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

Supplemental Information

Supplemental Methods and Materials

Lipid Extraction, Separation, and HPLC-MS-MS Analyses of Oxysterols in Cells and Tissues

This method has been described in detail previously (1,2). An appropriate amount of d_7-DHCEO standard was added to each sample before sample processing. Mouse brain tissues were homogenized in lysis buffer by blade homogenizer and the protein weight was measured as described previously. To the cell and tissue lysates were added NaCl aqueous solution (0.9%, 1 mL) and the resulting mixtures were extracted directly with the Folch solution (5 mL, chloroform/methanol = 2/1, containing 0.001M BHT and PPh_3) with vortexing (1 min) and centrifugation (5 min). The lower organic phase was recovered, dried under nitrogen, re-dissolved in methylene chloride (500 μL) and subject to separation with NH_2-SPE (500 mg; condition with 4 mL of hexanes and elution with 4 mL of chloroform/2-propanol (2/1)). The eluted fractions were then dried under nitrogen and re-constituted in methylene chloride (200 μL for cell samples and 400 μL for tissue samples) for high performance liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry (HPLC-APCI-MS-MS) analyses. HPLC conditions: Silica 4.6 × 150 mm column; 3 μm; 1.0 mL/min; elution solvent: 10% 2-propanol in hexanes. For MS, selective reaction monitoring was employed to monitor the dehydration process of the ion $[M+H]^+$ or $[M+H-H_2O]^+$ in the mass spectrometry.

RNA Preparation, and Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was isolated from the cells using Trizol (Life Technologies, Rockville, MD). Purification of total RNA was done using RNeasy Mini Kit (Qiagen), and on-column digestion of DNA was performed during the RNA purification step using RNase-Free DNase Set (Qiagen). The concentration of total RNA was measured on a Nanodrop instrument (Thermo Scientific, Wilmington, DE). Total RNA (200 ng) from each sample was reverse transcribed to cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real time PCR was performed with an ABI Prism 7300 System (Applied Biosystems) using cDNA (equivalent to 2 ng of input RNA) per 50 µl reaction volume, 2X SYBR green master mix, and gene-specific primers. All samples were run in triplicate. Data from the qPCRs was analyzed using the comparative cycle number determined as threshold (Ct) method (3). Differential expression was calculated as ΔΔCt against expression of several normalizers. We designed primers (~20 bp) to yield 85-110 bp PCR amplicons in Primer3 software (http://frodo.wi.mit.edu/) for different genes. For each gene, we designed several sets of primers. Each set was tested using no template and three different concentrations of a specific template. From this PCR we calculated the efficiency of PCR primers and R^2 value (coefficient of correlation). All human gene-specific primers used showed a slope between -3.10 and -3.58, with $R^2 > 0.99$. All qPCR amplicons were checked by gel electrophoresis, and all of the qPCR reactions gave rise to a single product of predicted size. In addition, the qPCR dissociation curve of the amplicons, performed after each qPCR run (using Dissociation Curve 1.0 software; ABI), also confirmed specific amplification. The primer sequences are: *Hmgcr*: aggactggatgaaaatgtgt, cttgttcaatatccatgctgatc; *Dhcr7*: ggacatctgggccaagact, gggagagacgtgtacagaag; *Srepb2*: atcgctcctccatcaatgac, ttcctcagaacgccagactt; *Scd*: ttggagaagcggtggataac, aaaaatcccacccaatcaca; *Fasn*: ttccgagattccatcctacg,

tgtcatcaaaggtgctctcg; *Fads1*: ttgagccaagaccaagtcct, cactgtgtgtccctttgtgg. The data reported in the Results section are the average of four independent cell culture experiments. The statistical significance was measured using two-tailed *t*-test in MS-Excel 2007. To compare the effect of antioxidant mixture and vitamin E on gene expression, we established first ΔCts for the control (CNT) and Smith-Lemli-Opitz Syndrome (SLOS) fibroblasts, then calculated the ΔΔCt for CNT sham treated – CNT treated and ΔΔCt for SLOS sham treated – SLOS treated. Finally, we compared the ΔΔCts of these two groups, which provided us the difference in response of CNT and SLOS fibroblasts to the treatments.

Western Blotting

At the end of experiment, the cultured fibroblasts were washed on ice in cold 1X phosphate-buffered saline (PBS) (Corning Cellgro) twice, and collected with a cell scraper in pre-cooled 1X PBS. The cell suspension was pelleted by centrifugation at 250 g for 10 min at +4°C. PBS was removed and the cells were resuspended in RIPA lysis buffer (Sigma Aldrich, Saint Louis, MO) supplemented with PMSF, protease inhibitor cocktail (Sigma Aldrich) and phosphatase inhibitor cocktail (Sigma Aldrich). After 30 min of lysis on ice and brief sonication cell lysate was cleared by centrifugation at 14,000 g for 5 min at +4°C. Protein concentration was determined using the DC^{TM} protein assay (Bio-Rad, Hercules, CA). Twenty µg of protein lysate was used for SDS-PAGE (NuPAGE 4-12% Bis-Tris gels, Novex, Life Technologies, Carlsbad, CA). After protein transfer and blotting with 5% non-fat milk, PDVF membranes were cut along 70 kDa mark. Top part of the membrane (>70 kDa) was incubated with an antibody against fatty acid synthase (sc-20140), 1:1,000, (Santa Cruz Biotechnology, CA) and the bottom part (<70 kDa) was incubated with an antibody against glyceraldehyde-3-phosphate

dehydrogenase (GAPDH, D16H11), rabbit mAb, 1:5,000, (Cell Signaling, Danvers, MA, USA) to assess the equal loading of proteins. Secondary antibody was anti-rabbit peroxidase conjugate A6154, 1:10,000 (Sigma Aldrich). The western blots were developed using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific, Rockford, IL). All blots were analyzed and normalized by measuring the pixel density of bands using UN-SCAN-IT (Silk Scientific Inc, Orem, UT). Differences in treatments and controls were analyzed using means, standard error and between group comparisons using Student's *t*-tests. Statistical significance was defined as $p < 0.05$.

Supplemental Results

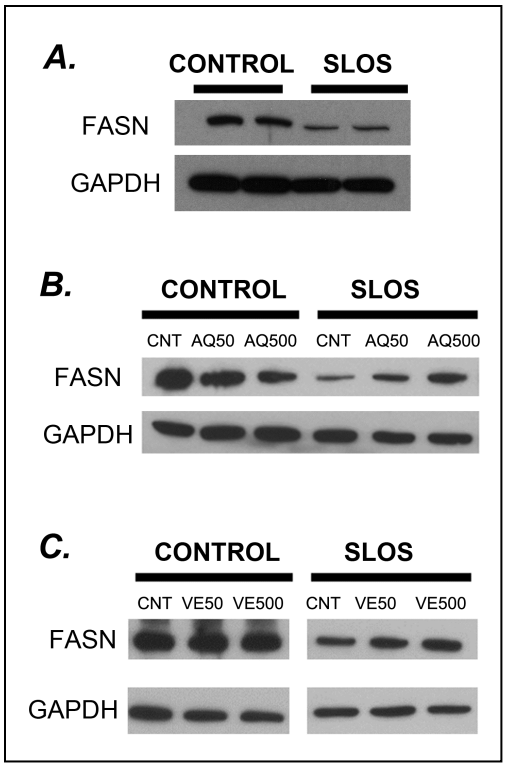

Figure S1. Western blot for FASN and GAPDH proteins in the human control fibroblast and human SLOS fibroblast cultures (**A**) at resting state, (**B**) treated with 50 nM and 500 nM antioxidant mixture and (**C**) treated with 50 and 500 nM vitamin E.

Supplemental References

1. Korade Z, Xu L, Mirnics K, Porter NA (2013): Lipid biomarkers of oxidative stress in a genetic mouse model of Smith-Lemli-Opitz syndrome. *J Inherit Metab Dis*. 36:113-122.

2. Korade Z, Xu L, Shelton R, Porter NA (2010): Biological activities of 7-dehydrocholesterol-derived oxysterols: implications for Smith-Lemli-Opitz syndrome. *J Lipid Res*. 51:3259-3269.

3. Doba T, Burton GW, Ingold KU (1985): Antioxidant and co-antioxidant activity of vitamin C. The effect of vitamin C, either alone or in the presence of vitamin E or a water-soluble vitamin E analogue, upon the peroxidation of aqueous multilamellar phospholipid liposomes. *Biochim Biophys Acta*. 835:298-303.