Lipidomics Analysis of Peroxisomal Disorders: Discovery of Deficits in Phosphatidylglycerol Levels in Rhizomelic Chondrodysplasia Type 1

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Abstract

Objectives: Decreases in the levels of plasmalogens, have been consistently demonstrated in rhizomelic chondrodysplasia type 1 (RCDP1), a genetic disorder of peroxisomal function. However, an in-depth lipidomics analysis has not been undertaken. We undertook such an analysis.

Study Design: We performed a high-resolution mass spectrometric shotgun lipidomics analyses of plasma and lymphoblasts from RCDP1 patients.

Results: We report for the first time, decrements in phosphatidylglycerol levels in plasma and lymphoblasts from RCDP1 patients. Phosphatidylinositol and phosphatidylserine levels also were unaltered in plasma and lymphoblasts. These data suggested that decrements in phosphatidylglycerol were due to increased catabolism, possibly in failed cellular attempts to restore deficient plasmalogen levels. This conclusion was further supported by supplementation of RCDP1 lymphoblasts with ether lipid plasmalogen precursors that bypass dysfunctional peroxisomes. These precursors augmented cellular levels of plasmalogens in control and RCDP1 lymphoblasts but only augmented phosphatidylglycerols in RCDP1 lymphoblasts.

Conclusions: Overall, our results indicate that the peroxisomal disorder, RCDP1, which is characterized by plasmalogen deficits, also possess decrements in phosphatidylglycerol levels, thereby also compromising mitochondrial function and pulmonary surfactant synthesis. Given the role of phosphatidylglycerols in surfactant, these new data potentially explain the severe respiratory compromise in RCDP children and may add a new parameter of mitochondrial dysfunction in these patients.

Keywords: Phosphatidylglycerol; RCDP; Lung Infections; Lipidomics; High Resolution Mass Spectrometry

Introduction

Rhizomelic chondrodysplasia punctata type 1 (RCDP1) is a peroxisome biogenesis disorder involving mutations in PEX7, the peroxisome transporter required for the import of enzymes involved in the synthesis of plasmalogens [1-3]. Insertion of a fatty alcohol via an ether linkage at sn-1 of the glycerol backbone of glycerophospholipids only occurs in peroxisomes. Deficiency in these critical glycerophospholipids results in the RCDP1 phenotype, which includes cataracts and shortened humeri and femurs (rhizomelia), punctate epiphyseal calcifications (chondrodysplasia punctate), microcephaly and dysmyelination (hypomyelination) which result in severe growth and neurological impairments.

Plasmalogen levels are routinely monitored in RCDP patients via gas chromatography (GC) and GC-MS assays which involve treating plasma samples with methanol under acidic conditions to yield the dimethylalcaleral derivatives of the ether linked fatty alcohols at sn-1. This yields a picture of the total plasmalogen pool but does not evaluate individual plasmalogens. We previously have quantitated individual ethanolamine plasmalogens in lymphoblasts from RCDP patients and in a murine Pex7 model of RCDP1 [4]. With this background, we designed studies utilizing high-resolution mass spectrometry to undertake a shotgun lipidomics analysis [5,6] of plasma and lymphoblasts from RCDP1 patients to both more broadly and more precisely evaluate additional lipid alterations in this severe childhood disorder. Since it is the goal of a number of investigators to evaluate plasmalogen precursors as potential therapeutics for peroxisomal disorders [4,7], increasing in our knowledge base of the lipidome in RCDP will be very valuable for future translational research efforts. Furthermore, recognition of a peroxisomal deficit in Alzheimer's disease [8-10], which may underlie cerebral white matter dysmyelination in Alzheimer’s disease [10-12], also support increasing our understanding of the full consequences of peroxisomal deficits on the human lipidome.
Materials and Methods

Clinical plasma samples

Plasma samples were obtained from 3 RCDP Caucasian patients aged 1, 5 and 6 years, while control plasma was obtained from 8 healthy Caucasian children aged 4 to 8 years. The PEX 7 mutations included, PEX7-p.Leu292* homozygote (c.875T>A); PEX7-Leu292*/Tyr40* (c.120C>G); and PEX7-Leu292*/Ala218Val. The clinical studies were approved by the Lincoln Memorial University Institutional Review Board. Plasma samples were processed as described previously, utilizing tert-butyl methylether and methanol for extraction of lipids [4-6,12]. The extraction solution contained [13C3]arachidonic acid, [13C3]phytanic acid, [13C6]hexacosanoic acid, [13C16]palmitic acid, [3H]cholesterol sulfate, [2H2]MAG 18:1, [13C2]DAG 36:2, [13H2]PtdEtn 34:1, [13H2]PtdEtN 28:0, [13H2]PtdCh 34:1, [13H2]PtdCh 28:0, [13H2]PtdCh 32:0, [13H2]SM 16:0, PtdSer 36:1, [13H2]PA 34:1, [13H2]PG 32.0 and CL(56:0) as internal standards. Extracts were dried by centrifugal vacuum evaporation and dissolved in isopropanol : methanol : chloroform 4:2:1 containing 7.5 mM ammonium acetate. Shotgun lipidomics were performed utilizing high-resolution (140,000 at 200 amu) data acquisition, with sub-ppm mass accuracy on an orbitrap mass spectrometer (Thermo Q Exactive) with successive switching between polarity modes [5,6].

Lymphoblast studies

Control (GM13072) and RCDP1 (GM09291; Pex7 c.870insCAA/875T>A or p.C290 E291insQ/L292X) lymphoblasts were obtained from the Coriell Inst. for Medical Research. Cells were cultured in 12 well plates as described previously [4]. For supplementation studies, batyl alcohol and chimyl alcohol were dissolved in ethanol (final ethanol concentration in culture was 0.2%). For precursor labeling studies, lymphoblasts in 12 well plates were incubated with 6 mg/100 ml media of either [13C3] glycerol or [13C16]palmitic acid for 24 hours. Lymphoblasts were harvested by centrifugation at 3,000 xg for 10 min and washed once with 15 ml of cold PBS. Lipid extraction was the same as for plasma except that the cells were sonicated [4].

Statistical analyses

Clinical data only involved 3 patients and are therefore presented as vertical scatter plots as % of control (N=8). For the lymphoblast studies, data are presented as % of control ([Tx mean]/(control mean) (1 ± SQRT((Tx SD/Tx mean)2 + (control SD/control mean)2) for 6 tissue culture wells in all experiments. For the labelling studies, atom percent excess is presented (mean ± SD) after correction for natural isotopic abundance. Data were analyzed by 1-way ANOVA, followed by the Tukey-Kramer test to determine differences between groups.

Results

RCDP1 plasma lipidomics

An initial shotgun lipidomics approach demonstrated the expected dramatic decrements in both choline and ethanolamine plasmalogens in the plasma of RCDP1 subjects Figure 1. We also monitored unexpected decreases in the circulating levels of phosphatidylglycerols (PG, Figure 1). While shotgun analyses cannot distinguish between phosphatidylglycerols and bis(monoacylglycerol)phosphates (BMP), the decrements in PG 36:1 indicate that the decreases we monitored are mainly constitute decrements the levels of phosphatidylglycerols since BMP 36:1 levels are less than 1% of the levels of PG 36:1 in human plasma [13].

Figure 1: Plasma levels of choline (PC) and ethanolamine (PE) plasmalogens and phosphatidylglycerols (PG). PC 38:6 (1), PC 36:4 (2), PC 34:1 (3), PC 40:6 (4), and PC 38:4 (5) all were decreased (panel PC) in RCDP I subjects (N=3). Data are presented as vertical scatter plots. PE 34:2 (6), PE 36:4 (7), PE 38:6 (8), PE 38:4 (9), and PE 40:6 (10) also were decreased in RCDP plasma (panel PE). Similarly, PG 34:0 (11), PG 34:1 (12), PG 34:2 (13), PG 36:0 (14), and PG 36:1 (15) were lower in RCDP plasma.
Lipidomics of RCDP1 lymphoblasts

Next, we took advantage of immortalized cell lines from RCDP1 patients. Using an RCDP1 lymphoblast cell line we validated decrements in plasmalogens and phosphatidylglycerols in cells Figure 2 similar to what we monitored in plasma samples from RCDP1 subjects Figure 1. To further explore other products of the de novo biosynthetic pathway for phosphatidylglycerols Figure 3, we measured phosphatidylinositols and found that the levels of these glycerophospholipids were not decreased Figure 2. Phosphatic acids, which are at a critical branchpoint interconnecting multiple glycerophospholipid pathways Figure 3 were elevated Figure 2. The very-long-chain fatty acid (VLCA), octacosanoic acid (26:0) also was elevated Figure 2 in RCDP lymphoblasts, another biomarker of peroxisomal dysfunction since this is the compartment where metabolism of VLCFA is initiated.

Glycerophospholipid biosynthesis in lymphoblasts

To monitor the dynamics of phosphatidylglycerol synthesis, we utilized stable isotope precursors [14]. In our first experiment, we utilized $^{13}$C$_{16}$ palmitic acid to label glycerophospholipid pools in lymphoblasts. Incorporation into plasmalogens Figure 4, panel A was drastically decreased in RCDP lymphoblasts compared to control lymphoblasts, as predicted by previous labeled precursor studies [4]. In contrast the incorporation of $^{13}$C$_{16}$ palmitic acid into phosphatidylglycerols Figure 4, panel B was not different from controls. $^{13}$C$_{16}$ palmitic acid incorporation into DAG 34:1 Figure 2, panel B was increased (p<0.01) while incorporation into DAG (32:0) was not different from control cells (data not shown).

Next, we evaluated $^{13}$C$_{3}$ glycerol incorporation into lipid pools Figure 4, panel C. With this precursor, incorporation rates as percent of the pool were unaltered for plasmalogens (PE and PC) as well as for phosphatidylserines. Increased incorporation into phosphatidylglycerols was monitored in RCDP lymphoblasts.

Phosphatidylglycerol metabolism

The data that we obtained for the steady-state levels and biosynthesis of phosphatidylglycerols led us to conclude that these lipids might be catabolized in a failed effort to supply precursors for plasmalogen synthesis. With this hypothesis in mind we first measured cardiolipins (CL 72:5, CL 72:6, and CL 77:8) in lymphoblasts and diacylglycerols (DAG 34:1, DAG 36:4, and DAG 34:1) in both lymphoblasts and patient plasma samples. In lymphoblasts, cardiolipins were decreased by 20 to 30% while diacylglycerols were increased by 30 to 50% Figure 2. These data were consistent with the initial hypothesis; however, diacylglycerols were not increased in patient’s plasma (data not shown).

Ether lipid supplementation

To further evaluate if increased catabolism of phosphatidylglycerols was potentially the result of failed attempts to restore plasmalogens via the metabolism of phosphatidylglycerols to augment diacylglycerol levels Figure 3, we investigated augmentation of plasmalogens with the ether lipid precursors, batyl alcohol and chimyl alcohol, which bypass peroxisomes in the synthesis of plasmalogens [7,15]. Chimyl alcohol was the most effective in augmenting a broad range of choline plasmalogens Figure 5, upper panel and ethanolamine plasmalogens (data not shown) in both control and RCDP1 lymphoblasts. While these increases in cellular plasmalogen levels did not alter phosphatidylglycerol levels in control lymphoblasts, this treatment did increase the levels of phosphatidylglycerols (32:0 and 32:1) in RCDP1 lymphoblasts Figure 5, lower panel. These are predominant phosphatidylglycerols involved in surfactant synthesis in humans [16-18].

Glycerophospholipid and very-long-chain fatty acid levels in RCDP1 lymphoblasts. The VLCA, octacosanoic acid (OA), phosphatic acid (PA 34:1), and diacylglycerol (DAG 34:1) were significantly elevated in RCDP lymphoblasts relative to control cells. Data are presented as % of control ± SD for 6 tissue culture wells. Phosphatidylinositols (PI 34:1) were not altered while significant decrements in the levels of phosphatidylglycerols (PG 32:0 and PG 34:1), ethanolamine plasmalogens (PE 36:4 and PE 38:6), choline plasmalogens (PC 36:4 and PC 38:6), and cardiolipins (CL 72:6) were measured. *, p<0.05.
Discussion

A key factor that negatively impacts the quality of lifestyle and ultimately limits longevity in RCDP1 patients is respiratory compromise with frequent lung infections and reactive airway disease [19,20]. In this regard, our observations of decreased phosphatidylglycerol levels in the plasma and lymphoblasts of RDDP1 patients is of significant notation. Phosphatidylglycerols are precursors to mitochondrial cardiolipins [21] and are essential components of lung surfactant [22,23]. ToF-SIMS studies have demonstrated that phosphatidylglycerols concentrate along the edges of the tubular proteolipids that form the tubular myelin of pulmonary surfactant and are dispersed throughout the interstitial space between the tubular networks [24]. Of further significance are the observations that phosphatidylglycerols regulate innate immunity against lung viral infections and the associated inflammatory processes [23-31]. Recent studies have also demonstrated in murine models that phosphatidylglycerol administration is effective both for postinfection treatment and for prophylaxis against respiratory syncytial viral infections [32], a predominant respiratory pathogen in young children. This combination of phosphatidylglycerol alterations in surfactant, the innate immune response, and mitochondrial function may contribute significantly to the incidence of respiratory infections in RCDP1 children.

Phosphatidylglycerols are the second most abundant phospholipids that are constituent in surfactant. The dominant phospholipid is dipalmitylphosphatidylcholine, a glycerophospholipid that was not decreased in plasma or lymphoblasts from RCDP1 patients, in our study. In contrast, we observed decreases in the levels of the major surfactant phosphatidylglycerol PG 34:1 [17] in both plasma and lymphoblasts from RCDP1 subjects. We observed no decrements in phosphatidylinositol levels nor in the incorporation of labeled glycerol or palmitic acid into phosphatidylglycerol pools, we conclude that the de novo CDP-DAG biosynthetic pathway [23] and acylation / transacylation remodeling of phosphatidylglycerols [33] are operational in RCDP1. The labeling with $^{13}$C$_{3}$glycerol also indicate that aquaporin channels are functional in RCDP1 and do not limit cellular access of glycerol [34].

The decreases in RCDP1 lymphoblasts of cardiolipins along with increases in diacylglycerols and in the synthesis of DAG 34:1, a major precursor of phosphatidylglycerols in surfactant, further suggest that phosphatidylglycerol catabolism is accelerated in RCDP1. This conclusion is also consistent with previous observations that lung phosphatidylglycerols are more susceptible to degradation and recycling than phosphatidylincholines [33]. While we did not monitor decreases in circulating levels of diacylglycerols in RCDP1 subjects, these measurements may be complicated by the complex role of diacylglycerols in the synthesis and metabolism of a vast array of glycolipids and glycerophospholipids in multiple compartments [23].

In toto, our data led us to hypothesize that phosphatidylglycerol catabolism is augmented in RCDP1 in a failed metabolic attempt to augment cellular phospholipids via increasing the diacylglycerol precursor pool. To evaluate this hypothesis we investigated augmentation of cellular phospholipids in lymphoblasts incubated with ether lipid precursors that bypass peroxisomes to augment cellular phospholipid levels. These data suggest that ether lipid supplementation to augment phospholipids [7,15] in RCDP1 patients also may augment phosphatidylglycerols. If this proves to be the case, then improved quality of lifestyle and longevity, relative to lung infections, may be a significant clinical benefit for RCDP children.
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Figure 5: Glycerophospholipid levels in control and RCDP1 lymphoblasts with ether lipid supplementation. Cellular choline plasmalogens (upper panel) and phosphatidylglycerols (lower panel) in control and RCDP1 lymphoblasts after a 24 hr incubation with vehicle, 50 μM batyl alcohol, or 50 μM chimyl alcohol. Chimyl alcohol significantly augmented choline plasmalogens in both control and RCDP1 lymphoblasts, while batyl alchol was less effective (upper panel). While ether lipid precursors had no effect on phosphatidylglycerol levels in control cells, augmentation of plasmalogens with chimyl alcohol in RCDP1 cells resulted in significant increases in cellular PG 32:0, PG 32:1, and PG 36:1 levels (lower panel). Batyl alcohol significant increased cellular levels of PG 36:1 (lower panel). *, p < 0.05 vs. control; #, p<0.05 vs. RCDP1.

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Competing Interests

The authors have no declared competing interests.

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