Neurobiology of Disease 45 (2012) 923–929

Contents lists available at SciVerse ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

DHCEO accumulation is a critical mediator of pathophysiology in a Smith–Lemli–Opitz syndrome model

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ARTICLE INFO

Article history:
Received 9 August 2011
Revised 25 October 2011
Accepted 4 December 2011
Available online 11 December 2011

Keywords:
7-Dehydrocholesterol
Oxysterol
DHCEO
Cortical neurons
Smith–Lemli–Opitz syndrome

ABSTRACT

Smith–Lemli–Opitz syndrome (SLOS) is an inborn error of metabolism caused by defective cholesterol biosynthesis. Mutations within the gene encoding 7-dehydrocholesterol reductase (DHCR7), the last enzyme in the pathway, lead to the accumulation of 7-dehydrocholesterol (7-DHC) in the brain tissue and blood of the SLOS patients. The objective of this study was to determine the consequences of the accumulation of an immediate cholesterol precursor, 7-DHC and its oxysterol metabolite, 3β,5α-dihydroxycholest-7-en-6-one (DHCEO), in the brain tissue of *Dhcr7*-KO mouse, a model for SLOS. We found that cholesterol, 7-DHC and DHCEO show region-specific distribution, suggesting that the midbrain and the cortex are the primary sites of vulnerability. We also report that neurons are ten fold more susceptible to a 7-DHC-derived oxysterol mixture than glial cells, and that DHCEO accelerates differentiation and arborization of cortical neurons. The overall results suggest that 7-DHC oxidative metabolites are critical contributors to altered neural development in SLOS. The future studies will test if antioxidant supplementation will ameliorate some of the clinical symptoms associated with this devastating disease.

Introduction

Cholesterol is an essential structural component of the central nervous system (CNS). Compared to the rest of the body, the brain contains proportionally the highest amount of cholesterol (Dietschy and Turley, 2001). However, cholesterol shows a highly regional distribution in the CNS, with the highest expression observed in the hippocampus and cortex (Korade et al., 2007). Smith–Lemli–Opitz Syndrome (SLOS) is a developmental disorder that arises from mutations in the gene encoding 7-dehydrocholesterol reductase (*DHCR7*), the last step in the cholesterol biosynthesis pathway (Irons et al., 1993; Tierney et al., 2000). The mutation leads to reduced levels of cholesterol and accumulation of 7-dehydrocholesterol (7-DHC) (Irons et al., 1993; Tierney et al., 2000). We recently demonstrated that 7-DHC is the most readily oxidizable lipid molecule in free radical-mediated oxidation reactions (Xu et al., 2009, 2010). As a result, 7-DHC is oxidized, giving rise to a number of metabolites, namely oxysterols (Xu et al., 2010). One of these metabolites, 3β,5α-dihydroxycholest-7-en-6-one (DHCEO) (Xu et al., 2011a) (Fig. 1), shows significant accumulation in fibroblasts of SLOS patients and in the brain of a SLOS mouse model (Xu et al., 2011a), raising the possibility that it might significantly contribute to SLOS pathophysiology in the brain of patients.

The regional differences in 7-DHC, DHCEO, and their physiological effects on brain development/function are not known to date. To better understand the mechanism by which 7-DHC and its metabolite, DHCEO, might contribute to the altered development and brain dysfunction in SLOS, we undertook a series of studies. We determined the distribution of cholesterol, 7-DHC and DHCEO across various brain regions and followed that with assessment of DHCEO effects on neuronal and glial cells.

Materials and methods

Dhcr7-KO Mice–Dhcr7-KO (*Dhcr7*$^{tm1Gst/J}$) mice were purchased from Jackson Laboratories (catalog # 007453). The mice were kept and bred in Division of Animal Care facilities at the Vanderbilt University. For the analysis of different brain regions embryos were dissected from pregnant females at E20 and the tail was removed from each embryo for genotyping. The genomic DNA from mouse tails was extracted using REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich). Genotyping was performed using the following PCR primers: forward — ggatcttctgagggcagcctt, reverse — tctgaacccttggctgatca, neo: ctagaccgcggctagagaat.

Embryonic heads were removed, brains and specific brain regions dissected and instantly frozen in pre-cooled 2-methylbutane (on dry ice) and stored at −80 °C until lipid extraction. We collected 5 litters of mice and compared specific brain regions in WT and KO mice

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Available online on ScienceDirect (www.sciencedirect.com).

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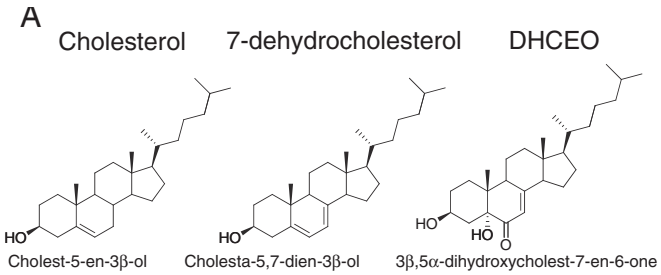
doi:10.1016/j.nbd.2011.12.011

A



B

Brain Region	Genotype	Cholesterol µg/mg protein	7DHC µg/mg protein	DHCEO ng/mg protein
Cortex	WT	32.0 ± 2.1	< 0.2	not detected
Cortex	KO	4.8 ± 0.7	22.4±3.3	17.2±1.8
Midbrain	WT	35.2 ± 2.7	< 0.2	not detected
Midbrain	KO	4.1 ± 0.7	36.6 ± 13.0	21.7±2.0
Hippocampus	WT	21.0 ± 1.1	< 0.2	not detected
Hippocampus	KO	6.9 ± 1.7	12.3 ± 2.6	4.8±0.9
Cerebellum	WT	21.7 ± 0.4	< 0.2	not detected
Cerebellum	KO	5.8 ± 0.8	10.5±2.2	9.8±1.9

Fig. 1. Structure and expression of cholesterol, 7-dehydrocholesterol and DHCEO in WT and *Dhcr7*-KO mouse brain tissue. A) Chemical structure of cholesterol, immediate cholesterol precursor 7-DHC, and DHCEO, which is derived from 7-DHC by oxidation. B) Expression of cholesterol, 7-DHC, and DHCEO levels in cortex, midbrain, hippocampus and cerebellum in E20 mouse brain tissue. 7-DHC levels < 0.2 µg/mg in WT are consistent with previously published measurements of 7-DHC in the mouse brain tissue (Fitzky et al., 2001; Wassif et al., 2001). DHCEO level in WT tissue was below the limit of detection for LC/MS (less than 2 ng). Note the high, but variable levels of 7-DHC and DHCEO across the various brain regions of the *Dhcr7*-KO mice.

within litter. The final number of replicates from different litters is shown in the Results section. 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 IACUC of the Vanderbilt University.

Primary neuronal and astrocytic cultures

Primary cortical neuronal cultures were prepared from E18 brain tissue as previously described (Korade et al., 2007). Briefly, the brain was isolated and the cortex dissected in pre-cooled dissection solution (HBSS). The cortex was cut into tiny pieces and incubated in Trypsin/EDTA for 20–30 min at 37 °C. Trypsin was inactivated by adding DMEM medium with 10% FBS (Thermo Scientific HyClone, Logan, UT). Tissue was centrifuged at 80 × *g* for 5 min and then rinsed with 5 ml of DMEM plus 10% FBS two times. After the second rinse, the tissue was triturated with a fire-polished Pasteur pipette, and the cells pelleted by centrifugation for 5 min at 80 × *g*. The cell pellet was resuspended in DMEM plus 10% FBS and the cells counted. For determining toxicity of 7-DHC oxysterol mixture the cells were plated on poly-L-lysine coated 96-well plate at density10^5/cm^2 and for testing the effects of DHCEO, the cells were plated on poly-L-lysine coated 24-well plates at density 10^4/cm^2. Two hours after plating, the plating medium was completely replaced with Neurobasal medium plus B-27 supplement (Gibco #17504-044) plus L-glutamine plus 5 µM cytosine arabinoside. To test the effects of DHCEO, 2–3 days after plating, the neuronal medium was completely replaced with either fresh medium or medium plus DHCEO (5 µM final concentration). After 48 h incubation in the cell culture incubator (37 °C, 5% CO_2), the cultures were processed for immunocytochemistry.

Primary astrocytic cultures were prepared from P1 brain tissue following the same procedure as for preparation of cortical neurons. After counting, the cells were plated in 60-mm dishes at density 1×10^6/plate and grown in DMEM medium plus 10% FBS for 10 days. During this time, the astrocytes completely filled the plate. The cultures were shaken overnight at 37 °C and washed with HBSS, trypsinized, resuspended in DMEM plus 10% FBS medium, counted and plated in 96-well plates to determine the toxicity of 7-DHC oxysterol mixture. After 48 h, the medium was replaced with fresh medium with or without oxysterol mixture and the astrocytes were incubated in cell culture incubator for additional 48 h. The viability of cells was determined using CellTiter 96®AQ_{ueous} One Solution Cell Proliferation Assay (Promega Corp., Madison, WI) as previously described (Korade et al., 2010).

Immunocytochemistry and Neurolucida®

At the end of the experiment, the neuronal cultures were washed 2× with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature. Following fixation, cultures were washed 3× with PBS and incubated in blocking buffer (PBS with 10% serum and 0.1% saponin) for 30 min. The blocking buffer was replaced with primary antibody diluted in the blocking buffer and the cultures were incubated at + 4 °C overnight. After removing primary antibody solution, the cultures were washed 3× in PBS and then incubated in secondary antibody diluted in blocking buffer for 1 h at room temperature. After incubation the cultures were washed 3× with PBS. The PBS was completely removed and tiny droplet of Flouromount was added to each well in 24-well plate. The round 12 mm glass coverslip was gently placed on the top of the cultures. The primary antibodies used in this study are: Tu-20 (mouse monoclonal to neuron specific beta III tubulin, Abcam #ab7751) and MAP2 (rabbit polyclonal to microtubule-associated protein 2, Cell Signaling Technology #4542). Secondary antibodies were: anti-rabbit IgG-Cy3 (Sigma, #C2306) and anti-mouse IgG-DyLight® 549 (Vector, #DI-2549). The cells were analyzed on the Zeiss AXIO Observer.Z1 inverted microscope equipped with Hamamatsu Digital Camera C10600 Orca R^2. Images were acquired using AxioVision Rel. 4.7 program. The objective lenses were Zeiss EC Plan-NEOFLUAR 10X/0,3 Ph1 (420341–9911) and Zeiss LD Plan-NEOFLUAR 20X/0,4 Ph2 Korr (∞/0-1,5). The images of individual neurons were saved as .tif files which were opened in Neurolucida® software for neuron reconstruction and morphometry. The individual neurons were traced and analyzed in NeuroExplorer with results presented in spreadsheet format and exported to Microsoft Excel®. We analyzed 10–14 images for each group (total 70 images). The statistical significance was measured using two tailed *t*-test in MS-Excel 2007.

Lipid extraction, separation, HPLC-APCI-MS-MS analyses, preparation of 7-DHC-derived oxysterols and DHCEO, were done as previously described (Korade et al., 2010; Xu et al., 2011a). Briefly, brain regions were homogenized in lysis buffer and the protein concentration was determined using Protein D_c photometric assay (BioRad). To the homogenates were added 5 mL Folch's solvent (chloroform/methanol = 2/1, containing 0.001 M BHT and PPh_3) and an appropriate amount of d_7-DHCEO standard. Aqueous NaCl solution (0.9%, 1 mL) was then added and the resulting mixture was vortexed for 1 min and centrifuged for 5 min. The lower organic phase was recovered, dried under nitrogen, re-dissolved in methylene chloride and subject to separation with NH_2-SPE (500 mg; the column was conditioned with 4 mL of hexanes and the neutral lipids containing oxysterols were eluted with 4 mL of chloroform/2-propanol (2/1)). The eluted fractions were then dried under nitrogen and re-constituted in methylene chloride (400 µL) for HPLC-APCI-MS-MS analyses (HPLC conditions: Silica 4.6 mm × 25 cm column; 5µ; 1.0 mL/min; elution solvent: 10% 2-propanol in hexanes). Selective reaction monitoring (SRM) was employed to monitor the dehydration process of $[M + H - H_2O]^+$ of the oxysterol (for DHCEO, fragmentation of *m/z* 399 to 381 was monitored). DHCEO was quantified by comparing the response of endogenous d_0-DHCEO to that of the d_7-DHCEO