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Analysis of lipoprotein-specific lipids in patients with acute coronary syndrome by asymmetrical flow field-flow fractionation and nanoflow liquid chromatography-tandem mass spectrometry

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ABSTRACT

A comprehensive lipid analysis was performed at the plasma lipoprotein level in patients with acute coronary syndrome (ACS) and stable coronary artery disease (CAD). Because the lipids in lipoproteins are related to the pathology of the cardiovascular system, lipoprotein-specific lipid analysis can be useful for understanding the mechanism of lipid-associated cardiovascular diseases. Lipoproteins were size-sorted into high density lipoproteins (HDL) and low density lipoproteins (LDL) using asymmetrical flow field-flow fractionation, then lipids of each lipoprotein were analysed using nanoflow ultrahigh performance liquid chromatography-electrospray ionization-tandem mass spectrometry. A total of 365 lipids were structurally identified and quantified by selected reaction monitoring method. Two high abundance lysophosphatidylcholines (16:0 and 18:0) were significantly increased only in the HDL of the ACS group (vs. the stable CAD group). Phosphatidylethanolamines (38:5 and 40:5) significantly increased in ACS by > 2-fold in both lipoproteins. (18:0, 22:6)-diacylglycerol increased in ACS by 3.5-fold only in LDL; however, most high abundance triacylglycerols decreased 2-fold in both lipoproteins. The present study revealed the usefulness of lipoprotein-specific analysis of lipids in distinguishing ACS from stable CAD, and the selected lipids analysed in this study may be useful in the development of lipid markers for the early detection of ACS.

1. Introduction

Coronary artery disease (CAD) is the most common form of heart disease and is caused by the accumulation of atherosclerotic plaque in the arterial wall [1]. In addition, acute coronary syndrome (ACS) includes unstable angina, myocardial infarction, and sudden cardiac death which can occur in the course of CAD when atherosclerotic plaques suddenly rupture within coronary arteries, leading to a decreased blood flow. Pathophysiology of ACS was known to be affected by factors including thrombosis-related molecules [2]. However, determinants of ACS have not been completely elucidated and still under investigation.

Lipoproteins are globular complexes containing proteins and lipids in the blood system, and they transport fats and cholesterols to the body. Because lipids play several roles including cell signaling, energy storage, and forming cellular structures, their abnormal metabolism has been reported to be linked with several metabolic diseases such as diabetes, obesity, and atherosclerosis [3,4]. Molecular lipid species with diverse biological functions have been associated with the physiology and pathology of the cardiovascular system. To date, a broad range of lipid classes, including phosphatidylcholine (PC), lysophosphatidylcholine (LPC), ceramide (Cer), and cholesteryl ester, have been linked to cardiovascular disease or recognized as risk factors for cardiovascular disease [5–7]. The levels of a few lipid species change after pharmacologic therapy [8] and these are considered potentially useful for clinical monitoring [9].

Most studies of unbiased high throughput lipid analysis, or lipidomics, have used entire blood samples as sources of metabolites [10]. Therefore, the characteristics or clinical implications of the overall or specific pattern of lipids found in each lipoprotein have not been completely determined. A few studies evaluated the differential characteristics of lipids found in specific lipoproteins according to their classes [11] or individual health conditions [12]. Recently, the profiling of oxidized phospholipids in different lipoproteins from patients plasma

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with CAD was determined using flow field-flow fractionation to sort the lipoproteins by size into high density lipoprotein (HDL) and low density lipoprotein (LDL), and oxidized phospholipids from each lipoprotein fraction of CAD patients were analysed in comparison to healthy controls by nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry (nLC-ESI-MS/MS) [13]. A feasibility study of a dual extraction strategy for the simultaneous analysis of lipids and proteins in lipoproteins used sequential density gradient ultracentrifugation to fractionate HDL and LDL in human and mouse plasma samples [14]. Because the development of CAD is closely associated with LDL oxidation [15,16], a comprehensive analysis of lipid profiles according to the type of lipoproteins can be useful to differentiate the pathogenesis of ACS from stable CAD. However, the lipidomic profiles at the lipoprotein level in those with ACS have not yet been examined.

In this study, a comprehensive lipidomic analysis was performed with different lipoproteins from patients with ACS and compared to those from patients with stable CAD. Plasma lipoproteins were size-sorted into HDL and LDL using semi-preparative scale asymmetrical flow field-flow fractionation (AF4). AF4 is an elution-based separation technique that can sort biological macromolecules, such as proteins, DNA, exosomes, organelles, and cells, by size in an open channel space [17–21] and it provides narrow size fractions of intact sample components that are suitable for further analysis with mass spectrometry or other biological methods [20–23]. Lipids in each lipoprotein fraction were analysed to identify the untargeted lipid molecular structures first and were quantified using high speed nanoflow ultrahigh performance LC-ESI-MS/MS (nUPLC-ESI-MS/MS) with the selected reaction monitoring (SRM) method. Finally, changes in lipoprotein-specific lipid profiles of patients with ACS were compared to those of patients with stable CAD.

2. Materials & methods

2.1. Chemicals and reagents

A total of 32 lipid standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA): 17:0- LPC, 18:1-LPC, 13:0/13:0- PC, 16:0/16:0-PC, 18:0p/18:1-PC, 17:1-lysophosphatidylethanolamine (LPE), 18:0-LPE, 12:0/12:0-phosphatidylethanolamine (PE), 14:0/14:0-PE, 17:0/17:0-PE, 17:1-lysophosphatidylglycerol (LPG), 14:0-LPG, 18:0-LPG, 12:0/12:0-phosphatidylglycerol (PG), 14:0/14:0-PG, 15:0/15:0-PG, 17:1-lysophosphatidylinositol (LPI), 17:0/20:4-phosphatidylinositol (PI), 16:0/18:2-PI, 17:0-lysophosphatidic acid (LPA), 17:0/17:0-phosphatidic acid (PA), d18:1/17:0-sphingomyelin (SM), d18:1/16:0-SM, d18:1/18:0-SM, d18:1/17:0- Cer, d18:1/14:0-Cer, d18:1/17:0-monohexosylceramide (MHC), d18:1/12:0-MHC, d18:1/16:0-dihexosylceramide (DHC), 17:0/17:0-diacylglycerol (DG), 17:0/17:1/17:0-triacylglycerol (TG), and d18:1/24:0-sulfatide (ST). Standard lipids with odd numbered fatty acyl chains were added to lipid extracts as a mixture of internal standards for quantification. Chemicals, including $CHCl_3$, NH_4OH, and NH_4HCO_3, were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade solvents (CH_3CN, CH_3OH, isopropanol, and MS grade water) and methyl-*tert*-butyl ether (MTBE) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Fused silica capillaries (20, 75, 100, and 200 μm inner diameter and 360 μm outer diameter) for plumbing and preparing capillary columns were purchased from Polymicro Technology (Phoenix, AZ, USA).

2.2. Patients and plasma sampling

Plasma samples were collected from patients with CAD after obtaining informed consent according to the permission of the Institutional Review Board of the Severance Hospital (Seoul, Korea). The study was conducted in accordance with the current version of the Declaration of Helsinki. From 30 participants with CAD, 10 with ACS (referred to ACS) (age = 55.2 ± 5.5) and 10 with stable CAD without ACS (age = 57.3 ± 6.9) were selected by excluding patients not taking a statin. Demographic data for the plasma samples are listed in Table 1. All plasma samples were kept at −80 °C.

Table 1
Demographic data for CAD with ACS (ACS) and CAD without ACS (stable CAD).

Factor	CAD without ACS (stable CAD, n = 10)	CAD with ACS (ACS, n = 10)
Age (years)	57.3 ± 6.9	55.2 ± 5.5
Sex, n (%) male	4 (40%)	6 (60%)
TG (mg/dL)	116.3 ± 35.1	107.1 ± 37.9
Total-cholestetol (mg/dL)	160.4 ± 42.9	182.4 ± 40.6
HDL-cholesterol (mg/dL)	39.5 ± 5.97	46.1 ± 10.47
LDL-cholesterol (mg/dL)	93.8 ± 44.0	112.6 ± 37.4
Final efflux (%)	18.74 ± 4.94	18.86 ± 7.10
Hyperlipidemia, n(%)	3 (30%)	3 (30%)
Smoker, n(%)	4 (40%)	1 (10%)
Diabetes, n(%)	2 (20%)	4 (40%)
BMI (kg/m^2)	24.65 ± 2.53	24.94 ± 2.15

2.3. Separation of HDL and LDL

Prior to sorting the lipoproteins from plasma samples by size, albumin and immunoglobulin G (IgG) were depleted using a ProteoPrep® Immunoaffinity Albumin & IgG Depletion Kit from Sigma Aldrich. Semi-preparative scale AF4 was used to separate lipoproteins by size with a polyvinyl chloride channel spacer: 26.6 cm (length) × 250 μm (thickness) with a trapezoidal decrease in channel width from 4.4 to 0.4 cm (Wyatt Technology Europe GmbH, Dernbach, Germany). A regenerated cellulose membrane (MWCO 10 kDa) purchased from Millipore (Danvers, MA, USA) was placed at the accumulation wall. The carrier solution used for AF4 was 0.1 M PBS buffer prepared with deionized (> 18 MΩ) water and filtered using a 0.22 μm nitrocellulose membrane filter from EMD Millipore (Billerica, MA, USA) prior to use. The carrier solution was delivered to the channel using an SP930D HPLC pump from Young-Lin Instruments (Seoul, Korea) via a model 7125 loop injector from Rheodyne (Cotati, CA, USA). For each injection, 250 μL of depleted sample (equivalent to 50 μL of raw plasma) was injected. The flow rates were 3.6 and 0.4 mL/min for the crossflow and outflow rates, respectively. Eluting lipoproteins were monitored with a model UV730D UV detector from Young-Lin at wavelengths of 280 nm for lipoprotein standards and 600 nm for plasma samples stained with Sudan black B (SBB) as shown in Fig. 1. Plasma samples were stained with SBB to determine the collection periods of the HDL and LDL fractions.

2.4. Lipid extraction

Each lipoprotein fraction collected from the AF4 separation was concentrated, then mixed with 300 μL of CH_3OH. The tube containing the mixture was placed in an ice bath for 10 min, then 1000 μL of MTBE was added and the solution was vortexed for 1 h. Thereafter, 250 μL of MS-grade H_2O was added to the tube, then the tube was vortexed for 10 min and centrifuged for 10 min at 1000 × *g*. The upper organic layer was transferred to a separate tube, then 300 μL of CH_3OH was added to the remaining bottom layer. The mixture was sonicated for 2 min, then centrifuged for 10 min at 1000 × *g*. The upper layer was removed and mixed with the previously collected organic layer. The tube containing the final mixture was sealed with a 0.45 μm MilliWrap PTFE membrane from Millipore to avoid lipid evaporation while it was vacuum dried for 12 h. The dried lipids were weighed and reconstituted in CH_3OH:H_2O (9:1, v/v) at a concentration of 5 μg/μL for nLC-ESI-MS/MS analysis.

2.5. Lipid analysis by nUPLC-ESI-MS/MS

The lipid molecular structures from each lipoprotein fraction were identified using a Dionex Ultimate 3000 RSLCnano System with an