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Lipidomic analysis of plasma lipoprotein fractions in myocardial infarction-prone rabbits

Hiroaki Takeda,[1,4] Tomonari Koike,[2,3] Yoshihiro Izumi,[1,4] Takayuki Yamada,[4] Masaru Yoshida,[5,6] Masashi Shiomi,[2,3] Eiichiro Fukusaki,[4] and Takeshi Bamba[1,4,*]

Division of Metabolomics, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan,[1] Institute for Experimental Animals, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan,[2] Division of Comparative Pathophysiology, Department of Physiology and Cell Biology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan,[3] Department of Biotechnology, Graduate School of Engineering, Osaka University, 2–1, Yamadaoka, Suita, Osaka 565-0871, Japan,[4] Division of Gastroenterology, Department of Internal Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan,[5] and Division of Metabolomics Research, Department of Internal Medicine, Kobe University Graduate School of Medicine, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan[6]

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Lipids play important roles in the body and are transported to various tissues via lipoproteins. It is commonly assumed that alteration of lipid levels in lipoproteins leads to dyslipidemia and serious diseases such as coronary artery disease (CAD). However, lipid compositions in each lipoprotein fraction induced by lipoprotein metabolism are poorly understood. Lipidomics, which involves the comprehensive and quantitative analysis of lipids, is expected to provide valuable information regarding the pathogenic mechanism of CAD. Here, we performed a lipidomic analysis of plasma and its lipoprotein fractions in myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits. In total, 172 lipids in plasma obtained from normal and WHHLMI rabbits were quantified with high throughput and accuracy using supercritical fluid chromatography hybrid quadrupole-Orbitrap mass spectrometry (SFC/Q-Orbitrap-MS). Plasma levels of each lipid class (i.e., phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, lysophosphatidylcholine, lysophosphatidylethanolamine, sphingomyelin, ceramide, triacylglycerol, diacylglycerol, and cholesterol ester, except for free fatty acids) in 21-month-old WHHLMI rabbits were significantly higher than those in normal rabbits. High levels of functional lipids, such as alkyl-phosphatidylcholines, phospholipids including ω-6 fatty acids, and plasmalogens, were also observed in WHHLMI rabbit plasma. In addition, high-resolution lipidomic analysis using very low density lipoprotein (VLDL) and low density lipoprotein (LDL) provided information on the specific molecular species of lipids in each lipoprotein fraction. In particular, higher levels of phosphatidylethanolamine plasmalogens were detected in LDL than in VLDL. Our lipidomics approach for plasma lipoprotein fractions will be useful for in-depth studies on the pathogenesis of CAD.

[**Key words:** Lipidomics; Lipoprotein; Supercritical fluid chromatography mass spectrometry; Coronary artery disease; Myocardial infarction-prone Watanabe heritable hyperlipidemic rabbit; Phosphatidylethanolamine plasmalogen]

Lipids, which have a wide variety of structures with a hydrophilic head group and hydrophobic fatty acid (FA) tails, play important roles in energy storage, the cell membrane, and cellular signal transduction (1). Lipids such as triacylglycerol (TAG) are stored in subcutaneous, internal, and intramuscular adipose tissues, and these lipids are transported throughout the body in lipoprotein particles. Lipoproteins consist of a core of hydrophobic lipids, including cholesterol ester (CE) and TAG, covered by a layer of apoproteins and phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM), which allows them to be transported in the aqueous environment of blood (2). Based on their lipid composition and physical structure, these lipoproteins are classified as chylomicron (CM), very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). VLDL and LDL are involved in the same metabolic pathway and transport lipids from the liver to peripheral tissues. In contrast, HDL returns excess lipids to the liver. In this manner, lipids play important roles in the body after being transported to various tissues via lipoproteins.

Coronary artery disease (CAD), including myocardial infarction and cardiac angina, is the major cause of death in developed countries. The basic pathogenesis of CAD involves abnormal lipid metabolism and a maladaptive immune response associated with chronic inflammation of the arterial wall (3). CAD has very specific symptoms (e.g., angina) in the advanced stages but does not have any symptoms in the early stages. Although low levels of HDL-cholesterol (HDL-Cho) and high levels of LDL-cholesterol (LDL-Cho) are considered risk factors for CAD, reliable diagnostic markers for measuring the severity of CAD have not been found. Additionally, the regulatory mechanism of lipid metabolism in CAD remains unclear.

Recently, lipidomics, which is the mass spectrometry-based comprehensive study of lipid molecular species, has been developed. Lipidomic analysis has attracted attention not only for

* Corresponding author. Tel.: +81 92 642 6170; fax: +81 92 642 6172.
E-mail address: bamba@bioreg.kyushu-u.ac.jp (T. Bamba).

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elucidating the cellular processes involved in health and diseases such as cancer and atherosclerosis but also for enabling personalized medicine (4). In our previous studies, we developed a lipidomic analytical system using supercritical fluid chromatography mass spectrometry (SFC/MS) (5–7). We can achieve high-speed analysis while maintaining the resolution of chromatography, thus enabling profiling of hundreds of different lipid molecular species including glycerolipids (e.g., monoacylglycerol, MAG; diacylglycerol, DAG; and TAG), glycerophospholipids (e.g., lysophosphatidylcholine, LPC; lysophosphatidylethanolamine, LPE; phosphatidylinositol, PI; PC; and PE), sphingolipids (e.g., ceramide, Cer and SM), and sterol lipids (e.g., CE) in mouse plasma within only 20 min (7). Although a plasma or serum lipidomics approach will be useful for biomarker discovery for CAD, the details of the lipid metabolism including the role of each lipoprotein are poorly understood. Because plasma contains various lipoproteins (CM, VLDL, LDL, and HDL), lipid profiling of plasma samples currently provides only general information. Therefore, the analysis of lipid class composition and species pattern of each lipoprotein (i.e., high-resolution lipidomic analysis) is expected to shed light on the pathogenic mechanism of CAD. Recently, only a few groups reported studies using lipidomic techniques for human lipoproteins of hyperlipidemic patients (8) or CAD patients (9), as alteration of lipid levels in lipoproteins leads to cardiovascular disease. However, because the only target lipids were phospholipids, the available lipidomic information for lipoproteins is limited.

Pathological animal models are important for preclinical analyses that elucidate pathological features, toxicity, and drug effects in detail. Myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits, which are developed by selective breeding of Watanabe heritable hyperlipidemic (WHHL) rabbits, are an animal model of familial hypercholesterolemia in humans (10). In previous studies, WHHLMI rabbits were used to investigate the progression of coronary atherosclerotic lesions (11) to observe the process of LDL oxidation with a radioiodinated short peptide probe developed (12), and to investigate the relationship between mesenteric fat accumulation and insulin resistance, hyperlipidemia and atherosclerosis (13). These studies revealed that WHHLMI rabbits are potentially useful for the study of myocardial infarction. However, to date, the levels of various lipid molecular species associated with myocardial infarction in WHHLMI rabbits remains unclear. Therefore, it is expected that high-resolution lipidomic analyses using lipoprotein fractions will be able to elucidate the lipid metabolism in myocardial infarction. In the present study, we therefore aimed to demonstrate the utility of high-resolution lipidomic analysis using plasma lipoprotein fractions in WHHLMI rabbits.

MATERIALS AND METHODS

Chemicals and reagents JIS special grade sodium chloride and sodium bromide, sodium hydroxide solution (1 mol/L), and ethylenediaminetetraacetic acid disodium salt dihydrate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ammonium acetate was obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). MS grade methanol was purchased from Kanto Chemical Co., Ltd (Tokyo, Japan), HPLC grade chloroform was obtained from Kishida Chemical (Osaka, Japan), and HPLC grade distilled water was purchased from Wako Pure Chemical Industries. An authentic sample of diacyl 12:0/12:0 phosphatidylcholine (PC 12:0/12:0) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Carbon dioxide (99.99% grade, Nerikigasu, Osaka, Japan) was used as the SFC mobile phase.

Animals All animal procedures were approved by the Kobe University Animal Care Committee. Five WHHLMI rabbits (21 months old) and three Japanese white (JW) rabbits (23 months old, Kitayama Labes, Co. Ltd., Ina, Japan) were used. The rabbits were housed individually in metal cages in a room with constant temperature (22 ± 2°C) and a consistent lighting cycle (12 h light/dark), and they were fed standard rabbit chow (120 g per day; LRC4, Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum (11).

Sample preparation Plasma lipoproteins were fractionated by ultracentrifugation with a stepwise method (VLDL, $d < 1.006$ g mL^{-1}; LDL, 1.006 g mL^{-1} $< d <$ 1.063 g mL^{-1}; and HDL, $d >$ 1.063 g mL^{-1}) (14,15).

Lipid extraction from rabbit plasma, LDL, and VLDL was performed using Bligh and Dyer's method (16) with minor modifications. In short, 40 μL of plasma or each lipoprotein fraction was mixed with 150 μL of a solvent mixture (methanol:chloroform = 2:1, v/v) containing PC 12:0/12:0 dissolved in methanol as an internal standard (20 ng mL^{-1} for plasma in JW rabbits, 200 ng mL^{-1} for plasma in WHHLMI rabbits, 20 ng mL^{-1} for VLDL in WHHLMI rabbits, and 200 ng mL^{-1} for LDL in WHHLMI rabbits), and then the solution was vortexed at the maximum setting for 30 s. After 50 μL of chloroform and 50 μL of distilled water were added, the solution was vortexed again. Phase separation of aqueous and organic layers was performed by centrifugation at 800 ×g for 10 min at 4°C. Aliquots (100 μL) of the organic layer were transferred to clean tubes. The plasma lipid extract (100 μL) for JW rabbits and WHHLMI rabbits was diluted to a final volume of 200 μL and 2000 μL, respectively, with a solution of 2:1 (v/v) methanol:chloroform for SFC/MS analysis. VLDL and LDL lipid extracts (100 μL) from WHHLMI rabbits were diluted to final volumes of 200 μL and 2000 μL, respectively, as described above.

Biochemical analysis for total cholesterol and total TAG Total cholesterol (Cho) and TAG levels were measured using a Cholesterol E-Test Assay Kit and Triglyceride E-Test Assay Kit (Wako Pure Chemical Industries, Ltd.) following the manufacturer's instructions.

SFC/Q-Orbitrap-MS conditions Lipid molecular species were separated using an Analytical SFC Method Station (Waters, Milford, MA, USA) controlled by SuperChrom software (Waters). The SFC conditions were as follows: column, Inertsil ODS-4 column (4.6 × 250 mm, particle size of 5 μm, GL Sciences, Tokyo, Japan); mobile phase, carbon dioxide (A) and 0.1% (w/v) ammonium acetate in methanol as modifier (B); flow rate, 3.0 mL min^{-1}; gradient curve, 10% B at 0 min, 30% B at 15 min, 10% B at 20 min; injection volume, 5 μL; column temperature, 35°C; and back pressure, 10 MPa.

Individual lipid molecular species were identified using hybrid quadrupole-Orbitrap mass spectrometer, Q Exactive MS fitted with an electrospray ionization (ESI) ion source (Thermo Scientific, Waltham, MA, USA) and controlled by Xcalibur software version 4.1 (Thermo Scientific). The Q Exactive MS parameters for high-mass-accuracy MS^1 and data-dependent MS^2 (dd-MS^2) analysis were described previously (7) and used here with minor modifications. The modified ionization conditions were as follows: capillary temperature, 380°C; and heater temperature, 350°C. For lipidomic analysis of plasma and lipoproteins, full MS/dd-MS^2 (top 3) was used. The experimental conditions for the data-dependent product ion scanning were as follows. The isolation width, normalized collision energy (NCE), and stepped NCE were set to m/z 2.0, 25 eV, and 20% in the positive ion mode, and m/z 2.0, 30 eV, and 20 % in the negative ion mode, respectively.

Data analysis Identification of lipid molecular species was performed using Lipid Search software (Mitsui Knowledge Industry, Tokyo, Japan) (17), and the product search mode, which identifies using accurate mass and MS^2 spectrum pattern, was used. The precursor-ion and product-ion tolerances were set to 5 and 10 ppm mass windows, respectively. The absolute intensity threshold for precursor ions and the relative intensity threshold for product ions were set to 5000 and 10%, respectively.

The chromatographic peak area for each analyte (precursor ion) was calculated using Xcalibur software ver. 2.2. (Thermo Scientific) based on the results obtained from Lipid Search. The quantitative values were calculated using the ratio of the chromatographic peak area of each analyte to that of the internal standard, PC 12:0/12:0. Furthermore, lipid data obtained from lipoprotein fractions were normalized to total cholesterol levels in each lipoprotein sample.

RESULTS

Characteristics of JW rabbits and WHHLMI rabbits In the present study, we performed a lipidomic analysis of plasma and its lipoprotein fractions (LDL and VLDL) in WHHLMI rabbits at the age of 21 months. Table 1 shows the age, sex, weight, total plasma Cho level, and total plasma TAG level in JW rabbits and WHHLMI rabbits. No significant difference in weight was observed between the two rabbit strains, whereas total plasma Cho and total plasma TAG

TABLE 1. Characteristics of JW rabbits and WHHLMI rabbits.

	JW rabbit ($n = 3$)	WHHLMI rabbit ($n = 5$)
Age (month)	23 ± 0	21 ± 0
Sex (% male)	100	40
Weight (kg)	3.5 ± 0.1	3.4 ± 0.4
Plasma total Cho (mg dL^{-1})	13 ± 2	914 ± 220***
Plasma total TAG (mg dL^{-1})	59 ± 38	305 ± 171

Values are mean ± standard deviation. Statistical significance was determined using Student's t-test ($^{***}p < 0.001$).