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Lipidomic alterations in lipoproteins of patients with mild cognitive impairment and Alzheimer's disease by asymmetrical flow field-flow fractionation and nanoflow ultrahigh performance liquid chromatography-tandem mass spectrometry

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ABSTRACT

Alzheimer's disease (AD) is an irreversible neurodegenerative disorder with the clinical symptom of the progressive loss of cognitive function and mild cognitive impairment (MCI) is a translational state between cognitive changes of normal aging and AD. Lipid metabolism and pathogenesis of Alzheimer's disease (AD) are closely linked. Despite obviously discrete lipidome constitutions across lipoproteins, lipidomic approaches of AD has been mostly conducted without considering lipoprotein-dependent alterations. This study introduces a combination of asymmetrical flow field-flow fractionation (AF4) and nanoflow ultrahigh performance liquid chromatography-tandem mass spectrometry (nUHPLC-ESI-MS/MS) for a comprehensive lipid profiling in different lipoprotein level of patients plasma with AD and amnestic MCI in comparison to age-matched healthy controls. Lipoproteins in plasma samples were size-sorted by a semi-preparative scale AF4, followed by non-targeted lipid identification and high speed targeted quantitation with nUHPLC-ESI-MS/MS. It shows 14 significantly altered high abundance lipids in AD, exhibiting >2-fold increases ($p < 0.01$) in LDL/VLDL including triacylglycerol, ceramide, phosphatidylethanolamine, and diacylglycerol. Three lipid species (triacylglycerol 50:1, diacylglycerol 18:1_18:1, and phosphatidylethanolamine 36:2) showing a strong correlation with the degree of brain atrophy were found as candidate species which can be utilized to differentiate the early stage of MCI when simple Mini-Mental State Examination results were statistically incorporated. The present study elucidated lipoprotein-dependent alterations of lipids in progression of MCI and further to AD which can be utilized for the future development of lipid biomarkers to enhance the predictability of disease progress.

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1. Introduction

Alzheimer's disease (AD) is an irreversible neurodegenerative disorder that is the most common cause of dementia in adults older than 65 years of age; notably, it presents a challenging task for health care in the developed countries, since it will dramatically increase in the future [1–3]. The clinical symptoms of AD comprise a progressive loss of cognitive function, typically memory; AD is pathologically distinguished by an extensive loss of synapses and neurons, as well as by the presence of neuritic plaques enriched with amyloid β (Aβ) peptides in brain [4] and of neurofibrillary tangles composed of hyperphosphorylated tau proteins [5]. Mild cognitive impairment (MCI) refers to a transitional state between the cognitive changes of normal aging and AD. Because 80% of MCI has been reported to progress to AD over a period of 6 years [6], and changes in the brain caused by AD pathogenesis may occur earlier than the onset of MCI symptoms [7], the identification of the earliest signs of disease progression is important in establishing preven-

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tive treatments for potential AD patients [8,9]. Therefore, reliable and convenient blood biomarkers, which clinicians could use to predict the potential of AD development, are crucial to enable the commencement of preventive intervention at the earliest stages.

Although several AD biomarkers, such as Aβ peptides and tau protein have been developed using cerebrospinal fluid (CSF) [10–13], lumbar puncture to obtain CSF is rather invasive. Few plasma proteins, based on proteomic approaches have been reported to predict the progression of MCI to AD [9,14,15]; however, most of them require further clinical validation. Alterations in lipid levels are known to modulate the generation of Aβ [3,16,17]. Studies show decreased levels of phosphatidylethanolamine plasmalogen and sulfatide, but increases in ceramide and diacylglycerol, in the AD-brain or CSF [3,18–20], as well as few putative lipid signatures from AD-plasma [21]. Also reported are higher levels of low-density lipoprotein (LDL) cholesterol, which is closely linked with AD [22]. While the roles and consequences of lipids in AD have been described, detailed profiles of blood lipids with AD, especially those of different lipoproteins, have not yet been thoroughly investigated.

Lipid analysis at the molecular level is often complicated, due to the diversity in lipid molecular structures. Rapid growth in mass spectrometry (MS) has facilitated lipidomic analysis. Direct analysis of lipids using electrospray ionization-tandem MS (ESI-MS/MS) provides the high throughput analysis and the accurate determination of lipid molecular structures [23,24], however, it can not avoid the ion suppression effect caused by high abundance lipid species and the difficulty in identifying isobaric lipids. Lipid analysis has been empowered by hyphenating chromatographic methods with ESI-MS/MS such as reversed phase liquid chromatography (RPLC) in most cases, hydrophilic-interaction liquid chromatography (HILIC), supercritical fluid chromatography (SFC), two-dimensional LC, and etc. [25–31]. Lately, we have demonstrated that nanoflow ultrahigh performance liquid chromatography-electrospray ionization-tandem mass spectrometry (nUHPLC-ESI-MS/MS) can facilitate the identification of lipid structures at low fmol levels with a high speed quantitation: lipid profiles in the plasma of patients with Gaucher diseases upon enzyme replacement therapy, in the muscle tissues of diabetic rats upon physical exercise, and in urinary exosomes from patients with prostate cancer [32–34].

In this study, a comprehensive targeted profiling of lipids has been performed with human plasma lipoproteins of patients who were diagnosed with AD and MCI in comparison to age-matched healthy controls. Lipoproteins were size-sorted into high density lipoprotein (HDL) and LDL including very low-density lipoprotein (VLDL) using semi-preparative scale asymmetrical flow field-flow fractionation (AF4), an elution-based size-separation technique [35]. Then, lipids in HDL and LDL/VLDL were analyzed for non-targeted identification of molecular structures, followed by targeted quantitation using nUHPLC-ESI-MS/MS based on selective reaction monitoring method and statistical evaluation of the dependence of lipids with brain damage. This study aimed to elucidate the lipoprotein-dependent lipids in the progression of MCI and AD, which can be utilized in the future as candidate molecules to enhance the predictability of disease by engaging with the mini-mental state examination (MMSE) results.

2. Experimental

2.1. Materials & reagents

A total of 25 lipid standards were used to optimize nUHPLC-ESI-MS/MS run conditions, together with 14 internal standards having odd-numbered acyl chains. These were lysophosphatidylcholine (LPC) 12:0, phosphatidylcholine (PC) 13:0/13:0, PC 16:0/14:0, PC 16:0/16:0, PC 18:1/18:0, PC 20:0/20:0, lysophophatidylethanolamine (LPE) 14:0, phophatidylethanolamine (PE) 12:0/12:0, phophatidylethanolamine plasmalogen (PEp) 18:0p/18:1, lysophosphatidic acid (LPA) 14:0, phosphatidic acid (PA) 12:0/12:0, lysophosphatidylglycerol (LPG) 14:0, phosphatidylglycerol (PG) 15:0/15:0, PG 16:0/16:0, lysophosphatidylinositol (LPI) 18:0, phosphatidylinositol (PI) 16:0/18:2, lysophosphatidylserine (LPS) 18:1, phosphatidylserine (LPS) 18:0/18:1, sulfatide (SulfoHexCer) d18:1/12:0, ceramide (Cer) d18:1/14:0, monohexosylceramide (HexCer) d18:1/16:0, dihexosylceramide (Hex2Cer) d18:1/16:0, cardiolipin (CL) $(14:0)_4$, diacylglycerol (DG) 16:0/18:1, and triacylglycerol (TG) 16:0/16:0/14:1. Lipids used for internal standards at selected reaction monitoring (SRM) were LPC 17:0, LPE 17:1, PE 17:0/17:0, LPA 17:0, PA 17:0/17:0, LPG 17:1, PI 17:0/20:4, SulfoHexCer d18:1/17:0, SM d18:1/17:0, Cer d18:1/17:0, HexCer d18:1/17:0, CL $(14:1)_3(15:1)$, DG 17:0/17:0-D_5, and TG 17:0/17:1/17:0-D_5. All lipid standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Standard materials of bovine serum albumin (BSA), lipoprotein standard high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were purchased from Sigma-Aldrich Co. (St. Louis, MO), and very low-density lipoprotein (VLDL) was from Merck Millipore (Darmstadt, Germany). Methyl-*tert*-butyl ether (MTBE), $CHCl_3$, NH_4HCO_2, and NH_4OH were purchased from Sigma-Aldrich. HPLC grade H_2O, CH_3CN, $(CH_3)_2CHOH$, and CH_3OH were purchased from J.T. Baker, Inc. (Phillipsburg, NJ, USA). Capillary tubes (inner diameter: 25 and 100 μm, outer diameter: 360 μm) were purchased from Polymicro Technologies, LLC (Phoenix, AZ).

2.2. Patient's plasma sample

From a total of 172 participants recruited in a memory clinic of University-affiliated general hospital (Seoul, Korea) with informed consent according to the permission of the Institutional Review Boards, 13 individuals (age = 71.5 ± 3.6) with normal cognition, 23 (age = 72.5 ± 3.1) with MCI, and 14 (age = 72.6 ± 3.1) with AD were finally selected. Inclusion criteria were female (65–80 years) with Clinical Dementia Rating (CDR) of 0–1.0. Exclusion criteria were 1) history of major psychiatric or neurologic illness (stroke, head trauma, epilepsy), 2) significant medical condition that may affect mental functioning, 3) no current diagnosis of diabetes, and 4) CDR ≥ 2.0. In addition, patients with hyperlipidemia or a high level of BMI were not included in order to reduce the influences of obesity or hyperlipidemia on the lipid profiles. The study was conducted in accordance with the current version of the Declaration of Helsinki. Demographic data for selected plasma samples are listed in Table S1 of Supplementary material.

Overnight fasting blood samples were collected in the morning (around 10 a.m.) by venipuncture at antecubital vein. Plasma was immediately prepared by centrifuge (2000 × *g*) for 15 min from whole blood collected in a heparin tube after gently mixing. Plasma was allocated into several Eppendorf tubes by 0.7 cc each and immediately stored in −80 °C. Blood sample for genotyping was collected into an EDTA tube, allocated into Eppendorf tubes and stored in −80 °C. APOE ε4 genotyping was performed from whole blood as described previously [36]. Rest of blood sample was immediately sent to clinical laboratory for routine blood tests including complete blood cell count, BUN/creatinine, albumin, liver function tests, fasting serum glucose, glycated hemoglobin (HbA1c), serum lipids, homocysteine, thyroid function test etc.

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