Neuroscience Letters 552 (2013) 71–75

Contents lists available at ScienceDirect

Neuroscience Letters

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

Altered phospholipid molecular species and glycolipid composition in brain, liver and fibroblasts of Zellweger syndrome

Celine Miyazaki[a], Makiko Saitoh[a,*], Masayuki Itoh[b], Sumimasa Yamashita[c], Makoto Miyagishi[d], Sachio Takashima[e], Ann B. Moser[f], Masao Iwamori[g], Masashi Mizuguchi[a]

[a] Department of Developmental Medical Sciences, Graduate School of Medicine, University of Tokyo, Japan
[b] Department of Mental Retardation and Birth Defect Research, National Center of Neurology and Psychiatry, Japan
[c] Division of Child Neurology, Kanagawa Children's Medical Center, Japan
[d] Molecular Composite Medicine Research Group, Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology, Japan
[e] Yanagawa Institute for Developmental Disabilities, International University of Health and Welfare, Japan
[f] Peroxisomal Diseases Laboratory, The Hugo W Moser Research Institute, The Kennedy Krieger Institute, United States
[g] Department of Biochemistry, Faculty of Science and Technology, Kinki University, Japan

HIGHLIGHTS

- We analyzed the lipid molecular species of five patients' tissues with Zellweger syndrome.
- The relative amount of sphingomyelin with shorter chain fatty acid was slightly increased in patients' cerebellum.
- Increase of glycolipids was confirmed in Zellweger syndrome.
- *ACOX1* and *GNPAT* RNAi study suggested that both of them could modulate glycolipid metabolism in neuronal cells.

ARTICLE INFO

Article history:
Received 22 May 2013
Received in revised form 9 July 2013
Accepted 29 July 2013

Keywords:
Peroxisome
Zellweger syndrome
Sphingomyelin
Plasmalogen
Ganglioside
RNAi

ABSTRACT

We studied the altered molecular species of lipids in brain and liver tissues, and fibroblasts from patients with Zellweger syndrome (ZS). ZS cerebellum samples contained a higher amount of sphingomyelin with shorter chain fatty acids compared to that in normal controls. The amount of phosphatidylethanolamine (PE) was less than half of that in controls, with the absence of the PE-type of plasmalogen. Gangliosides were accumulated in the brains and fibroblasts of ZS patients. To investigate whether or not impaired beta-oxidation of very long chain fatty acids and/or plasmalogen synthesis affects glycolipids metabolism, RNAi of peroxisomal acylCo-A oxidase (*ACOX1*) and glyceronephosphate O-acyltransferase (*GNPAT*) was performed using cultured neural cells. In neuronal F3-Ngn1 cells, *ACOX1* and *GNPAT* silencing up-regulated ceramide galactosyltransferase (*UGT8*) mRNA expression, and down-regulated UDP-glucose ceramide glucosyltransferase *(UGCG)*. These results suggest that both impaired beta-oxidation of very long chain fatty acids and plasmalogen synthesis affect glycolipid metabolism in neuronal cells.

1. Introduction

Patients with Zellweger (ZS) syndrome and D-bifunctional protein (DBP) deficiency suffer from severe neurological impairment [1,4,6]. Dysfunction of the peroxisome affects not only the metabolism of long-chain polyunsaturated fatty acids (LCPUFA) but also ether-phospholipid biosynthesis [2,10,23]. ZS patients show VLCFA accumulation and a marked plasmalogen deficiency due to a *PEX* gene defect. On the other hand, DBP deficiency impedes the oxidation process of VLCFA [5,21]. The mechanism of central nervous system (CNS) abnormality in ZS and DBP deficiency has not yet been clarified.

We previously reported increases of ceramide monohexoside (CMH) and dipalmitoyl glycerophospholipids in the cerebral gray

Abbreviations: ZS, Zellweger syndrome; DBP, D-bifunctional protein; PE, phosphatidylethanolamine; VLCFA, very long chain fatty acid; PC, phosphatidylcholine; ACOX1, peroxisomal acyl coenzyme-A oxidase; GNPAT, glyceronephosphate O-acyltransferase; CNS, central nervous system; CMH, ceramide monohexoside; CDH, ceramide dihexoside; CTH, ceramide trihexoside; UGCG, ceramide glucosyltransferase; UGT8, ceramide galactosyltransferase; G6PDH, glucose-6-phosphate dehydrogenase; PBS, phosphate-buffered saline.

* Corresponding author at: Department of Developmental Medical Sciences, Graduate School of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan. Tel.: +81 3 5841 3615; fax: +81 3 5841 3628.
E-mail address: makisaito-tky@umin.ac.jp (M. Saitoh).

0304-3940/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neulet.2013.07.045

Table 1
(US) Samples were obtained from Peroxisomal Diseases Lab, Kennedy Krieger Institute, Baltimore, United States and NICHD Brain and Tissue Bank at the University of MD, United States. (JP) Samples were originated from Japanese patients [16–18]. NI; not identified, NA; not available.

Patients	Mutated genes	Age, sex	Samples
ZS1 (US)	*PEX 26*	6 months, female	Cerebellum; liver
ZS2 (US)	*PEX 1*	7 months, female	Cerebellum; liver; fibroblasts
ZS3 (JP)	NI	7 months, male	Frontal lobe gray and white matter
ZS4 (US)	*PEX 5*	NA	Fibroblasts
ZS5 (US)	*PEX 5*	NA	Fibroblasts
DBP (JP)	*HSD17B4*	1 year and 7 months, male	Frontal lobe gray and white matter

Controls	Cause of death	Age, sex	Samples
C1 (JP)	Micrognathia	0 years, female	Cerebellum
C2 (JP)	RDS	0 years, male	Cerebellum
C3 (JP)	Sudden infant death syndrome	6 months, female	Cerebellum; frontal lobe gray and white matter
C4 (JP)	Sudden infant death syndrome	1 year and 9 months, male	Cerebellum; frontal lobe gray and white matter; liver
C5 (US)	Burn	6 years, male	Cerebellum; liver
C6 (US)	–	2 months, male	Fibroblasts

matter of a ZS patient [17]. In a DBP deficiency patient, the lipid composition in white matter myelin, as well as the molecular species of plasmalogen-PE in the gray matter, was altered [18]. In the present research, we conducted lipid analysis of ZS patients' brains, livers, and fibroblasts to extend our previous findings [16,17].

We hypothesized that impaired peroxisomal beta oxidation and plasmalogen synthesis may modulate glycolipids synthesis via ceramide metabolism. Ceramides mediate apoptosis triggered by numerous mechanisms. Fatty acids of various chain lengths are bioactive molecules that reportedly exhibit in vitro antiproliferative actions including induction of oxidative stress and modification of the intracellular signaling pathway [3]. Accumulated VLCFA may induce an increase of ceramide, followed by the accumulation of ceramide monohexoside (CMH). In the CNS, polyunsaturated fatty acids and gangliosides are extremely abundant, and these functional lipids play an important role in the intercalation of lipoproteins, such as glycolipid-enriched microdomains, in the lipid bilayer of the cell membrane [9,11,12]. Therefore, the alteration in glycolipid metabolism may be related to the pathomechanism in CNS of ZS patients. To better understand the altered glycolipid metabolism in ZS patients' tissues, we targeted glycolipid synthesizing enzyme expression by treating human neural cell lines with peroxisomal acyl-coenzyme A oxidase 1 (*ACOX1*) and glyceronephosphate O-acyltransferase (*GNPAT*) RNAi.

2. Materials and methods

2.1. Control and patient samples

Six patients and six control (C1-C6) samples were examined in this study. Patients ZS1 (GM16866), ZS2 (101885), ZS3, ZS4 (GM13268) and ZS5 (GM13266) were diagnosed as having ZS, and one patient (DBP) as having DBP deficiency (Table 1). Cerebellum and liver tissue samples from ZS1, ZS2 and C5 were obtained from the NICHD Brain and Tissue Bank at the University of Maryland in Baltimore, MD and cell lines from ZS4, ZS5, and C6 were provided by the Peroxisomal Diseases Laboratory, Kennedy Krieger Institute (Baltimore, MD, US). Other tissues were obtained in Japan. This study was approved by the Ethical Committee of the University of Tokyo (No. 3188).

2.2. Lipid extraction and TLC analysis

Lipids extracted from brain and liver tissues were preserved at −70 °C until use. To quantify each fraction, lipids were applied to glass-coated silica gel TLC plates (Merck, Darmstadt, Germany) as described previously [14–18,22]. The developing solvent for cholesterol, phospholipids and glycolipids analyses were hexane/diethyl ether/acetic acid (80:20:1, by vol.), chloroform/methanol/water (65:35:8, by vol.), and chloroform/methanol/0.5% calcium chloride (60:35:8, by vol.), respectively. To detect neutral lipids/phospholipids and glycolipids, cupric acetate-phosphoric acid and orcinol-sulfuric acid were used, respectively. The lipid band intensity was measured with Image J (NIH 1.42q software) for quantification. We performed TLC-immunostaining as previously reported [17] with a slight modification, using a 3,3′-diaminobenzidine (DAB) staining solution (15 ml distilled water/0.75 ml of 1 M Tris HCl (pH 7.6)/3 μg DAB/3 μl of 30% H_2O_2). Plasmalogen assaying of the brain and liver crude lipid samples was performed as described previously [14].

2.3. Neural cell culture

The neuronal cells used were immortalized human neural stem cell clone of HB1.F3 transfected with neurogenin 1 (F3-Ngn1) [19]. Human glioblastoma/astrocytoma cells (HTB-14) were purchased from the ATCC™ Biological Resource Center. F3-Ngn1 cells were cultured in DMEM medium (Life Technologies) with the addition of 10% heat inactivated fetal bovine serum and 2% penicillin/streptomycin, and HTB-14 cells in MEM medium (Life Technologies) with 10% fetal bovine serum and 2% penicillin/streptomycin. Both types of cells were cultured at 37 °C in a 5% CO_2 incubator and were harvested with 0.05% trypsin.

2.4. Vector preparation and transfection

shRNA *ACOX1* and *GNPAT* were inserted into the GeneEraser pGE-2-hrGFP II shRNA Expression Vector (Stratagene, CA, US) according to the manufacturer's manual. The sequences were as follows; *ACOX1*sense, 5′-GCCTCAGATTACACAAGTAAA-3′; antisense, 5′-TTTACTTGTGTAATCTGAGGC-3′; *GNPAT*sense, 5′-GCCAAGACATTGACTCCTAAA-3′; antisense, 5′-TTTAGGAGTCAATTGTCTTGGC-3′. Cells were transfected with 2.5 μg of shRNA for 48 h using MultiFectam (Promega, Fichburg, US), and re-suspended in PBS containing 1 mM EDTA, 25 mM HEPES (pH7.0), and 1% dialyzed fetal bovine serum. GFP-positive cells were collected with BD FACSAria (Becton, Dickinson and Company, NJ, US) with 488-nm laser option. The FACS results were analyzed by BD FACSDiVa software.

2.5. Quantitative real-time PCR

Total RNA was isolated from shRNA transfected cells with TRIzol reagent (Life Technologies), and reversely transcripted to cDNA