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Detection of unusual very-long-chain fatty acid and ether lipid derivatives in the fibroblasts and plasma of patients with peroxisomal diseases using liquid chromatography-mass spectrometry

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ABSTRACT

Metabolic changes occur in patients with peroxisomal diseases owing to impairments in the genes involved in peroxisome function. For diagnostic purposes, saturated very-long-chain fatty acids (VLCFAs) such as C24:0 and C26:0, phytanic acid, pristanic acid, and plasmalogens are often measured as metabolic hallmarks. As the direct pathology of peroxisomal disease is yet to be fully elucidated, we sought to explore the fatty acid species that accumulate in patients with peroxisomal diseases. We developed a method for detecting a range of fatty acids implicated in peroxisomal diseases such as Zellweger syndrome (ZS) and X-linked adrenoleukodystrophy (X-ALD). To this end, we employed an ultra-performance liquid chromatography-mass spectrometry (LC-MS) coupled with negatively charged electrospray ionization. Fatty acids from patients and control subjects were extracted from total lipids by acid-hydrolysis and compared. In accordance with previous results, the amounts of VLCFAs, phytanic acid, and pristanic acid differed between the two groups. We identified extremely long and highly polyunsaturated VLCFAs (ultra-VLC-PUFAs) such as C44:12 in ZS samples. Moreover, three unknown molecules were prominent in control samples but scarcely detectable in ZS samples. LC-MS/MS analysis identified these as 1-alkyl-*sn*-glycerol 3-phosphates derived from ether lipids containing fatty alcohols such as C16:0, C18:0, or C18:1. Our method provides an approach to observing a wide range of lipid-derived fatty acids and related molecules in order to understand the metabolic changes involved in peroxisomal diseases. This technique can therefore be used in identifying metabolic markers and potential clinical targets for future treatment.

1. Introduction

Very-long-chain fatty acids (VLCFAs) consist of a hydrocarbon chain containing 22 or more carbon atoms. Unlike shorter fatty acids, which are catabolized in the mitochondria, VLCFAs are catabolized in peroxisomes through beta-oxidation [19,46]. Peroxisomes are single-layered subcellular organelles distributed in the cytoplasm that are also required for the biosynthesis of bile acids [12], ether lipids such as plasmalogens [9], and for the degradation of phytanic acid into pristanic acid [52]. Levels of VLCFAs, phytanic acid, pristanic acid, bile acid, and plasmalogens are altered in patients with peroxisomal diseases. Because of this, they are used as biochemical hallmarks in diagnosis [44,48]. Patients diagnosed as having peroxisomal diseases such as Zellweger syndrome (ZS) and X-linked adrenoleukodystrophy (X-ALD) are unable to catabolize VLCFAs effectively leading to their accumulation in tissue and plasma. Patients with ZS also accumulate phytanic acid and pristanic acid, while being deficient in bile acid and ether lipid synthesis [48,44].

ZS is the most severe form of peroxisomal biogenesis disorder (PBD), a group of impairments that includes Zellweger spectrum disorder (ZSD), rhizomelic chondrodysplasia punctata (RCDP) type 1 and type 5 [4]. The ZSD is a group of diseases all sharing the same pathogenic genes [11]. It includes, from most to least severe; ZS, neonatal adrenoleukodystrophy (NALD), and infantile Refsum disease (IRD) [21,44]. Symptoms of ZS include craniofacial dysmorphism, glaucoma, retinitis pigmentosa, hepatomegaly, jaundice, severe hypotonia, renal cysts, chondrodysplasia punctata, psychomotor retardation, and the inability to eat. Most patients with ZS die within 12 months of birth. Microgyrus, delayed myelination, and neuronal migration defects are the anatomical abnormalities seen in the brains of patients with ZS. Patients with NALD show clinically milder phenotypes with less prominent craniofacial dysmorphism, eye abnormalities, hepatomegaly, and rare occurrence of renal cysts or chondrodysplasia punctata. These patients usually survive up to 2 years following birth, although those cases are at risk of demyelination and brain leukodystrophy. The typical symptoms of IRD are

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visible facial abnormalities, retinal degeneration, hearing impairment, hepatomegaly, and mild psychomotor retardation. Many patients with IRD live to adulthood with some living up to 30 years of age. Moreover, the discovery of patients with milder, later onset symptoms has led to a widening of the clinical definition of ZSD [13,39,40,42,55]. The clinical severity and type of ZSD (ZS, NALD, or IRD) are likely to reflect the magnitude of the mutation on the causal gene [21,44].

PBD is attributable to autosomal mutations in one of 13 disease-related *PEX* genes, any of which are essential for either recruiting peroxisomal membrane proteins (*PEX3*, *PEX13*, and *PEX19*) or importing peroxisomal matrix proteins (*PEX1*, *PEX2*, *PEX5*, *PEX6*, *PEX7*, *PEX10*, *PEX12*, *PEX14*, *PEX16*, *PEX26*) during peroxisomal biogenesis [45,48]. In patients with PBD, functional peroxisomes fail to develop, leading to either metabolite accumulation (e.g. VLCFAs, phytanic acid, and pristanic acid) or depletion (ether lipids/plasmalogens and bile acids). However, defects in patients with RCDP type 1 or 5 (defects in *PEX7* or *PEX5 long isoform*, respectively) are limited to depletion in plasmalogens and accumulation of phytanic acid. This is because *PEX7* and *PEX5 long isoform* are only responsible for importing proteins with a peroxisome targeting signal 2 (PTS2) that are required for plasmalogen and phytanic acid processing. Unfortunately, the direct pathological influence of the peroxisomal metabolite(s) that leads to the development of PBD symptoms is largely unknown [48,44].

Mutations in *ATP-binding cassette transporter type D1 (ABCD1)* gene, located on the X-chromosome and cording for ALDP, is responsible for X-ALD [31]. ALDP transfers VLCFAs from the cytoplasm to the peroxisomal matrix in the form of acyl-CoA (VLCF acyl-CoA) leading to their degradation through β-oxidation. Patients with X-ALD fail to efficiently catabolize VLCFAs, resulting in increasing tissue and plasma VLCFA levels [18,26,28]. X-ALD exhibits a wide range of clinical severity. The cerebral form of X-ALD manifests as demyelination and subsequent inflammation of the cerebral white matter resulting in severe brain damage; adrenomyeloneuropathy (AMN), in which motor neurons and the autonomic nervous system are affected by the axonal neuropathy accompanied by demyelination; Addison's disease, in which the symptoms are limited to adrenal insufficiency. In some female carriers, locomotive insufficiency develops with aging, similar to the symptoms of AMN [20]. The age of pathological onset also varies from patient to patient, and the types of symptoms are, so far, unpredictable before the onset [20,30]. The mutated nucleotide or the type of mutation (missense, nonsense, or deletion) in the *ABCD1* gene has no correlation with the types of symptoms experienced or the timing of pathological onset [5,47]. Despite such pathological variety, blood VLCFA levels remain high since birth in male patients with X-ALD presenting with various clinical conditions.

Plasma VLCFAs such as hexacosanoic acid (also known as cerotic acid; C26:0) and tetracosanoic acid (lignoceric acid; C24:0) are reliable markers for diagnosing ZS and X-ALD [28,48]. Gas chromatography-mass spectrometry (GC-MS) has been widely used for measuring VLCFAs, as well as phytanic acid and plasmalogens after methyl-esterification [23,29]. We previously reported a GC-MS method for measuring fatty acids including VLCFAs, phytanic acid, and C16:0-DMA (a plasmalogen derivative formed by the methyl esterification) in a single assay for diagnostic purposes [50]. The GC-MS method detects fatty acids with hydrocarbon chains ranging from C14 to C22, which are normally abundant in human tissue, with high sensitivity, however, the detection of relatively rare VLCFAs with hydrocarbon chains of length C24 or more is less efficient.

Liquid chromatography-mass spectrometry (LC-MS) has been used to complement GC-MS in many applications, including the measurement of fatty acids. Several reports have described the analysis of VLCFAs by LC-MS. For example, in one such study fatty acids were derivatized with 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE) and measured by LC-MS [3]. In another study, fatty acids constituting each lipid species were analyzed by LC-MS/MS [14]. A lysophosphatidyl choline (1-acyl-2-lyso-*sn*-*glycero*-3-phosphorylcholine, lyso-PC) containing VLCFA (26:0-lyso-PC) was measured as a metabolic marker in screenings for newborns with ZS and X-ALD [15,16].

In the current study, we employed LC-MS to measure a repertoire of non-derivatized fatty acids, including VLCFAs and branched fatty acids (phytanic acid and pristanic acid). We examined the fatty acid profiles of serum and fibroblasts taken from patients with peroxisomal diseases. Specifically, we focused on the levels of saturated and unsaturated VLCFAs. Compared with relatively shorter fatty acids such as C20:0 and C22:0, VLCFAs longer than C24:0 can be efficiently detected in this method. Mono- and polyunsaturated fatty acids can also be detected by the same assays. Combined with 2-D map analysis, we were able to identify derivatives of ether lipids, which are the precursors of plasmalogens. Our method provides a standard, single assay method for analyzing a variety of fatty acids, including those affected in patients with peroxisomal diseases. The methods described here can be used for the diagnosis of a wide variety of peroxisomal diseases as well as for future research in the field.

2. Materials and methods

2.1. Reagents

The following chemicals were used in this study: myristic acid (tetradecanoic acid; C14:0; Sigma-Aldrich, St. Louis, MO; #70079), palmitic acid (hexadecanoic acid; C16:0; Sigma-Aldrich, #76119), stearic acid (octadecanoic acid, C18:0, Sigma-Aldrich, #85679), nonadecylic acid (nonadecanoic acid, C19:0, Sigma-Aldrich, #72332), arachidic acid (eicosanoic acid; C20:0; Sigma-Aldrich, #A-3631), behenic acid (docosanoic acid, C22:0, Tokyo Chemical Industry/TCI, Tokyo, Japan, #B1747), lignoceric acid (tetracosanoic acid, C24:0, TCI, #T0076), pentacosylic acid (pentacosanoic acid, C25:0, TCI, #P0882), cerotic acid (hexacosanoic acid, C26:0, TCI, #C0829), montanic acid (octacosanoic acid, C28:0, Sigma-Aldrich, #284432), phytanic acid (Sigma-Aldrich, #P4060), pristanic acid (Santa Cruz Biotechnology, Dallas, TX, #sc-281137), 1-(1Z-octadecenyl)-2-oleoyl-*sn*-*glycero*-3-phosphoethanolamine (C18(Plasm)-18:1 PE; plasmenyl-ethanolamine; Avanti polar lipids, Alabaster, AL, #852758P), high-performance liquid chromatography (HPLC) grade *tert*-butyl methyl ether (*tert*-BME, Sigma-Aldrich, #34875), HPLC grade acetonitrile (Wako, Osaka, Japan, #019-08631), HPLC grade acetone (Wako, # 014-08681), HPLC grade 1 mM ammonium acetate (Wako, #018-21041), and 10% ammonia solution (Wako, #013-17505). Each fatty acid compound was dissolved in two volumes of chloroform and one volume of methanol, containing 0.05% butylated hydroxytoluene (Nacalai Tesque, Kyoto, Japan, #11421-92), and stored at −20 °C until use.

2.2. Lipid purification and fatty acid extraction

Lipid extraction was performed following the methods described in [17,24]. One hundred microliters (μL) of serum or fibroblast pellet collected from a 10-cm dish culture was mixed with 400 μL of acetonitrile and 50 μL of 5 M hydrochloric acid in a 15 mL glass tube with a Teflon-lined plastic cap, and then vigorously mixed using a vortex for 1 min. The sample was subsequently kept at 100 °C in an oil bath for 1 h. After cooling to room temperature (25 °C), 800 μL of *t*-butyl methyl ether (*t*-BME), 100 μL of methanol containing C19:0 (internal standard; 1 μg/mL), and 400 μL of water were added to the sample and vortexed again for 1 min. After allowing to settle for 5 min, the upper phase was collected and 800 μL of water was added. After 5 min centrifugation, the upper organic phase (*t*-BME) containing the fatty acids was collected in a glass sample vial (Waters, Milford, MA, #186000327c), and the organic solvent was evaporated in a stream of nitrogen gas. The dried sample was re-suspended in 100 μL of acetone and analyzed by LC-MS. The recovery rate of fatty acids with this method (examined using a standard mixture) was around 80%. We used C19:0 as an internal

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