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Top-down and bottom-up lipidomic analysis of rabbit lipoproteins under different metabolic conditions using flow field-flow fractionation, nanoflow liquid chromatography and mass spectrometry

Seul Kee Byeon[a], Jin Yong Kim[a], Ju Yong Lee[a], Bong Chul Chung[b], Hong Seog Seo[c], Myeong Hee Moon[a,*]

[a] Department of Chemistry, Yonsei University, Seoul 120-749, Republic of Korea
[b] Future Convergence Research Division, Korea Institute of Science and Technology, Seoul 136-791, Republic of Korea
[c] Cardiovascular Center, Korea University Guro Hospital, Seoul 152-703, Republic of Korea

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ABSTRACT

This study demonstrated the performances of top-down and bottom-up approaches in lipidomic analysis of lipoproteins from rabbits raised under different metabolic conditions: healthy controls, carrageenan-induced inflammation, dehydration, high cholesterol (HC) diet, and highest cholesterol diet with inflammation (HCI). In the bottom-up approach, the high density lipoproteins (HDL) and the low density lipoproteins (LDL) were size-sorted and collected on a semi-preparative scale using a multiplexed hollow fiber flow field-flow fractionation (MxHF5), followed by nanoflow liquid chromatography-ESI-MS/MS (nLC-ESI-MS/MS) analysis of the lipids extracted from each lipoprotein fraction. In the top-down method, size-fractionated lipoproteins were directly infused to MS for quantitative analysis of targeted lipids using chip-type asymmetrical flow field-flow fractionation-electrospray ionization-tandem mass spectrometry (cAF4-ESI-MS/MS) in selected reaction monitoring (SRM) mode. The comprehensive bottom-up analysis yielded 122 and 104 lipids from HDL and LDL, respectively. Rabbits within the HC and HCI groups had lipid patterns that contrasted most substantially from those of controls, suggesting that HC diet significantly alters the lipid composition of lipoproteins. Among the identified lipids, 20 lipid species that exhibited large differences (>10-fold) were selected as targets for the top-down quantitative analysis in order to compare the results with those from the bottom-up method. Statistical comparison of the results from the two methods revealed that the results were not significantly different for most of the selected species, except for those species with only small differences in concentration between groups. The current study demonstrated that top-down lipid analysis using cAF4-ESI-MS/MS is a powerful high-speed analytical platform for targeted lipidomic analysis that does not require the extraction of lipids from blood samples.

1. Introduction

Lipoproteins are globular complexes present in blood that are composed of lipids with few proteins and are classified into density-dependent subclasses, including high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) [1]. The major roles of lipoproteins are transportation of lipids and cholesterols in the bloodstream and their functions within the human body are related to cellular structure, intercellular signaling, cell proliferation, and apoptosis [2,3]. Since altered metabolism of lipids can often trigger the pathogenesis of metabolic disease [4,5], methods for lipid analysis have become of increasing interest in clinical fields for diagnostic and prognostic applications. Among the various risk factors for metabolic diseases, consumption of an unhealthy diet is a major factor [6]. The various types of unhealthy diet and their influences are discussed below. High cholesterol diets often cause metabolic diseases that lead to obesity and diabetes, and can even lead to the development of cancer [7,8]. Accumulation of carrageenan, a common additive utilized in the food industry to improve taste and keep ingredients from separating, can eventually cause inflammation in the human body, which is a known contributor to serious metabolic diseases

* Corresponding author. Tel.: +82 2 2123 5634; fax: +82 2 364 7050.
E-mail address: mhmoon@yonsei.ac.kr (M.H. Moon).

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such as heart diseases [9]. Dehydration, caused by insufficient intake of water, promotes low blood pressure and decreases the overall amount of blood in the human body. In extreme cases, it can affect the brain and cause minor or major headaches, along with diminished memory and impaired nerve function [10]. Since decreased levels of HDL and increased levels of LDL, along with a size reduction in LDL, are common signs of an increased risk for various types of metabolic disease, it is important to study the relative levels of lipoproteins. Therefore, a separate lipid profiling of HDL and LDL is necessary to discover the specific lipid species involved in the development of metabolic disorders.

Analysis of lipids from lipoproteins has been performed in using a bottom-up method, which requires the isolation of different lipoproteins, extraction of lipids from the HDL/LDL fractions, and analysis of the lipids with sophisticated mass spectrometry (MS) methods in conjunction with liquid chromatography (LC). While HDL and LDL can be separated by size exclusion chromatography (SEC), gel electrophoresis, and ultracentrifugation [11–14], flow field-flow fractionation (FlFFF) methods provide robust and high speed size separation of HDL/LDL in an empty channel without the concern of sample interaction with the packed bed or gels [15–18]. Recently, multiplexed hollow fiber FlFFF (MxHF5) [19] was successfully utilized for size-sorting of HDL and LDL particles from plasma samples on a semi-preparative scale (~50 μL plasma per injection) and quantitative analysis using nanoflow LC-ESI-MS/MS of the extracted phospholipids (PLs) in different lipoproteins was performed using samples from patients with coronary artery disease (CAD) [20]. Lipid analysis can also be made using a top-down approach in which plasma or serum lipoproteins can be injected directly into electrospray ionization-tandem MS (ESI-MS^n) during the separation of lipoproteins. Development of a chip-type asymmetrical FlFFF (or AF4) channel offered an on-line hyphenation with MS (cAF4-ESI-MS/MS) to carry out a direct analysis of the lipids in different lipoproteins [21,22]. Use of miniaturized FlFFF channel prior to ESI-MS offers great advantages such as the on-line desalting of plasma samples, which can enhance the ionization of lipid species, and a high-speed lipid screening capability (~200 min per sample) with the bypass of the time consuming analytical steps (~36 h): isolation of the HDL/LDL fraction and extraction of the lipids from each lipoprotein fraction prior to LC–MS/MS analysis. Moreover, lipid extraction from each lipoprotein fraction, an essential step for chromatographic separation, is not necessary in the top-down analysis method. While sophisticated LC–MS/MS analysis of lipids is a beneficial platform that provides a comprehensive analysis of lipids, including isomeric forms of lipids, with the minimization of ion suppression from highly abundant species, the pre-chromatographic steps limit the throughput, and this often becomes a problem when the sample set is large. However, top-down lipidomic analysis by cAF4-ESI-MS/MS offers great potential for high speed screening of targeted lipids with increased throughput.

In this study, comprehensive lipid profiling was carried out for the HDL and LDL fractions of serum samples from New Zealand white rabbits raised under different metabolic conditions: normal diet (control), inflammation induced by injecting carrageenan, dehydrated diet, high cholesterol (HC) diet, and high cholesterol diet with inflammation (HCI). Changes in the lipid distribution in rabbit sera under these different metabolic conditions were characterized by both the bottom-up and top-down analysis methods: an off-line combination of MxHF5 with nLC-ESI-MS/MS and a direct analysis using cAF4-ESI-MS/MS, respectively, as illustrated in Fig. 1. Initially, qualitative and quantitative lipid profiling was carried out using pooled serum samples from each of the different diet groups of rabbits using an off-line combination of MxHF and nLC-ESI-MS/MS. Then, lipid species showing greater than 10-fold differences among the groups were selected and these targeted

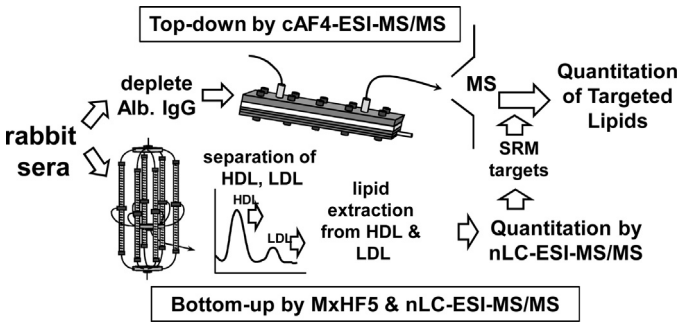

Fig. 1. Schematic of the top-down and bottom-up approaches for lipoprotein specific lipid analyses from rabbit serum samples using an on-line cAF4-ESI-MS/MS and an off-line combination of MxHF5 with nLC-ESI-MS/MS.

species were quantified by cAF4-ESI-MS/MS from individual samples using the selected reaction monitoring (SRM) method in order to confirm the differences and to validate the top-down approach as an alternative high speed screening platform for the analysis of lipids from lipoproteins.

2. Experimental

2.1. Materials and reagents

Bovine serum albumin (BSA), apoferritin, thyroglobulin, carbonic anhydrase (CA), NH_4HCO_2 and NH_4OH as ionization modifiers for MS, and methyl-*tert*-butyl ether (MTBE) for lipid extraction were purchased from Sigma–Aldrich (St. Louis, MO, USA). Serum samples from New Zealand white rabbits raised under five different metabolic conditions were obtained from Korea University Guro Hospital (Seoul, Korea). The first animal group consisted of four 12-week-old healthy rabbits, the second group consisted of five rabbits with inflammation induced by hypodermic injection with 3 mL of 1% carrageenan solution for 3 weeks, the third group of three rabbits was given 1/3 of the water supply given to the other groups to induce a mild dehydration effect, the fourth group of five rabbits was maintained on a high cholesterol diet, and the last group consisted of six rabbits with inflammation induced by carrageenan and maintained on a high cholesterol diet. Serum samples from a healthy human control and a patient with coronary artery disease (CAD) were obtained under informed consent from Severance hospital (Seoul, Korea) to allow for a comparison of the concentrations and sizes of lipoproteins with those of rabbits. The ProteoPrep® Immunoaffinity Albumin & IgG Depletion Kit from Sigma–Aldrich was used to deplete albumin and immunoglobulin G (IgG) from rabbit serum, as these can interfere with lipoproteins or lipids in UV detection and on-line MS analysis. HPLC grade H_2O, $CHCl_3$, CH_3CN, C_3H_7OH, and CH_3OH for LC were purchased from Avantor Performance Materials (Center Valley, PA, USA). Nineteen lipid standards (16:0-lysophosphatidylcholine (LPC), 18:0-lyso phosphatidylethanolamine (LPE), 13:0/13:0-phosphatidylcholine (PC) as an internal standard (IS), 18:0/18:0-PC, 18:0/18:0-phosphatidylethanolamine (PE), 18:0-lysophosphatidic acid (LPA), 18:0-lysophosphatidylglycerol (LPG), 18:0-lysophosphatidylserine (LPS), 16:0/16:0-phosphatidic acid (PA), 18:0/18:0-phosphatidylglycerol (PG), 15:0/15:0-PG, 16:0/18:1-phosphatidylinositol (PI), 16:0/16:0-phosphatidylserine (PS), $(18:1)_4$-cardiolipin (CL), d18:1/12:0-sphingomyelin (SM), d18:1/16:0-monohexosylceramide (MHC), d18:1/16:0-dihexosylceramide (DHC), d18:1/14:0-ceramide (Cer), d18:1/22:0-ceramide (Cer), and d18:1/24:0-trihexosylceramide (THC)) used to establish the run conditions of nLC-ESI-MS/MS were purchased from Avanti