Twenty-four to 48 h after transfection, cells were used for further experiments. For the isolation of CHO-K1 cells harboring pFLAG-CH25Htet-on (CHO-CH25Htet-on cells), CHO-K1 cells seeded into 6-well plate were cotransfected with pFLAG-CH25Htet-on (2 µg/well) and pMAM-BSD (0.1 µg/well). Twenty-four hours after transfection, cells were grown in medium containing blasticidin (7 µg/ml) for 5 days [7].

## Result and Discussion

### Radioimmunoprecipitation Assay

Blasticidin-resistant clones were isolated by limited dilution, and clones that were positive for Dox-dependent FLAG-CH25H expression were selected by immunoblotting with anti-FLAG antibody. RNA isolation and quantitative PCR Total RNA was isolated using ISOGEN II

(Nippon Gene). cDNA was then reverse synthesized using total RNA (2 µg/reaction) isolated and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). mRNA levels of a gene were analyzed by quantitative real-time PCR using FastStart Universal SYBER Green Maser (ROX) (Roche) and a specific primer set. Quantitative PCR was performed using a Quant Studio 6 Flex Real-Time PCR System or a StepOnePlus instrument (Applied Biosystems). mRNA levels were normalized to 18S ribosomal RNA levels. radioimmunoprecipitation assay buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, and 0.5% Protease inhibitor cocktail) as described (53). After determination of protein concentration using bicinchoninic acid assay, equal amounts of proteins were subjected to SDS-PAGE and immunoblot analysis. Primary antibodies used are as follows; anti-Insig1 polyclonal antibody (PAB8786) from Abnova, anti-FLAG monoclonal antibody [8].

### Immunostaining and immunofluorescence microscopy

Cells were seeded onto a 18 mm × 18 mm glass coverslip (Matsunami) placed in a 6-well plate and grown overnight. Cells were then transfected with plasmid as specified in figure legends. Afterward, cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. After blocking with 5% FBS in PBS for 1 h, cells were incubated with primary antibodies for 1 h. After washing with 1% FBS in PBS three times, specimens were incubated with Alexa Fluor 488-conjugated antimouse immunoglobulin G and Alexa Fluor 568-conjugated anti-rabbit immunoglobulin G (Invitrogen; A11036) (1:800 dilution) in 2% FBS in PBS for 45 min. Nuclei were stained with 4',6-diamidino-2-phenylindole. Specimens were washed with PBS for three times and mounted with ProLong Diamond Antifade Mountant. Immunofluorescence confocal images were acquired using a Zeiss LSM800 with Airyscan equipped with a Plan-Apochromat 63×/1.40 Oil DIC M27 objective [9].

Accumulating evidence indicates that disturbances in the retinal cholesterol homeostasis contribute to pathogenesis of such major eye diseases as age-related macular degeneration and diabetic retinopathy. Hence, our finding that the serum LDL to HDL ratio affects, at least in part, retinal cholesterol uptake from the systemic circulation raises a question whether this uptake is even higher in humans who have the LDL to HDL ratios higher than those in hamsters, at least 5-fold as exemplified by normolipidemic subjects, which are not on a cholesterol-lowering medication (Table 1). While it is difficult to make any quantitative predictions, different types of studies suggest that normally, in situ biosynthesis is likely the major source of cholesterol for human retina. The results also suggest that de novo synthesis of 25-HC preferentially regulates SREBP-2 activity. Finally, we quantitatively determine the specificity of the four cholesterol hydroxylases in living cells. Based on our current findings, we conclude that endogenous side-chain oxysterols primarily regulate the activity of SREBP-2, not LXR.

### Analysis of fatty acid

Analysis of fatty acid composition Gas chromatography was used to determine the fatty acid profiles of the lipids lard (LO), FO-120, FO-150, and RO in the plasma and liver, as previously described (Li et al., 2019). In a word, the examples were disintegrated in 0.5 M potassium hydroxide-methanol for saponification and methylated with a boron trifluoride-methanol arrangement (1:3, v/v). N-hexane, a saturated sodium chloride solution, and anhydrous sodium sulfate were then added after this. The supernatant was collected for gas chromatographic (GC) analysis after sufficient shaking and rest. The normalization

method was used to quantify the individual fatty acid retention times in comparison to those of commercial standards. The relative percentage of each fatty acid's peak area is used to represent the findings [10].

## Conclusion

A hostile microenvironment in tumor tissues disrupts endoplasmic reticulum homeostasis and induces the unfolded protein response (UPR). A chronic UPR in both cancer cells and tumor-infiltrating leukocytes could facilitate the evasion of immune surveillance. However, how the UPR in cancer cells cripples the anti-tumor immune response is unclear. Here, we demonstrate that, in cancer cells, the UPR component X-box binding protein 1 (XBP1) favors the synthesis and secretion of cholesterol, which activates myeloid-derived suppressor cells (MDSCs) and causes immunosuppression. Cholesterol is delivered in the form of small extracellular vesicles and internalized by MDSCs through macropinocytosis. Genetic or pharmacological depletion of XBP1 or reducing the tumor cholesterol content remarkably decreases MDSC abundance and triggers robust anti-tumor responses. Thus, our data unravel the cell-non-autonomous role of XBP1/cholesterol signaling in the regulation of tumor growth and suggest its inhibition as a useful strategy for improving the efficacy of cancer immunotherapy.

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