Global changes of phospholipids identified by MALDI imaging mass spectrometry in a mouse model of Alzheimer’s disease

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Running Title: Imaging Mass Spectrometry (IMS) of Lipids in a Mouse Model of Alzheimer’s Disease

Abbreviations
Abstract

Alzheimer’s disease (AD) is the most common form of dementia; however, at the present time there is no disease-modifying drug for AD. There is increasing evidence supporting the role of lipid changes in the process of normal cognitive aging and in the etiology of age-related neurodegenerative diseases. AD is characterized by the presence of intraneuronal protein clusters and extracellular aggregates of β-amyloid (Aβ). Disrupted Aβ kinetics may activate intracellular signaling pathways including tau hyperphosphorylation and proinflammatory pathways. We analyzed and visualized the lipid profiles of mouse brains using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Direct tissue analysis by MALDI-TOF imaging mass spectrometry (IMS) can determine the relative abundance and spatial distribution of specific lipids in different tissues. We used 5XFAD mice that almost exclusively generate and rapidly accumulate massive cerebral levels of Aβ-42 (1). Our data showed changes in lipid distribution in the mouse frontal cortex, hippocampus, and subiculum, where Aβ plaques are first generated in AD. Our results suggest that MALDI-IMS is a powerful tool for analyzing the distribution of various phospholipids and that this application might provide novel insight into the prediction of disease.
Introduction

Alzheimer’s disease (AD), the most common form of dementia, is primarily caused by abnormal protein kinesis and the accumulation of aggregated proteins in the brain. Several hypotheses regarding the cause of AD have been proposed, among which the amyloid hypothesis is the most widely accepted (2). According to this hypothesis, AD is caused by abnormal accumulation of misfolded β-amyloid (Aβ), a sequential cleavage product produced from amyloid precursor protein (APP) by β- and γ-secretases, and hyperphosphorylated tau protein. Several lines of evidence support the idea that Aβ can trigger the hyperphosphorylation of tau, resulting in neuronal degeneration in the brain (3, 4).

There is evidence that free fatty acids (FFA) are associated with several signaling processes related to the pathogenesis of AD. In particular, palmitic and stearic acid induce AD by triggering hyperphosphorylation of tau protein (5). Membrane phospholipids are a prominent source of FFAs. Under disease conditions, phospholipase enzymes are produced in excess, inducing the hydrolysis of phospholipids and the release of FFAs. It is reasonable to hypothesize that alterations in phospholipid concentrations are closely related to the pathogenesis of AD as phospholipids perform a number of important cellular roles including stabilization of membrane ion channels, neurotransmission, and the localization of β-amyloid plaques to phospholipid cores (6, 7).

As a result of progress in mass spectrometry, and MALDI imaging mass spectrometry (IMS) in particular, the analysis of phospholipid changes during disease progression has become feasible. MALDI-IMS is a unique tool that integrates molecular and histological information together with information attained using traditional optical microscopy. This technology allows us to directly determine the localization, structure, and relative quantity of molecules in a single experimental tissue (8). Since its introduction by Caprioli and colleagues, IMS has proven to be a powerful tool for analyzing and visualizing different classes of biological molecules, including metabolites, peptides, and proteins, in tissue sections.

Lipids, particularly phospholipids (PL), have important cellular functions due to their prominent roles in
energy storage, cell membrane formation, and cell signaling pathways. As phospholipids constitute the majority of the biological molecules in cells and whole tissues that are analyzed in MALDI-MS experiments (9-11), this is a useful method to monitor PL changes in certain biological states. After adipose tissue, brain tissue contains the second highest concentration of PLs (20–25%) (12-14). MALDI IMS has emerged as the prime choice for PL analysis because it provides advantages over other ionization methods such as electrospray ionization (ESI) mass spectrometry, including simple sample preparation and the inclusion of spatial information (15, 16). During the MALDI procedure, PLs are positively or negatively charged according to their head group polarity. Therefore, it is beneficial to use a matrix that is capable of ionizing PLs in both positive and negative ion modes in order to improve the efficiency of PL identification in a single analysis. Recently, several binary matrices have been developed to ionize PLs effectively, including one that we developed (17, 18). With the introduction of a binary matrix, this has become the popular approach to the profiling and visualization of PLs using MALDI and there have been various applications of binary matrices for IMS of phospholipids in pathological tissues (19-23). In this study, we profiled and acquired images of the phospholipid changes associated with AD development using AD model mice and age-matched controls. Our results showed changes in the distribution of various lipid species including phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylinositol (PI), and sulfatide (ST). In particular, IMS data showed remarkable changes in lysophosphatidylcholine (LPC) and ST in the brain tissues of 9-month-old 5XFAD Tg mice. Our results indicate that MALDI-IMS is a powerful tool for analysis of the distribution of various PLs and that this application might provide novel insight into the prediction of disease.
Materials and Methods

The 5XFAD transgenic mouse model

The 5XFAD mouse line (Tg6799, Stock Number 006554, Jackson Laboratory, ME, USA) contains two human PSEN1 mutations (PSEN1 M146L, L286V) and three human APP mutations (APP KM670/671NL, I716V, V717I). Consequently, 5XFAD Tg mice rapidly develop severe amyloid pathology compared with other familial AD mouse models. Amyloid plaques accumulate early within the frontal cortex and the subiculum regions and start to aggravate the molecular pathology in the brain. All animal experiments and maintenance were performed under the guidelines of the Institutional Animal Care and Use Committee and Seoul National University.

Preparation of the binary matrix solution

The binary matrix used was a mixture of 2,5-dihydroxybenzoic acid (2,5-DHB) and α-cyano-4-hydroxycinnamic acid (CHCA) (7 mg/ml each). Trifluoroacetic acid (0.1% TFA) and 1% piperidine were added to the matrix mixture as ion pairing agents (17). The 2,5-DHB and CHCA matrices were purchased from Bruker Daltonics (Bremen, Germany) and used without purification. TFA and piperidine were purchased from Sigma Aldrich (Steinheim, Germany). All other materials were laboratory grade and purchased from Sigma Aldrich or Abcam Biochemicals (Cambridge, UK).

Sample Preparation for MALDI-IMS

All brain tissue samples (n = 3 in each group) were dissected from mice, immediately transferred to liquid nitrogen, and stored at -80°C. Subsequently, the frozen brains were sectioned at -20°C along the anterior-posterior axis into 12-µm thick sections using a Cryo-cut microtome (Leica Instruments GmbH, Hubloch, Germany). The frozen tissue sections were then thaw-mounted on glass slides coated with indium tin oxide (ITO) (Bruker Daltonics). The prepared slides were dried in desiccators for approximately 20 min and...
stored at -80°C until use. An ImagePrep instrument (Bruker Daltonics) was used to spray a total of 3 mL of the binary matrix solution onto each tissue section. The optimal method, DHB_Proteins, of the ImagePrep instrument was set to achieve a homogeneous matrix crystal on the tissue according to the manufacturer's standard protocol in the automatic mode. After matrix application, the homogeneity of matrix on the tissue was assessed using the Chip-1000 (Shimadzu Biotech, Kyoto, Japan) scanning image. The MTP slide adapter (Bruker Daltonics, Bremen, Germany) mounted with the tissue section ITO slide was directly transferred to the MALDI mass spectrometer and analyzed.

**IMS analysis**

Both profiling and IMS experiments on tissue sections were performed using an UltrafleXtreme MALDI MS instrument equipped with a 1 kHz SmartBeamII Nd:YAG/355 nm as an ionization source. (Bruker Daltonics, Bremen, Germany). Mass calibration was performed with external standards using lipid-mixed calibration standards with m/z ranges of 674–834 Da (positive ion mode) and 564–906 Da (negative ion mode) prior to data acquisition to achieve a mass accuracy of better than 50ppm (12, 24). A mass resolution of M/ΔM ~20,000 was typically achieved in the mass window of phospholipids. For IMS data acquisition, the 300–2,000 m/z range was analyzed in both positive and negative ionization modes by averaging 500 consecutive laser shots/pixel with followed parameters: laser focus set: small; delay: 180 ns; ion source 1: voltage, 25 kV, ion source 2: voltage, 21.65 kV; lens voltage: 9.2 kV. The spatial resolution for the imaging data was 150 µm. No modifications were made for the laser parameters (i.e., laser attenuation offset and focus setting) were not modified.

Software from Bruker Daltonics (FlexImaging, FlexAnalysis, and ClinproTools) was used for data analysis. Data analysis was done by “Processing methods” for peak picking, baseline subtraction, and smoothing operations. Centroid algorithm was used for peak picking. We compared the brains of Tg mice and age-matched controls (LT) at 3 and 9 months of age to obtain a list of phospholipids that showed significant
differences in their distribution. The regions of interest (ROI) selected for the principal components analysis (PCA) were in the area where plaques first formed as previously confirmed by 4G8 staining. Differences in the intensities between Tg and age-matched control mice were assessed by ANOVA test (p<0.05) with molecular ion peaks with more than 1.5-fold difference between groups and t-test for verification (p<0.05). The PCA clearly separated the four experimental groups based on their individual phospholipid profile. Ion intensities of each m/z value were normalized by the ClinproTools and FlexImaging software algorithms. All spectral intensities were divided by the total ion count value. Ion intensities were evaluated by comparing ion pairs from injured and non-injured tissue attained from the same brain section after normalization. The Top Hat baseline with a 10% minimal baseline width was used for baseline subtraction. Data reduction was performed at a factor of six.

MALDI-LIFT (MS/MS) analysis was performed to confirm the chemical structures of the selected lipids of interest. It was directly performed on the sample tissue after MALDI-MS. We precede the lipid identification using Lipidomics Gateway (http://www.lipidmaps.org) based on major fragment ions of MS/MS spectrum after performing manual mono isotope selection. LIFT mode condition: CID mode = false; precursor ion selector (PCIS) mass limit = 2–4 Da; Ion source volt-age 1/2 = 8kV/7.1 kV; LENS voltage = 3.6 kV; LIFT voltage1/2 = 19 kV/4.3 kV in positive mode. CID mode = false; PCIS mass limit = 2–4 Da; Ion source voltage 1/2 = 8 kV/7.1 kV; LENS voltage = 3.6 kV; LIFT voltage 1/2 = 19 kV/4.2 kV in negative mode.

**Immunohistochemistry (IHC)**

For staining of amyloid plaques in brains, 5XFAD mice brains were cut into 30-µm cryosections. Brain slices were washed in PBS and thawed in 70% formic acid solution for 20 min, followed by incubation with biotinylated anti-Aβ antibody (4G8, 1:1,000; Covance ImmunoTechnologies) in blocking solution containing 0.3% Triton X-100 at 4°C overnight and then with secondary antibody (Streptavidin Alexa Fluor 488, 1:500, Life Technologies). For staining of secretory PLA₂, the sections were incubated overnight with
anti-sPLA₂ antibody (1:100, Santa Cruz Biotechnology) in blocking buffer. Sections were washed in PBS and incubated with Alexa-488–conjugated secondary antibody followed by staining with methoxy-x04 dye. Brain samples were examined by confocal microscopy (Fv10i, Fluoview, Olympus), and analyzed using a FV10-ASW 2.1 viewer.
Results

Comparison of profiling spectra between brains of normal and AD model mice

Differences in phospholipid content between AD brains and age-matched control brains were investigated by employing IMS followed by statistical analysis using a combination of tools. To determine the overall changes in phospholipid composition, we took tissue sections from the anterior and posterior regions of brains from 5XFAD mice and age-matched LT controls at 3 and 9 months of age. Figure 1A shows images of sectioned anterior brain tissue confirming the uniform deposition of matrix. The applied binary matrix composed of DHB and CHCA (17) was used to improve the detection of phospholipids in both modes of polarity. Figure 1B shows 4G8-stained images comparing the level of plaque formation in 3-month-old versus 9-month-old 5XFAD mouse brains. The accumulation of β-amyloid (Aβ) was evident at 3 months of age in both anterior and posterior regions of the brain. A greater number of Aβ plaques were observed in the brains of 9-month-old 5XFAD mice. In previous AD studies, Aβ was found to accumulate initially in the hippocampus region of the subiculum and then spread to the whole brain, as shown in Figure 1B. In the anterior brain tissue sections of 5XFAD mice, the plaques were primarily localized to the cortex region, whereas plaques were widely distributed throughout the posterior region.

To investigate phospholipid composition changes with respect to AD progression, we performed IMS in both positive and negative ion modes on two consecutive tissue sections from anterior and posterior regions of the brain. Brains from three mice were analyzed for each experimental group. Following the whole tissue IMS analysis, the data were analyzed using Fleximaging and ClinProTools as described previously (12). Figure 2 presents a comparison of the average spectra obtained from the whole brain tissue sections after normalization using ClinproTools 2.2 in positive (Figure 2A) and negative (Figure 2B) ion mode. The ion peaks corresponding to phospholipids that showed differences in their levels among the analyzed tissues (p value <0.001) are marked with an asterisk. Table 1 shows the list of identified phospholipids with their differential levels.
Total MS spectra analysis by PCA and selection of putative markers for Alzheimer’s disease

To differentiate the profiles of each brain group, we conducted a principle component analysis (PCA) using ClinproTools 2.2 (Figure 3). The IMS data sets of cortex regions and subiculum regions acquired in both positive and negative ionization modes were analyzed by PCA. PCA analysis showed a clear separation between Tg and LT (Figure 3A and B, Supplementary Figure 2) and revealed a number of ion peaks with specific m/z values that showed significant changes in their distribution within the brain. Box plots for the analysis were included in Supplementary Figure 3. Before the PCA, we defined the regions of interest (ROI) in the cortex and the subiculum of each brain and collected the spectra of each ROI. A larger distinction between Tg and age-matched control mice was observed in the 9-month-old group than in the 3-month-old group, with a 95% confidence interval. We selected differentially expressed lipids from experimental groups by ANOVA test (p<0.05) with molecular ion peaks with more than 1.5-fold difference between groups and we performed t-test for verification (p<0.05). The molecular ion images contributing to the separation between Tg and age-matched control groups are shown in Figure 3C and D.

The phospholipids from the tissue sections were subsequently validated using tandem mass spectrometry (MS/MS) and a LIPID MAPS database search. In positive ion mode, fragment ion m/z 184 was derived from the head group of phosphatidylcholine, and fragment ions of m/z 147 and m/z 163 were the sodiated or potassiated cyclic 1,2-phosphodiester ion, respectively (25). These three fragment ions are characteristic for PC species or SM species. Also observed in the spectra is major ion produced through neutral head group loss of PC 32:0 and PE 32:0 species leading to fragment ion of m/z 551 corresponds to the diglyceride like ion. Such diglyceride like ion is a commonly observed fragment ion in positive ion mode analysis of phospholipids (26). From the MS/MS spectra and combined database search, these ions were identified as three lysophospholipids (m/z 496.5, m/z 522.5 and m/z 534.4), one SM (m/z 725.5) eight PCs (m/z 722.5, m/z 734.6, m/z 760.7, m/z 772.6, m/z 782.6, m/z 798.6, m/z 826.7, m/z 848.7), two PIs (m/z 885.7, m/z 892.6), one PS (m/z 834.7), two ST-OHs (m/z 850.8, m/z 878.8), and three STs (m/z 862.8, m/z 888.7 and m/z 890.7) as listed in Table 1. The representative fragment spectra of four different phospholipid classes
(LPC, PC, ST-OH, and PI) are shown in Figure 4 and other fragment spectra are provided in Supplementary Figure 1.

**Tissue distribution analysis of selected phospholipids by IMS**

The molecular ion images that showed differential distribution in all experimental groups (3- and 9-month-old 5XFAD mice and age-matched controls) are displayed in Figure 5. We reconstructed molecular images based on the intensities of the corresponding m/z peaks using Fleximaging software (Figure 5) and selected phospholipids that showed differential distributions in the brain tissues from 3- and 9-month-old 5XFAD mice that were distinct from those in the age-matched controls. Of the phospholipids identified in positive ion mode, m/z 496.5 (LPC 16:0, [M+H]⁺), m/z 534.4 (LPC 16:0, [M+K]⁺), m/z 722.5 (PC 30:3, [M+Na]⁺), m/z 725.5 (SM d34:1, [M+Na]⁺), m/z 772.6 (PC 32:0, [M+K]⁺), and m/z 798.6 (PC 34:1, [M+K]⁺) showed unique distributions in the brain tissues of the four experimental groups. As shown in Figure 5A, m/z 772.6 (PC 32:0, [M+K]⁺) in the frontal cortex region and m/z 725.5 (SM d34:1, [M+Na]⁺), m/z 772.6 (PC 32:0, [M+K]⁺), and m/z 798.6 (PC 34:1, [M+K]⁺) in the subiculum region were markedly decreased in 9-month-old 5XFAD mice brains. Interestingly, a significant increase in m/z 496.5 (LPC 16:0, [M+H]⁺) and m/z 534.4 (LPC 16:0, [M+K]⁺) was observed in amyloid plaque regions of both the frontal cortex and subiculum of 9-month-old 5XFAD mice. Previous studies showed a decrease in sphingomyelin (SM) in AD resulting from the activation of sphingomyelinase (SMase), which hydrolyzes SM to ceramide (27). Ceramides enhance the transmission of signals that induce neuronal apoptosis. In this study, the level of SM, including m/z 725.5 (SM d34:1, [M+Na]⁺) in the subiculum region, was reduced in 9-month-old Tg brains relative to age-matched LT controls, as shown in Figure 5.

In the negative ion mode, there was a noticeable reduction of sulfatide (ST) species in both the cortex and hippocampus region of brain tissues of 5XFAD mice relative to LT age-matched controls. The molecular ion images of m/z 850.8 (hydroxylated sulfatide d18:1/h20:0), m/z 862.8 (sulfatide d18:1/22:0), m/z 878.8 (hydroxylated sulfatide d18:1/h22:0), m/z 888.7 (sulfatide d18:1/24:1), and m/z 890.7 (sulfatide
d18:1/24:0) are displayed in Figure 5B. Sulfatide is synthesized from galactosylceramide in the central nervous system (CNS) by galactosylceramide sulfotransferase (GST) and is a major component of the myelin sheath of axons. Recently, several studies reported abnormal ST accumulations and deficiencies in the brains of neurodegenerative disease patients; brain ST levels have been shown to be dramatically reduced in AD (28) but are not reduced in individuals experiencing dementia with Lewy bodies and elevated in different brain regions in individuals with Parkinson’s disease (29).

Previous work has demonstrated that lysophospholipid is formed by hyperactivated phospholipase A$_2$ (PLA$_2$) in patients with neurodegenerative diseases (12). To confirm the role of PLA$_2$ in AD, we performed immunohistochemical staining of sPLA$_2$ in 5XFAD mouse brains. Immunostaining with sPLA$_2$ antibody showed partial accumulation of sPLA$_2$ localized around the A$_\beta$ plaques (Figure 6). We hypothesized that, under the influence of the inflammatory pathway component PLA$_2$, LPCs are abundant in the brains of 9-month-old Tg mice.

Discussion

In this study, we explored changes in phospholipid composition in the brains of mice with induced AD using MALDI–TOF. We identified several species of phospholipids, including LPC, PC, SM, PS, PI, and ST, in both positive and negative ion modes and demonstrated changes in their distribution.

β-amyloid plaques (Aβ), the most common lesions associated with AD, are caused by accumulation of misfolded proteins. Aβ and intracellular neurofibrillary tangles are induced by phosphorylated tau protein. Oakley et al. generated 5XFAD mice possessing mutations in the genes encoding the amyloid precursor protein (APP) and presenilin (PS1, PS2) proteins. These mutants rapidly accumulate Aβ in the subiculum and deep cortical layers (1). In this study, we used the 5XFAD mouse model and initially performed 4G8 staining to confirm plaque formation in the AD brains (Figure 1). We also compared the degree of plaque formation in 3- and 9-month-old 5XFAD mice brains. Plaques in the anterior cortex of the brain were more developed in the older 5XFAD mice. In addition, a greater number of plaques appeared in the subiculum of
the brains from 9-month-old 5XFAD mice brains than in those from 3-month-old 5XFAD mice. Therefore, we can be confident that our mouse model possesses the essential features of AD.

PC is the most abundant phospholipid, and is present in the outer leaflet of the cellular membrane. Previous reports showed that glycerophosphatidylcholine concentration typically increases with age; moreover, this increase is more pronounced in aged AD patients and those with enhanced β-amyloid plaque formation (29-32). Breakdown of APP protein and aggregation of amyloid plaques are the major causes of neuronal deterioration in AD. However, cellular calcium influx and calcium overload can also cause membrane damage by increasing the enzyme activity of phospholipase, which hydrolyzes membrane phosphatidylcholine (33). The increase in LPC and decrease in PC in the AD brains detected in our positive ion mode analysis (Figure 5) directly resulted from the activation of PLA₂, which is reported to be hyperactivated in various disease conditions, including AD, Parkinson’s disease, ischemia, spinal cord trauma, and head injury (34-40). It was also reported that increased neuronal activity of PLA₂ converts PC to LPC, which stimulates glial cells and subsequently induces neuroinflammation (34). Similar to previous reports, our data suggest that hyperactivated PLA₂ affected phosphatidylcholine in our study, converting m/z 798.6 (PC 34:1, [M+K]⁺) and m/z 760.7 (PC 34:1, [M+H]⁺) to m/z 534.4 (LPC 16:0, [M+K]⁺) and m/z 496.5 (LPC 16:0, [M+H]⁺). As shown in Figure 5, in the posterior brain of 9-month-old 5XFAD mice m/z 798.6 (PC 34:1, [M+K]⁺) showed a marked reduction whereas m/z 534.4 (LPC 16:0, [M+K]⁺), which is derived from PC 34:1, was enhanced. Activation of PLA₂ was confirmed in a validation experiment (Figure 6). Furthermore, in the positive ion mode analysis we observed that sphingomyelin, m/z 725.5 (SM d34:1, [M+Na]⁺), tended to be substantially decreased in brain tissue obtained from older mice with induced AD (Figure 5A). Sphingomyelin is a major component of neuronal myelin membranes, which are involved in several signaling pathways including cell growth, differentiation, and senescence. There are 3 types of sphingomyelinases: neutral sphingomyelinase (N-SMase), acidic sphingomyelinase (A-SMase), and alkaline SMase. Both neutral and acidic sphingomyelinase are activated in AD by soluble oligomers of Aβ. Sphingomyelinases break down sphingomyelin into ceramide, which functions as a second messenger.
molecule to induce apoptosis and neuronal cell death (41). We suggest that the reduction of SM in the AD brains is caused by overactive SMases and may induce neuroinflammation and neuronal apoptosis (27, 28, 42, 43). As we found the SM level was decreased, it is expected postulation that ceramide which is an important metabolite of SM, may be changed accordingly. However, it is not possible to detect and quantify interesting degradation products of SM such as Cer under normal IMS of lipids. The ionization efficiency of low abundant Cer during MALDI is extremely low to be detected or quantified without special high resolution MS and treatment of tissue samples. Recently, a novel MALDI MSI protocol for on-tissue detection and structural confirmation of ceramides and other sphingolipids were proposed (44). They used high-resolution MALDI-FTICR in combination with on-tissue ceramidase and sphingomyelinase enzyme digestions to detect and visualize the distribution of ceramides and sphingomyelin in tissues. A reduction in ST has been reported previously (28, 45). Earlier reports showed that ST levels were reduced by approximately 90% and 50% in gray and white matter of AD brains, respectively (46). In Figure 5B, we provide the images from IMS analysis in the negative ion mode. Of the phospholipids analyzed, the expression levels of several STs differed depending on the experimental group. STs play an important role in various biological processes including myelination, signal transduction, modulation of cell adhesion, neuronal plasticity, and cell morphogenesis (47, 48) and a change in ST levels is likely to be an indicator of the pathogenesis of various human diseases. AD patients exhibit a dramatic loss of myelin and axons and increased DNA fragmentation (49-51). Han et al. suggested that ST deficiency in AD might contribute to neuronal dysfunction and AD pathogenesis (28, 52, 53).

Various biochemical analysis methods are now widely used to investigate changes in the amounts of particular phospholipid molecules. Some techniques are able to show a quantifiable alteration of specific phospholipid composition but are unable to perform local imaging, whereas other visualization techniques can produce a local image of phospholipids but cannot provide quantification. The use of IMS overcomes these problems for biochemical analysis. We applied IMS to simultaneously detect and quantify local alterations of phospholipids in brain samples in an attempt to link amyloid plaques and phospholipids in a

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spatial and temporal manner within the brain. This is the first study to show the correlation between amyloid plaque deposition and phospholipid characterization in the brain of an AD model. Our findings might contribute to the underlying mechanism of plaque formation and AD pathogenesis focusing on phospholipid biogenesis.
Acknowledgments

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References


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179.
Figure legends

Figure 1. Representative brain sections immunostained for amyloid plaques using mAb 4G8.

(A) The image of sectioned anterior brain tissue demonstrates the uniform deposition of matrix. The anatomical map of a mouse brain (A) is applied from Paxinos et al. (54). (B) Brains from 3- and 9-month-old animals (n = 3 in each group) were analyzed by histological staining with an anti-Aβ (4G8) antibody. 4G8-stained images clearly show that Aβ accumulation is initiated at 3 months of age in both the anterior and posterior regions of the brain. The areas corresponding to the cortex region (circle) and subiculum (square) were indicated.

Figure 2. Comparison of MS spectra in positive ionization mode (A) and negative ionization mode (B) obtained from whole brain tissue sections after normalization. Ion peaks that show differences in their levels among the analyzed tissues (p value <0.001) are marked with an asterisk. Spectra from 3-month-old LT and 5XFAD mice are represented by red and green, respectively. Spectra from 9-month-old LT and 5XFAD mice are represented by blue and yellow, respectively.

Figure 3. PCA and visualization of the molecular distribution of major components of specific regions.

PCA results of the acquired MS spectra from the cortex regions of anterior brain (A) and the subiculum regions of posterior brain (B) in positive ion mode reveal clear separation between the groups. PCA results of data obtained from negative ion mode are listed as Supplementary Figure 2. Representative molecular ion images from the anterior brain (C) and the posterior brain (D).

Figure 4. Validation of selected phospholipids by MS/MS analysis of tissue.

MS/MS spectra of LPC 16:0 [M+K]+ at m/z 534 and PC 32:0 [M+H]+ at m/z 734 in positive ion mode (A). MS/MS spectra of sulfatide at m/z 878 and phosphatidylinositol at m/z 885 in negative ion mode (B).
Figure 5. Differential distribution of phospholipids according to Aβ plaque formation in brain tissues.

Representative MALDI images of LPC, SM, and PC (A) and ST-OH and ST (B) show alterations in their levels according to Aβ plaque formation.

Figure 6. Immunohistochemistry of sPLA₂

Immunostaining with sPLA₂ antibody (green) showed that partial accumulation of sPLA₂ localized around amyloid plaques (blue). Amyloid plaques are indicated with asterisks. Scale bar, 20 μm.
## Tables

### Table 1. Phospholipids identified by MALDI TOF throughout the whole brain.

<table>
<thead>
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<th>m/z</th>
<th>Phospholipid Class</th>
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<th>Adduct Formation</th>
<th>Fragment ion</th>
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<td>K</td>
<td>163</td>
</tr>
<tr>
<td>848.7</td>
<td>PC&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>38:4</td>
<td>K</td>
<td>163</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fatty acid (the number of carbon atoms in fatty acids : the number of double bonds in fatty acids)

<sup>b</sup> Identified by MALDI-MS/MS fragmentation

<sup>b</sup> Structure suggested by LIPID MAPS database
## Negative ion mode

<table>
<thead>
<tr>
<th>m/z</th>
<th>Phospholipid Class</th>
<th>FA (c:db)</th>
<th>Fragment ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>834.7</td>
<td>PS&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>40:6</td>
<td>M – 87 (Head group)</td>
</tr>
<tr>
<td>850.8</td>
<td>ST-OH&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>d18:1/h20:0</td>
<td>241, 257/259, 97, 300</td>
</tr>
<tr>
<td>862.8</td>
<td>ST&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>d18:1/22:0</td>
<td>241, 257/259, 97, 300</td>
</tr>
<tr>
<td>878.8</td>
<td>ST-OH&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>d18:1/h22:0</td>
<td>241, 257/259, 97, 300</td>
</tr>
<tr>
<td>885.7</td>
<td>PI&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>18:0/20:4</td>
<td>241</td>
</tr>
<tr>
<td>888.7</td>
<td>ST&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>d18:1/24:1</td>
<td>241, 257/259, 97, 300</td>
</tr>
<tr>
<td>890.7</td>
<td>ST&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>d18:1/24:0</td>
<td>241, 257/259, 97, 300</td>
</tr>
<tr>
<td>892.6</td>
<td>PI&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>38:1</td>
<td>241</td>
</tr>
</tbody>
</table>

* Fatty acid (the number of carbons : the number of double bonds in fatty acid chain)

<sup>a</sup> Identified by MALDI-MS/MS fragmentation

<sup>b</sup> Structure suggested by LIPID MAPS database

<sup>c</sup> Structure suggested by reference
Figures

Figure 1.

A. Sectioned frozen brain by 12μm Sprayed with matrix on sectioned brain

B. Anterior brain Frontal Cortex
   Posterior brain
   Posterior Subiculum

3months Tg brain 9months Tg brain
Figure 2.

A. Positive ion mode

B. Negative ion mode

Legend:
- LT_3 month
- TG_3 month
- LT_9 month
- TG_9 month
Figure 3.

A. Anterior brain cortex region

B. Posterior brain subiculum region

C. Anterior brain (9 months old)

D. Posterior brain (9 months old)
Figure 4.

A. Positive ion mode

m/z 534 LPC 16:0 [M+K]^+

m/z 734 PC 32:0 [M+H]^+

B. Negative ion mode

m/z 878 ST-OH d18:1/h22:0

m/z 885 PI 18:0/20:4
Figure 5.
Figure 6.