Antioxidant Supplementation Ameliorates Molecular Deficits in Smith-Lemli-Opitz Syndrome

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Background: Smith-Lemli-Opitz syndrome (SLOS) is an inborn error of cholesterol biosynthesis characterized by diminished cholesterol and increased 7-dehydrocholesterol (7-DHC) levels. 7-Dehydrocholesterol is highly reactive, giving rise to biologically active oxysterols.

Methods: 7-DHC-derived oxysterols were measured in fibroblasts from SLOS patients and an in vivo SLOS rodent model using high-performance liquid chromatography tandem mass spectrometry. Expression of lipid biosynthesis genes was ascertained by quantitative polymerase chain reaction and Western blot. The effects of an antioxidant mixture of vitamin A, coenzyme Q10, vitamin C, and vitamin E were evaluated for their potential to reduce formation of 7-DHC oxysterols in fibroblast from SLOS patients. Finally, the effect of maternal feeding of vitamin E enriched diet was ascertained in the brain and liver of newborn SLOS mice.

Results: In cultured human SLOS fibroblasts, the antioxidant mixture led to decreased levels of the 7-DHC-derived oxysterol, 3β, 5α-dihydroxycholest-7-en-6-one. Furthermore, gene expression changes in SLOS human fibroblasts were normalized with antioxidant treatment. The active ingredient appeared to be vitamin E, as even at low concentrations, it significantly decreased 3β, 5α-dihydroxycholest-7-en-6-one levels. In addition, analyzing a mouse SLOS model revealed that feeding a vitamin E enriched diet to pregnant female mice led to a decrease in oxysterol formation in brain and liver tissues of the newborn *Dhcr7*-knockout pups.

Conclusions: Considering the adverse effects of 7-DHC-derived oxysterols in neuronal and glial cultures and the positive effects of antioxidants in patient cell cultures and the transgenic mouse model, we believe that preventing formation of 7-DHC oxysterols is critical for countering the detrimental effects of *DHCR7* mutations.

Key Words: Antioxidants, 7-dehydrocholesterol, DHCEO, Dhcr7, oxysterol, Smith-Lemli-Opitz Syndrome

Smith-Lemli-Opitz syndrome (SLOS) is characterized by multiple congenital malformations and defects (with 2/3 toe syndactyly being the most common), photosensitivity, impaired cognitive function, and behaviors of autistim spectrum disorder (1–4). Smith-Lemli-Opitz syndrome is caused by mutations in the gene encoding the last enzyme in cholesterol biosynthesis—7-dehydrocholesterol (7-DHC) reductase (5–7), resulting in accumulation of 7-DHC in various tissues (8,9). The physiological concentration of 7-DHC in healthy human plasma is very low (.005 to .05 mg/dL), while in persons with SLOS it is greatly elevated (mean = 25 mg/dL) (9–11). Smith-Lemli-Opitz syndrome as a single-gene disorder is recognized as being an appropriate model for understanding the genetic causes of autism (3,12). In addition, it provides a model to study the role of cholesterol in autism spectrum disorder (13,14).

7-Dehydrocholesterol was found to be the most reactive lipid molecule toward free radical peroxidation (15,16), producing over a dozen oxidation products (i.e., oxysterols) in vitro and in vivo (17–20). These 7-DHC-derived oxysterols exert cytotoxicity, reduce cell proliferation, and induce cell differentiation and gene transcript changes (21,22). One of the major 7-DHC-derived oxysterols, 3β,5α-dihydroxycholest-7-en-6-one (DHCEO), was established as a biomarker of the peroxidation of 7-DHC in cells and in animal models of SLOS (17,22). However, DHCEO by itself is also more than a biomarker, as it alters gene expression, promotes differentiation, and induces arborization of mouse cortical neurons (23).

Our previous studies suggest that the accumulation of 7-DHC and its related oxysterols may contribute significantly to the pathogenesis of SLOS, which points to a new direction of therapeutic approach—inhibition of the formation of 7-DHC and/or 7-DHC-derived oxysterols. In the current study, we report on the effects of antioxidant supplementation on the accumulation of 7-DHC oxysterols in human fibroblasts from SLOS patients and an in vivo rodent model (*Dhcr7*-knockout [KO] mice). In summary, we find that antioxidants, and specifically vitamin E supplementation, effectively inhibit the peroxidation of 7-DHC in SLOS human fibroblasts and newborn *Dhcr7*-KO mice and reverse the most critical lipid biosynthesis gene expression changes in the fibroblasts.

Methods and Materials

Materials

AquADEKs and Aqua-E were purchased from Yasoo Health Inc, Johnson City, Tennessee. Vitamin C, vitamin A, coenzyme Q, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri), the highest quality grades available. The aqueous solution of vitamins C and A were prepared fresh before each use in distilled water and then added immediately to cell culture medium. All cell culture reagents were from MediaTech (Manassas, Virginia) and Invitrogen (Life Technologies, Grand Island, New York). High-pressure liquid chromatography (HPLC) grade solvents (hexanes and 2-propanol) were purchased from Thermo Fisher

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Scientific Inc (Waltham, Massachusetts). Syntheses of [25,26,26,26,27,27,27- d_7]-7-DHC, d_7-DHCEO, 4α-hydroxy-7-DHC, 4β-hydroxy-7-DHC, and 7-keto-cholesta-5,8-dien-3β-ol were described elsewhere (18,22,24). NH_2-SPE cartridges (55 μm, 70Å, 500 mg/3 mL) were purchased from Phenomenex, Inc (Torrance, California).

Cell Cultures

Control (GM05399, GM05565) and SLOS (GM05788, GM03044) human fibroblasts were purchased from the Coriell Institute (Camden, New Jersey). GM05788 donor subject is a compound heterozygote: one allele has a G>T transversion at nucleotide 413 of the 7-DHC reductase gene (c.413G>T) resulting in the substitution of valine for glycine at codon 138 (Gly138Val [G138V]), and the second allele has a C>T transition at nucleotide 1213 (c.1213C>T) resulting in the substitution of tyrosine for histidine at codon 405 (His405Tyr [H405Y]). GM03044 has 45,XY,t(13;14)(13qter>cen>14qter), unbalanced in fibroblasts, clinically affected, increased 7-DHC/cholesterol ratio. All cell lines were maintained in DMEM supplemented with L-glutamine, 10% fetal bovine serum (FBS) (Thermo Scientific HyClone, Logan, Utah) and penicillin/streptomycin at 37°C and 5% carbon dioxide. For antioxidant supplementation, all human fibroblasts were plated in 60 mm cell culture dishes and left to adhere overnight in the cell culture incubator, and the following day the medium was completely replaced with fresh DMEM medium without phenol red containing 10% cholesterol-deficient serum (Thermo Scientific HyClone Lipid Reduced FBS) with or without antioxidants. This FBS medium did not have detectable cholesterol level. The cell culture medium was replaced three times a week and the fibroblasts were cultured for 5 to 7 days in the presence or absence of antioxidants. AquADEKs is an antioxidant-rich multivitamin and mineral supplementation. The nominal concentration of all isoforms of tocopherols in AquADEKs was calculated to be approximately 100 mmol/L, and it was used as a stock solution. This stock was further diluted in cell culture medium to the final concentrations shown in the figures. AquADEKs contains in mmol/L: β-carotene 6.4, vitamin C 255, total tocopherol 112 (77 mmol/L of d-α-tocopherol and 35 mmol/L of other mixed tocopherols), coenzyme Q10 2.3, vitamin D_3 .03, vitamin K .9, niacin 49, and vitamin B_6 2.4. Other ingredients include thiamin, riboflavin, biotin, pantothenic acid, selenium, sodium, and zinc. The concentration of Aqua-E was determined to be 67 mmol/L of total vitamin E (ca. 32 mmol/L of d-α-tocopherol and 35 mmol/L of other mixed tocopherols). This stock was further diluted in cell culture medium to the final concentrations shown in the figures. Different batches of cultured cells were used for oxysterol measurement, RNA extraction, and protein analysis. For each type of experiment, three to five independent batches of cultures were prepared and the data presented in the graphs show the average of different experiments. For oxysterol measurements, we used a wide range of concentrations of antioxidant mixture and water soluble vitamin E: 1 nmol/L to 10 μmol/L. For gene expression and protein analysis, we used 50 nmol/L and 500 nmol/L of antioxidant mixture and vitamin E. All cultured SLOS and control human fibroblasts used were passage 8-20. The statistical significance was measured using two tailed *t* test in MS Excel 2007 (Microsoft, Redmond, Washington).

Animals

Dhcr7-heterozygous (HET) (*Dhcr7*^{tm1Gst}) mice were purchased from Jackson Laboratories (catalog # 007453; Bar Harbor, Maine). The mice were kept and bred in the Division of Animal Care facilities at Vanderbilt University. Fifteen female mice, 2 to 3 months of age, were paired with male mice (two female mice with one male mouse), and at that time, mice were randomly allocated to one of three dietary treatments: 1) control (CNT) Laboratory Rodent Diet 5001 (LabDiet); 2) control diet D10001 (vitamin E containing [VEC]) (Research Diets, Inc., New Brunswick, New Jersey) containing standard vitamin mix (V1001), including vitamin E (50 IU/kg diet as vitamin E acetate) and no vitamin C; and 3) vitamin C and E rich diet (VER) D04101103 (Research Diets, Inc) containing 1g vitamin C and 350 IU vitamin E per kg diet in addition to the standard vitamin mix for a total of 400 IU/kg diet vitamin E. Although mice are capable of synthesizing their own vitamin C in the liver, vitamin C is included in the diet to decrease the possibility that high levels of vitamin E could begin to play a pro-oxidant role since vitamin C can recycle vitamin E from its radical form (25,26). Excess vitamin C is excreted in urine. Samples were obtained from multiple litters for each treatment (VEC diet: 4 litters with total 34 pups [6 WT, 19 HET, 9 KO]; VER diet: 5 litters with total 41 pups [8 WT, 22 HET, 11 KO], CNT diet: 5 litters with total 30 pups [8 WT, 17 HET, 5 KO]). Pups were collected shortly after birth at postnatal day 0 (and in some cases at embryonic day 20) because *Dhcr7*-KO pups do not survive long past birth, and brain and liver were harvested and frozen instantly in dry ice-precooled 2-methyl butane at −80°C until needed for analyses. Tails were saved for genotyping. The female mice were allowed 1 week of rest after delivery and mated again to obtain more litters. This process took about 6 months, during which time all mice were kept on the diet that was initially assigned. The oxysterols were measured in whole brains and livers from WT and KO mice. The vitamin E content was measured in heterozygous littermates of *Dhcr7*-WT and *Dhcr7*-KO mice used for oxysterol analysis. This was done because there is not a sufficient amount of tissue to reliably measure both oxysterols and vitamin E content from the same sample (the average wet weight of the brain from newborn KO mouse is ∼60 mg). The genomic DNA from mouse tails was extracted using RED Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich). Genotyping was performed using the following polymerase chain reaction primers: forward: GGATCTTCTGAGGGCAGCCTT, reverse: TCTGAACCCTTGGCTGATCA, neo: CTAGACCGCGGCTAGAGAAT. The statistical significance was measured using two-tailed *t* test in MS Excel 2007. All procedures were performed in accordance with the Guide for the Humane Use and Care of Laboratory Animals. The use of mice in this study was approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

Lipid Extraction, Separation, and High-Performance Liquid Chromatography Tandem Mass Spectrometry Analyses of Oxysterols in Cells and Tissues

This method has been described previously (18,22) and we provide a detailed description in Supplement 1.

RNA Preparation, Quantitative Polymerase Chain Reaction, and Western Blotting

These methods have been described previously (21,27), and we provide a detailed description in Supplement 1. Statistical analyses of the quantitative polymerase chain reaction (qPCR) data were performed using pairwise Student *t* test in MS Excel 2010, while false discovery for multiple testing was performed by calculating the individual q-value (28) for transcript using the Benjamini-Hochberg approach (29).

Determination of α-Tocopherol and Ascorbic Acid Levels

Vitamin E was measured as α-tocopherol in the cortex and liver from heterozygous mice, from at least two litters, from dams