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Lipidomic profiling of plasma and urine from patients with Gaucher disease during enzyme replacement therapy by nanoflow liquid chromatography–tandem mass spectrometry

Seul Kee Byeon [a], Ju Yong Lee [a], Jin-Sung Lee [b,**], Myeong Hee Moon [a,*]

[a] Department of Chemistry, Yonsei University, Seoul 120-749, South Korea
[b] Department of Pediatrics, Yonsei University College of Medicine, Seoul 120-752, South Korea

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ABSTRACT

Gaucher disease (GD) is a rare genetic disorder that arises from lipid species, especially monohexosylceramide (MHC), accumulating in different organs. GD results from a β-glucocerebrosidase deficiency, causing metabolic or neurologic complications. This study comprehensively profiled lipids from patients and healthy controls to discover active lipid species related to GD. Most studies have evaluated lipids from one type of biological sample, such as plasma, urine, or spinal fluid, which are the main sources of lipids in human bodies. The purpose of this study, however, was to collect and assess both plasma and urine samples from a group of individuals, explore the lipids, and select characteristic species that show significant differences between controls and patients from the two sources. Also, the response of lipids to enzyme replacement therapy (ERT), which is targeted to reduce excessive lipid accumulation within lysosomes, was investigated by obtaining plasma and urine from patients after receiving the therapy. Most lipid species were found in both plasma and urine but their concentrations differed, and some species were found in either plasma or urine only. Out of 125 plasma and 105 urinary lipids that were identified by nLC–ESI–MS/MS, 20 plasma and 10 urinary lipids were selected as characteristic species for having average concentrations that were significantly increased or decreased in patients by greater than 2-fold. Moreover, the concentrations of most lipids that showed greater than 2-fold of difference in patients decreased after ERT indicating that these species were directly or indirectly affected by the therapy.

1. Introduction

Gaucher disease (GD) is a rare genetic disorder that is inherited recessively and caused by the deficiency or absence of an enzyme called β-glucocerebrosidase. β-Glucocerebrosidase degrades glucosylceramide (GluCer), a type of monohexosylceramide (MHC), inside lysosome [1]. GluCer accumulation in lysosomes eventually gives rise to Gaucher cells in the spleen or liver, causing the affected organs to grow abnormally large. GD is differentiated into three types clinically. Type I GD is the least serious as it causes no visible changes in appearance and no neurological complications. Types II and III involve neurodegenerative problems such as dementia, epilepsy, autism, and Parkinson's disease [2,3]. Type II is the most serious form because it involves severe neurodegenerative problems early in life, and patients tend to die before they reach adolescence. Type III develops during adulthood, so it is not as severe as type II. The incidence of GD is reported to be from approximately 1/111,111 to 1/855 in the Ashkenazi Jewish population, with most of these patients having type I GD. Types II and III are often observed in the Middle East and Asia [4]. GD causes multiple physical and neurological problems that may result in premature death or reduced quality of life. Because GD is of a very specific nature, no complete cure has been developed, but enzyme replacement therapy (ERT), which involves routinely administering the deficient enzyme to a patient, is used to lower levels of the excessive substrate and prevent further organ damage [5]. While GD can be readily detected by a genetic test or protein biomarkers, it is related to altered lipid metabolism, resulting in abnormal

* Corresponding author. Tel.: +82 2 2123 5634; fax: +82 2 364 7050.
** Co-Corresponding author.
E-mail addresses: jinsunglee@yuhs.ac (J.-S. Lee), mhmoon@yonsei.ac.kr (M.H. Moon).

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MHC accumulation, and is also reported to be associated with phosphatidylcholine (PC) and sphingomyelin (SM) increases [6]. Therefore, detailed lipid profiling of patient body fluids can provide a new perspective on how these lipids are related. Moreover, monitoring lipidomic change during drug intervention will be useful.

The roles of lipids range from storing energy to serving a structural role and signal transduction across cell membranes [7,8]. As many metabolic diseases, such as diabetes, obesity, and atherosclerosis, have been reported to be related to lipids [9,10], lipid chemistry has captured increased attention in the medicinal and lipidomics fields. Of the various classes of lipids, phospholipids (PLs) are the main components of biological membranes. PLs vary in terms of the polar head group, length of the acyl chain, and degree of unsaturation. Sphingolipids (SL) such as SM and ceramides (Cer), including MHC and dihexosylceramides (DHC), are the second most abundant lipid class. As lipids are directly or indirectly involved in the development of diseases, studies have been performed to unveil the relationship between lipids and diseases, such as prostate cancer [11,12], breast cancer [13,14], ovarian cancer [15], colorectal cancer [16], and coronary artery disease [17].

Analysis of lipids has rapidly evolved with the development of analytical methods such as gas chromatography (GC), thin-layer chromatography (TLC), and liquid chromatography (LC). The conventional GC and TLC methods require pre- or post-column derivation for detection [18–20]. The development of sophisticated MS has played a critical role in identifying and characterizing lipids from various biological samples over the last few decades. ESI–MS with tandem MS has dramatically affected omics studies analyzing the structure of complicated but biologically active molecules. While ESI–MS/MS provides a rapid analysis of lipids through a direct infusion, LC [21,22] before MS expands the ability to separate complicated lipid mixtures with minimal ionization suppression during MS analysis. In addition, comprehensive structural determination of lipid molecules by data-dependent MS/MS analysis [14,23–25] and the use of a capillary column at nanoflow rate scale for LC-ESI-MS/MS offers increased sensitivity with a ~1 fmol limit of detection from plasma samples [26].

In this study, relative abundance of lipids from plasma and urine samples from patients with GD were profiled both qualitatively and quantitatively with nanoflow LC–ESI–MS/MS. This work focused on distinguishing PLs, especially SLs (in particular MHC, DHC, and SM), from patients with GD that differed significantly from healthy controls. The change in targeted lipid levels in plasma and urine samples both from same GD patients was examined before and after each ERT treatment.

2. Experimental

2.1. Materials and reagents

Fourteen PL standards (16:0-lysophosphatidylcholine (LPC), 18:0-lyso phosphatidylethanolamine (LPE), 18:0/18:0-PC, 18:0/18:0-PE, 18:0-lysophosphatidic acid (LPA), 18:0-lysophosphatidylglycerol (LPG), 18:0-lysophosphatidylserine (LPS), 16:0/16:0-PA, 18:0/18:0-PG, 15:0/15:0-PG, 16:0/18:1-phosphatidylinositol (PI), 16:0/16:0-PS, d18:1/12:0-SM, and $(18:1)_4$-cardiolipin (CL) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), and six Cer standards (d18:1/16:0-GalCer, d18:1/18:0-GluCer, d18:1/16:0-LacCer, d18:1/14:0-Cer, d18:1/22:0-Cer, and d18:1/24:0-THC) were from Matreya, LLC (Pleasant Gap, PA, USA). 15:0/15:0-PG was directly added to urine and plasma samples as an internal standard (IS). All lipid standards were diluted to 30 pmol/μL in CH_3OH:CH_3CN (9:1, v/v). The mixture was stored at 4 °C until use. All organic solvents for lipid extraction including CH_3OH, $CHCl_3$, and methyl-*tert*-butyl ether (MTBE) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All solvents for the LC mobile phase (CH_3CN, isopropanol, and CH_3OH) were of HPLC grade and purchased from Avantor Performance Materials (Center Valley, PA, USA). NH_4HCO_2 and NH_4OH for ionization modifiers were purchased from Sigma–Aldrich.

Plasma and urine samples from 15 healthy controls and 3 patients with GD were collected under informed consent from Severance Hospital (Seoul, Korea) at Yonsei University on the day of the clinical intervention before and 2 h after treatment and this study was approved by Institutional Review Board. All patients with GD in this study were diagnosed with type I disease.

2.2. Evaluation of lipid extraction methods

Because the efficiency of simultaneously extracting different categories of lipids, such as PLs and SLs, varies among methods, 3 methods (Bligh and Dyer [27], Folch with $CHCl_3$ [28], and the modified Folch with MTBE/CH_3OH [29]) were tested using lipid standards added to human urine. Fifteen microliters of 30-pmol/μL lipid standard mixture was added to 985 μL of urine from a healthy control and dried by vacuum centrifugation overnight.

2.2.1. Bligh and Dyer

The dried lipids from the urine sample spiked with standard lipids were dissolved in 300 μL of CH_3OH and 250 μL of $CHCl_3$. After vortexing for an hour, 250 μL of $CHCl_3$ and 450 μL of MS-grade H_2O were added, followed by vortexing for 10 min. The solution was then centrifuged at 1000 × *g* for 10 min, resulting in a distinct separation between the organic and aqueous layers. The lower organic layer was carefully pipetted into a pre-weighed Eppendorf tube, dried under vacuum centrifugation, dissolved in $CHCl_3$:CH_3OH (1:1, v/v), and diluted to 2 pmol/μL in CH_3OH:CH_3CN (9:1, v/v) for nLC–ESI–MS/MS.

2.2.2. Folch with $CHCl_3$

The dried lipid mixture was dissolved in 300 μL of CH_3OH and vortexed briefly before adding 600 μL of $CHCl_3$. After an hour of vortexing, 180 μL of MS-grade H_2O was added to the mixture and vortexed again for 10 min. The rest of the extraction steps were the same as the Bligh and Dyer method.

2.2.3. Modified Folch with MTBE/CH_3OH

The dried lipid mixture was dissolved in 300 μL of CH_3OH and vortexed briefly before adding 1000 μL of MTBE. After an hour of vortexing, 250 μL of MS-grade H_2O was added and vortexed for 10 min to induce phase separation. After centrifuging for 10 min at 1000 × *g*, the upper organic layer was pipetted to a new tube. Three hundred microliters of CH_3OH was added to the lower layer and sonicated for 2 min. After centrifuging at 1000 × *g* for 10 min, the upper organic layer was again collected and combined with the previously collected organic layer. This mixture was dried under vacuum centrifuge overnight. The final dried powder was diluted for nLC-ESI-MS/MS analysis as described in Section 2.2.1.

2.3. Lipid extraction from human plasma and urine samples

Lipids were extracted from 50-μL aliquot of plasma and 2000 μL of urine (15 controls and 3 patients with GD) according to the modified Folch with MTBE/CH_3OH procedure. At the final de-solvation step, weights of dried plasma and urinary lipids of each sample were measured, dissolved, and diluted to 20 μg/μL for plasma and 30 μg/μL for urine by using CH_3OH:CH_3CN (9:1, v/v) as a dispersing solvent. The reason for leveling each individual sample extract to the same concentration rather than dissolving all samples in the same volume of CH_3OH:CH_3CN was to avoid any possibility of low abundant lipid species from not being detected by MS because they