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Improved method for quantitative analysis of methylated phosphatidylethanolamine species and its application for analysis of diabetic mouse liver samples

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Abstract

N-monomethyl phosphatidylethanolamine (MMPE) and *N,N*-dimethyl phosphatidylethanolamine (DMPE) species are intermediates of phosphatidylcholine (PC) *de novo* biosynthesis through methylation of phosphatidylethanolamine (PE). This synthesis pathway for PC is especially important in the liver when choline is deficient in the diet. In spite of some efforts on the analysis of MMPE and DMPE species, cost effective and high throughput method for determination of individual MMPE and DMPE species including their regioisomeric structures is still missing. Therefore, we adopted and improved the “mass-tag” strategy for determining these PE-like species by methylating PE, MMPE, and DMPE molecules with deuterated methyl iodide to generate PC molecules with 9, 6, and 3 deuterium atoms, respectively. Based on the principles of multidimensional mass spectrometry-based shotgun lipidomics, we could directly identify and quantify these methylated PE species including their fatty acyl chains and regiospecific positions. This established method provided remarkable sensitivity with a limit of detection at 0.5 fmol/μl, high specificity, and a broad linear dynamics range of > 2500 folds. By applying this method to the liver samples of streptozotocin (STZ)-induced diabetic mice and their controls, we found that the levels of PC species had the trends to decrease and the amounts of PE species tended to increase in the liver of STZ-induced diabetic mice comparing to their controls, but not significant changes in MMPE and DMPE species were determined. However, remodeling of fatty acyl chains in these determined lipids was observed in the liver of STZ-induced diabetic mice with reduction of 16:1 and increases in 18:2, 18:1, and 18:0 acyl chains. These results demonstrated that the improved method would serve as a powerful tool to reveal the role of the PC *de novo* biosynthesis pathway through methylation of PE species in biological systems.

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Keywords

Diabetes; liver damage; mass spectrometry; *N*-monomethyl phosphatidylethanolamine; *N,N*-dimethyl phosphatidylethanolamine; phosphatidylcholine biosynthesis; shotgun lipidomics

Introduction

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are usually the most abundant cellular lipid classes in animals and plants, accounting for up to 70 to 80% of the total lipids, and it is obvious that they are the key building blocks of cellular membranes [1]. The difference between PC and PE is the choline and ethanolamine groups connecting to the glycerol-3-phosphate backbone [2]. PC makes up a major part of the outer leaflet of the plasma membrane and PE is mainly in the inner leaflet [3]. The cylindrical shape of PC molecular is an important structural component contributing to the integrity and function of membranes, whereas the relative small head group of PE can accommodate the need of inner membrane curvature and the insertion of proteins within the membrane without loss of the integrity of the membrane [4, 5]. One of the primary metabolic pathways for *de novo* synthesis of PC is the sequential methylation of PE, which is catalyzed by an enzyme, i.e., phosphatidylethanolamine *N*-methyltransferase (PEMT), with *N*-methyl- and *N,N*-dimethyl-phosphatidylethanolamine (MMPE and DMPE, respectively) as intermediates [3]. PEMT catalyzes all the three consecutive steps and spans in all the cellular membrane. This pathway is especially important in the liver when choline is deficient in the diet for appropriate secretion of the plasma lipoproteins since PC is the only phospholipid necessary for lipoprotein assembly and secretion [6]. The relative rates of the *de novo* synthesis pathway largely depend on the organism and the metabolic program of given cellular tissue types. However, the produced amounts of MMPE and DMPE are never found at greater than trace levels in animal tissues. At the meantime of producing intermediates, MMPE and DMPE, generated another by-product, S-adenosylhomocysteine, whose hydrolysis product in the liver, homocysteine, is related to cardiovascular disease and myocardial infarction [7-9]. In addition, the activity of the catalyzed enzyme, PEMT, for the PE methylation pathway might be an important predictor of non-alcoholic fatty liver disease in humans [10-12]. Therefore, increased MMPE or DMPE levels could result in more generation of homocysteine and indicate a high activity of PEMT, which are likely related to cardiovascular disease, myocardial infarction, or non-alcoholic fatty liver disease.

Analysis of MMPE and DMPE species has been performed by data dependent acquisition profiling on a hybrid quadrupole time-of-flight mass spectrometry (MS) instrument by emulated neutral loss scans (NLS) of 155.03 and 169.05 Da from their *N*-methyl phosphoethanolamine and *N,N*-dimethyl phosphoethanolamine head groups, respectively [13]. This analysis method could bring in false-positive identification and biased quantification from the interferences between protonated and sodiated species. Moreover, accurate quantification of MMPE and DMPE species requires spiking in separated internal standards for each class of lipid to avoid ionization discrimination and differences of fragmentation efficiency. In shotgun lipidomics, PE species are generally analyzed under weak basic condition (such as adding a small amount of LiOH) in negative mode [2] since

PE molecules become anionic under alkaline conditions. Currently, there is no reported tandem MS method with highly sensitivity and specificity for PE species analysis in the negative-ion mode. Therefore, many low abundant PE anions are buried in the baseline noise and could not be detected or profiled. Similarly, the low amounts of the whole classes of MMPE or DMPE species could not be determined by the same methods in the negative-ion mode. Although the strategy of PE derivatization with fluorenylmethoxycarbonyl (Fmoc) chloride through the MS analysis by NLS of the Fmoc moiety could greatly improve the sensitivity and could identify and quantify all PE species including the very low abundant PE in the negative-ion mode [14], this strategy is not suitable for the determination of MMPE or DMPE species since the hydrogen(s) on their amine moiety is/are replaced by methyl group(s). Ejsing and colleagues created a new “mass-tag” strategy to methylate DMPE, MMPE, and PE species with deuterated methyl iodide (CD_3I) to generate PC molecules with different deuterated degrees at the quaternary amine with a mass offset of 3, 6 and 9 Da, respectively [15]. This methodology allows characterizing DMPE, MMPE, and PE species as endogenous PC with specific mass offsets, since all of the investigated species have a phosphocholine head group and equal ionization efficiency. In addition, it is also possible to accurately quantify PC, DMPE, MMPE, and PE species using only PC and/or PE internal standards. However, this reported method by using multiple precursor ion scanning (PIS) of phosphocholine fragment ions of the protonated species failed to determine the fatty acyl chains of these species and their regioisomeric structures. Moreover, a much more excessive amount of CD_3I was used in the method whereas it is well known that methyl iodide exhibits acute toxicity and has resulted in nausea, vomiting, slurred speech, drowsiness, skin blistering, and eye irritation [16, 17].

Herein, by applying the principles of multidimensional MS-based shotgun lipidomics (MDMS-SL) [2, 18, 19] and employing an improved reaction condition to methylate DMPE, MMPE, and PE species, we could accurately, highly efficiently, and sensitively identify and quantify those DMPE, MMPE, and PE species directly from biological samples, including determination of the fatty acyl chains and their regiospecific positions. To demonstrate the capacity of the improved analytical strategy, we compared the lipid profiles of PC, DMPE, MMPE, and PE species in liver samples between streptozotocin (STZ)-induced diabetic mice and their controls. Moreover, from the analysis, remodeling of fatty acyl chains in those lipids was also observed in the STZ-induced diabetic mouse liver with reduction of 16:1 and increases in 18:2, 18:1, and 18:0 acyl chains. We believed that this improved method should extend the MDMS-SL platform and provide another powerful tool to identify and quantify PC, DMPE, MMPE, and PE species at the same time, thereby leading to further understanding the remodeling of these species during PC *de novo* biosynthesis through the PE methylation pathway.

Material and Methods

Materials

All of the synthetic phospholipid standards including di16:0 and di18:1 MMPE, di16:0 and di18:1 DMPE, di16:0, di18:3, and 16:0-22:6 PE, and di14:1 PC were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). All of these standards were used without further

purification. All organic solvents used for lipid extraction, sample preparation, and MS analysis were obtained from Burdick and Jackson (Muskegon, MI, USA). Ammonium hydroxide (NH₄OH) was purchased from Thermo Fisher Scientific, Inc. (Fair Lawn, NJ, USA). Deuterated methyl iodide (CD₃I), streptozotocin [*N*-(methylnitrosocarbamoyl)- α -D-glucosamine] (STZ), and other chemicals were obtained from Sigma-Aldrich Chemical Co. in their highest purity available (St. Louis, MO, USA).

Animal experiments

Wild type mice (male, C57BL/6, 7 to 8 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Diabetes in the mice was induced by a daily intravenous injection for 5 days (in the tail vein) of STZ (50 mg/kg body weight in 0.1 ml of 0.1 M citrate buffer, pH 4.5) as described previously [20]. Control mice received citrate buffer (0.1 ml) alone. Diabetes was confirmed within 48 h by blood glucose levels of > 300 mg/dl as measured by chemstrips (bG, Boehringer-Mannheim). Mice induced to diabetes by STZ for one week were euthanized by asphyxiation with CO₂. The livers were excised quickly, perfused with ice-cold PBS to remove blood, blotted with Kimwipes (Kimberly-Clark, Roswell, GA, USA) to remove excess buffer, and then immediately freeze-clamped at the temperature of liquid N₂. All of tissue samples were stored at -80 °C until lipid extraction. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Science, 2011) and were approved by the Institutional Animal Care and Use Committee at the Sanford-Burnham Medical Research Institute.

Lipid extraction

Liver tissues were pulverized into fine powder by a stainless steel biopulverizer at the temperature of liquid nitrogen. The tissue fine powders of 10 to 20 mg were weighed and homogenized in 10x diluted PBS by 10 cycles of 3 sec of pulse of sonication and 3 sec of pauses (Branson digital sonifier 450, Branson Ultrasonics Corp., Danbury, CT, USA) in a 2.0-ml cryogenic vial (Corning Life Sciences, Tewksbury, MA, USA). Protein assays on the liver homogenates were performed utilizing a bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, USA) with bovine serum albumin as a standard. All determined lipid levels (PE, MMPE, DMPE, and PC) were normalized to the protein contents of individual samples. An accurate volume of individual liver homogenate (equal to 1 to 2 mg protein of sample) was transferred into a disposable glass culture test tube. Before lipid extraction, an internal standard (di14:1 PC) for quantitation was added into individual test tube containing liver homogenate [21, 22]. A modified Bligh and Dyer procedure was performed to extract the lipid species as described previously [23]. The final lipid extracts were reconstituted into a volume of 200 μ l of CHCl₃/MeOH (1:1, v/v) per milligram of tissue protein, flushed with nitrogen, capped, and stored at -20 °C.

Lipid methylation

Lipid extracts (equivalent to approximately 0.05 to 0.1 mg of tissue protein content) were transferred into disposable 0.5-dram (1.8-ml) borosilicate glass vials. If the transferred sample volume is higher than 20 μ l, it was evaporated to lower than 20 μ l under a nitrogen

stream. To the containers with the transferred samples, 20 μ l of CD₃I and 20 μ l of saturated NH₄OH were added. After the reaction mixtures were vortexed for 20 sec, the reaction vials were flushed with nitrogen, capped, and incubated at 40 °C for 90 min. The reaction was terminated after washing the reaction solution into a 10-ml disposable borosilicate glass test tube with 1 ml of CHCl₃/MeOH (1:1, v/v). The washing step was further repeated 3 times, and the solution was combined. Into the same tube, 2 ml of LiCl (10 mM) solution was added and the reaction products were extracted utilizing the modified Bligh and Dyer procedure [23]. The CHCl₃ (bottom) layer was collected into another new glass tube, and then evaporated under a nitrogen stream to dryness. The residues were resuspended with 80 μ l of CHCl₃/MeOH (1:1, v/v), flushed with nitrogen, capped, and stored at -20 °C for ESI-MS analysis.

MS analysis of lipids

Prior to performing ESI-MS analysis in the positive-ion mode after direct infusion, individual methylated lipid solution was further diluted to a final concentration of 0.1-1 pmol/ μ l of the internal standard with CHCl₃/MeOH/isopropanol (1/2/4, v/v/v) containing 40 μ M of LiOH in a 96-well plate. MS analysis of the samples was performed on a QqQ mass spectrometer (Thermo TSQ Vantage, San Jose, CA, USA) equipped with an automated nanospray device (TriVersa NanoMate, Advion Bioscience Ltd., Ithaca, NY, USA) as previously described [24]. ESI spray voltage of 1.15 kV and gas pressure of 0.55 psi on the NanoMate apparatus were employed for all MS analyses, and controlled by Chipsoft 8.3.1 software. All MS or MS/MS analyses were operated under Xcalibur software as previously described [24]. Typically, a 2-min period of averaging signal with a scan rate of 1 sec/scan in the profile mode was used for each MS spectrum, and a 16-min period of signal containing 4 scan events with the neutral loss scan (NLS) of 59.1, 62.1, 65.1, and 68.1 was employed for identifying and quantifying PC, and deuterated DMPE, MMPE, and PE, respectively with a scan rate of 1 sec/event. Other tandem MS spectra were acquired with a 4-min period of averaging signal with a scan rate of 1 sec/scan. For all of tandem MS analysis in the NLS or product-ion mode, collision gas (argon) pressure was set at 1.0 mTorr or specified, and collision energy was specified. A mass resolution setting of 0.7 Th for Q1 and Q3 was used for both MS and tandem MS analyses.

Results and Discussion

Modified methylation of DMPE, MMPE and PE species with deuterated methyl iodide

Systematic analysis of PC using an MDMS-SL technology could identify the subclasses of PC (including plasmalogen) and individual molecular species, and discriminate the fatty acyl constituents and their regiospecificity of diacyl PC species [25]. Therefore, in order to take advantage of the high sensitivity, specificity, and accuracy of this method for systematic analysis of PC, we adopted the “mass-tag” strategy using CD₃I to methylate the amino groups of PE, MMPE, and DMPE into quaternary amines which have the identical structures as PC species, but contain 9, 6, or 3 deuterium atoms, respectively (see Electronic Supplementary Material Figure S1) [15]. The methylated products should behave identical to PC molecules in MS or tandem MS analysis with a specific mass offset of 9, 6, 3 Da for PE, MMPE, and DMPE species, respectively.

It was reported that PE, MMPE, and DMPE could be methylated with CD₃I at 90 °C to produce deuterated PC with a 99.5% or better reaction efficiency [15]. However, under our investigation, unreacted PE, MMPE, and DMPE, and the intermediate products (e.g., PE or MMPE with one tagged methyl group or PE with two tagged methyl groups) could still be detected by MS analysis (see Electronic Supplementary Material Figure S2) even though the amount of CD₃I was much higher than that of the reacted lipid species with extended reaction time. The other product of the methylation reaction is hydrogen iodide, which is a very strong acid. We thought that the accumulation of the produced hydrogen iodide in the reaction solution could affect the process of methylation, and the strong acidity might prevent the loss of the hydrogen from the primary or secondary amine groups. After saturated NH₄OH (20 µl) was added into the reaction system, mass spectrometric analysis of the reaction solution showed that the reactant lipids and intermediate products were not displayed in the mass spectra and only intensive deuterated PC species were detected.

In these experiments, we also optimized the amounts of CD₃I and saturated NH₄OH necessary for methylation. Because amine groups are common moieties in lipid extracts of biological samples, an excessive amount of CD₃I should be favorable to the completion of PE, MMPE, and DMPE methylation. The broad levels of amine-containing species including PE, MMPE, and DMPE present in biological samples require a high ratio of CD₃I vs. PE, MMPE, and DMPE species to warrant the completion of methylation, which also requires a high amount of NH₄OH as anticipated. However, if the ratio of CD₃I or NH₄OH to PE, MMPE, and DMPE species was too high, the resultant by-products or the extra basicity resulted from excessive NH₄OH could lead to ion suppression and affect the analysis of the resultant deuterated PC. Moreover, since methyl iodide exhibits moderate to high acute toxicity after inhalation [26], we should try to use a minimal amount of CD₃I in the reaction. In order to optimize the ratio between the reactants, we derivatized a standard mixture of PE, MMPE, DMPE, and PC at a total amount of 50 nmol and a lipid extract from mouse liver corresponding around 0.05 to 0.1 mg of tissue protein content, respectively, with varied volumes of CD₃I from 1.5 to 200 µl and of saturated NH₄OH from 1 to 35 µl. It was found that when 20 µl of either CD₃I or saturated NH₄OH was used for the reaction under the conditions, which corresponded to 3 to 10 thousand times more than what the methylation needed, the mass spectra showed the best signals of the species with a relatively low background noise. Since we did not detect any leftover reactants, including PE, MMPE, and DMPE, or partially methylated intermediate products, such as *N*-mono- or di- *d*₃-methyl PE (only one or two CD₃ reacted with PE) or *N*, *N*-methyl-*d*₃-methyl PE (only one CD₃ reacted with MMPE), it was concluded that the methylation efficiency on PE, MMPE, and DMPE species through CD₃I and saturated NH₄OH would be 99.8% or better.

In the study, we also investigated how the temperature and time of the reaction, the volume of the reaction container, and the follow-up washing solvent affected the methylation of PE, MMPE, and DMPE species. Specifically, a mixture of PE, MMPE, DMPE, and PC standards with methylation reagents was incubated at 40, 50, 70, and 90 °C for 0.5, 1, 1.5, 2, 3, and 15 h with a random combination of the reaction temperature and time. We found that under the examined temperatures, all the mass spectra showed a completed methylation without apparent differences. However, if the reaction lasted for 15 h, the mass spectra of

the methylated products were very noisy. Therefore, reaction temperature of 40 °C and reaction time of 1.5 h were selected for methylation. The size of reaction container was compared utilizing a 1.8-ml vial and a 6-ml glass tube. It was concluded that the smaller size of the container led to a better yield of methylation and the corresponding mass spectra showed better signals of deuterated PC species. A comparison between pure water and 10 mM of LiCl used in the final washing step by the modified Bligh and Dyer procedure demonstrated that utilization of a LiCl solution was more favored for identification and quantification of PE, MMPE, and DMPE species as lithium adducts (further discussed in the next section). It was also investigated if evaporation of the lipid extract was necessary before methylation. Mass spectrometric analysis of the reaction products did not show any apparent difference with or without pre-evaporation as long as the volume of the lipid extract used for reaction was not higher than 20 μ l, which is usually larger than the required amount of tissue lipid extract (corresponding to 0.05 to 0.1 mg protein).

Identical characteristics of the methylated DMPE, MMPE, and PE to PC species

Identical to the protonated PC species which show a predominant fragment ion peak at m/z 184.1, product-ion ESI-MS analysis of protonated methylated DMPE, MMPE, or PE species after collision induced dissociation (CID) also displayed a predominant fragment ion peak at m/z 187.1, 190.1, or 193.1 from their head groups, respectively (see Electronic Supplementary Material Figure S3). Therefore, the product ion MS analysis of protonated PC and methylated DMPE, MMPE, or PE did not provide any rich information about their fatty acyl chains or regioisomers, except that the precursor ion scans of 184.1, 187.1, 190.1, and 193.1 could be used to identify or quantify PC, DMPE, MMPE, and PE species, respectively.

It was reported that NLS of 59.1, 183.1, and 189.1 Da facilitated the identification and quantification of lithiated individual PC molecular species and that the information about fatty acyl chains and the regioisomers of PC species could be determined by NLS of fatty acids (FA) plus trimethylamine (59.1 Da) of the corresponding lithium adducts [25]. Since after methylation, DMPE, MMPE, and PE species were isotopologues of PC with 3, 6, or 9 additional deuterium atoms, respectively, we could also use the same strategies to identify individual species of DMPE, MMPE, and PE classes, quantify their contents, and discriminate their regioisomer structures with a mass offset of 3, 6, or 9 Da, respectively (Figure 1). For example, the product ion ESI-MS analysis of lithium adducts of methylated di16:0 DMPE at m/z 743.6 (Figure 1A) after CID showed the fragment ion peaks at m/z 681.5, 557.5, and 551.5, corresponding to the neutral losses of 62.1, 186.1, and 192.1 Da, which represented the loss of trimethylamine, phosphocholine, and lithiated phosphocholine containing one deuterated methyl group, respectively. Similarly, lithium adducts of methylated di16:0 MMPE (Figure 1B), and di16:0 and 16:0-22:6 PE (Figure 1C and D) yielded their fragment ions at their corresponding relative losses of 65.1, 189.1, and 195.1 Da, and 68.1, 192.1, and 198.1 Da, respectively, in their product ion ESI-MS mass spectra after CID. In conclusion, NLS of 62.1, 186.1, and 192.1 Da could be used to sensitively detect the molecular ions of lithiated methylated DMPE, so as NLS of 65.1, 189.1, and 195.1 Da, and NLS of 68.1, 192.1, and 198.1 Da to detect lithiated methylated MMPE, and PE, respectively.

It should be pointed out that we recognized the overlap between a few NLS for detecting the molecular species of PC, DMPE, MMPE, or PE. For example, NLS of 189.1 Da could represent the loss of lithiated phosphocholine from PC head group or the loss of phosphocholine moiety from deuterated methylated MMPE head group, so as NLS of 192.1 Da, which could be the loss of lithiated phosphocholine from deuterated methylated DMPE or the loss of phosphocholine moiety from deuterated methylated PE. However, since MDMS-SL analysis does not depend on any single scan, but relies on the entire set of building blocks, such an overlap does not affect the identification and quantification of individual species present in a specific class as extensively discussed previously [19]. Therefore, the combination of three specific NLS mentioned above was used together to identify PC, DMPE, MMPE, or PE species. For example, a peak with the exact m/z value of lithiated methylated DMPE needed to be detected contemporaneously in the mass spectra of NLS of 62.1, 186.1, and 192.1 Da in order to confirm the DMPE species, that is the same as MMPE need to be confirmed in all of the NLS of 65.1, 189.1, and 195.1 Da (see Electronic Supplementary Material Figure S4). The detection strategy by combining three NLS could successfully avoid any false-positive identification and even exclude the sodium adducts of DMPE, MMPE, and PE species.

It should be recognized that the product-ion ESI mass spectrum of methylated 16:0-22:6 PE (Figure 1D) showed a much stronger fragment ion corresponding to the loss of phosphocholine than the one from the loss of lithiated phosphocholine, which is different from the product ion mass spectra of other species (Figure 1A, B, and C). It is likely due to the presence of polyunsaturated FA chain which is likely more favored for an activated rearrangement to stabilize the generated intermediate ion during the loss of the phosphocholine head group as previously discussed [25].

As previously described, the two fatty acyl chains and their regiospecificity of PC species could be determined by NLS of fatty acids plus trimethylamine (i.e., NLS(59.1 + FA mass)) from their lithium adducts [25]. Logically, analyses of NLS(62.1 + FA mass), (65.1 + FA mass), and (68.1 + FA mass) could be performed to identify the fatty acyl constituents of methylated DMPE, MMPE, and PE and their regioisomers, respectively. It was demonstrated that the fragment ions resulting from NLS of trimethylamine plus FA mass were more intense than those from NLS of FA mass or NLS of lithiated FA mass under the optimized experimental conditions (Figure 1). For example, product ion MS analysis showed that the fragment ion at m/z 425.2 corresponding to the loss of (62.1 + 16:0 FA) was more intense than those ions at m/z 487.5 (loss of 16:0 FA) and m/z 481.3 (loss of lithiated 16:0 FA) (Figure 1A). A same conclusion could be drawn from the analyses of other PE-derived classes (Figure 1B, C, and D). It was worthy to mention that due to the presence of methylated PE species containing different fatty acyl chains, the fragment ion peak yielded from the neutral loss of trimethylamine plus either FA mass was more intense than its corresponding fragment ions from NLS of either FA mass or lithiated FA mass (Figure 1D). Specifically, the ion peaks at m/z 493.5 (loss of 22:6 FA) and m/z 487.3 (loss of lithiated 22:6 FA) were weaker than the one at m/z 425.2 (loss of 68.1 plus 22:6 FA), and the peaks at m/z 565.5 (loss of 16:0 FA) and m/z 559.3 (loss of lithiated 16:0 FA) were weaker than the one at m/z 497.2 (loss of 68.1 plus 16:0 FA). Therefore, identification of the fatty acyl

structure using NLS(trimethylamine + FA mass) was more sensitive and accurate. Utilizing these NLS of trimethylamine plus FA mass also avoided any interferences from the coexisting triacylglycerol species, even at an amount much lower than that of DMPE, MMPE or PE molecular species [27]. Moreover, this identification strategy offered different NLS by a mass offset of 3 Da for the same fatty acyl chain of PC, DMPE, MMPE, and PE, and avoided the interferences from each other since the amounts of PC and PE species were much higher than those of DMPE and MMPE molecular species in most biological samples.

It was previously demonstrated that the fragment ion intensities corresponding to the NLS of trimethylamine plus *sn*-1 FA mass were higher than those corresponding to the NLS of trimethylamine plus *sn*-2 FA mass [25]. Because we could not obtain DMPE or MMPE containing different fatty acyl chains, lithiated methylated 16:0-22:6 PE was used to demonstrate that the same strategy could also be used to identify the regioisomers of DMPE and MMPE species (Figure 1D). The intensity of the fragment ion peak at m/z 497.2 (loss of $68.1 + 16:0$ FA) is more abundant than the one at m/z 425.2 (loss of $68.1 + 22:6$ FA), which concluded that the PE species was 16:0-22:6 PE which matched with its original source. Therefore, relative intensity comparison of DMPE, MMPE, or PE species between the mass spectra corresponding to NLS of trimethylamine plus FA masses could be used to discriminate the regioisomers of these lipid classes.

Contemporary quantification of DMPE, MMPE, and PE

As aforementioned, some of the discovered NLS might include two different classes of PC, DMPE, MMPE, and PE. Lithium adducts of both PC and methylated MMPE displayed in the spectrum of NLS189.1 Da, and lithium adducts of both methylated DMPE and PE species were detected from the analysis of NLS192.1 Da. The natural amount of PC in biological samples is usually much higher than MMPE and the amounts of PE species are much higher than DMPE. Thus, in the same NLS mass spectra, the coexisting PC or PE species would affect the accurate quantification of MMPE or DMPE species, respectively. Therefore, the established two-step method for PC quantification [25] could not be used to measure the amounts of DMPE or MMPE species.

It could be recognized that PC, and methylated DMPE, MMPE, and PE would not interfere with each other in their NLS spectra of trimethylamine. Although the sensitivity of NLS(trimethylamine) is not as good as NLS(phosphocholine) or NLS(lithiated phosphocholine), (Figure 1), tandem MS analysis of PC, DMPE, MMPE, and PE with NLS(trimethylamine) showed an increased sensitivity at collision energy of 26 eV in comparison to those obtained from the higher collision energy. More importantly, in the NLS(trimethylamine) analysis, the equimolar mixture of di16:0 and di18:1 DMPE, di16:0 and di18:1 MMPE, and di16:0, di18:3, and 16:0-22:6 PE displayed nearly equal ion intensities of these standards (see Electronic Supplementary Material Figure S4). Since DMPE and MMPE species in biological samples are usually present in very low abundance and they overlap with the abundant PC and/or PE species after methylation, it is not possible to quantify DMPE and MMPE species by using survey scan spectra. Therefore, the NLS of trimethylamine, specifically, NLS62.1, 65.1, and 68.1 Da could be employed to quantify methylated DMPE, MMPE, and PE species due to the enhanced sensitivity and specificity of

NLS or PIS, which is widely performed in lipidomics [19]. In practice, methylated PE could also be quantified by using the established strategy for PC analysis, since the high abundance of PE species in normal biological samples would not be interfered by DMPE in the NLS192.1 Da in spite of their coexistence.

Since the methylated DMPE, MMPE, and PE species are the isotopologues of the corresponding PC with 3, 6 or 9 more deuterium atoms in the quaternary amine group, respectively, we could directly use the exogenously added internal standard, di14:1 PC [25] for PC analysis to quantify these methylated DMPE, MMPE and PE species. An advantage of the Thermo TSQ Vantage triple quadrupole instrument is capable of performing multiple NLS [28] by “event” scans. Mass spectra of NLS59.1, 62.1, 65.1, and 68.1 were sequentially and alternatively acquired at the rate of 1 scan/sec for a total of 16 min. In this case, the effects of any unstable ESI spray and ionization on the NLS acquisition could be minimized.

The linearity of dynamic range of the established method was determined by titrating the PC internal standard (di14:1 PC) solution (12.5 pmol/μL) into mixtures of DMPE, MMPE, and PE species (0.25 to 625 pmol/μl each at nearly equal amount). After methylation with CD₃I, the PC, DMPE, MMPE, and PE mixtures were diluted to a total lipid standard concentration of < 5 pmol/μl prior to direct infusion for tandem MS analysis. The concentration of each of DMPE, MMPE, and PE species was calculated by comparing their peak intensities in corresponding NLS to that of the internal standard of di14:1 PC in NLS 59.1 after ¹³C deisotoping. The linear plots of the DMPE, MMPE, and PE standards between the measured and theoretical concentrations were acquired with linear correlation coefficients from 0.997 to 0.999 and linear slopes from 0.93 to 1.04 (Figure 2). The high correlation of the measured ions corresponding to the individual DMPE, MMPE, and PE species by using PC internal standard with theoretical values indicated that all of the PC, DMPE, MMPE, and PE species present in biological samples could be measured by using one internal standard under the optimized conditions. The limit of detection of the established method was also assessed by the dilution experiment as low as 0.5 fmol/μl.

Determination of DMPE and MMPE in STZ-induced diabetic mouse liver

In order to evaluate the capability of the established method for analysis of methylated PE species, we determined and compared the levels of PC, DMPE, MMPE, and PE species present in liver samples of STZ-induced diabetic mice and their controls. It was reported that the levels of PC species were reduced and PE species were elevated in the liver of rats after four months of hyperglycemia induced by STZ and these lipid changes were concomitantly with signs of hepatic damage (i.e., connective tissue with cellular infiltration, numerous Kupffer cells) [29]. The investigators also observed the remodeling in phospholipid fatty acyl chains with a reduction of 16:0 FA and an increase in 18:2 and 18:0 FA in STZ-treated rat liver. By using our newly developed strategy, we could investigate if there are variations in the PC *de novo* biosynthesis pathway though PE methylation by determining the lipid profiles of DMPE and MMPE and if the remodeling is also observed in the fatty acyl chains of DMPE and MMPE species in the liver of STZ-induced diabetic mice at their very early stage (i.e., one week after STZ injection).

After the extracts of liver samples from STZ-induced diabetic mice and their controls were derivatized with CD₃I, we identified the molecular structures of all of the present DMPE and MMPE species by using 2D MS by the improved method. As an example, Figure 3 showed how the methyl PE molecule structures were identified. Since the abundances of DMPE and MMPE were in trace amounts, there was no apparent DMPE or MMPE peaks shown in the survey MS scan for the analysis of lithiated aminoglycerophospholipid species. However, NLS62.1 and 186.1 Da spectra were specific to lithiated methylated DMPE species with enhanced sensitivity. In combination with the NLS192.1 spectrum (which was interfered with methylated PE species), all of the present ions corresponding to DMPE species in the lipid extract could be identified. From the detected m/z values by these spectra, the numbers of their total carbon atoms and double bonds in their aliphatic chains of the DMPE or MMPE molecules could be deduced. Their individual fatty acyl chains were determined by NLS of 62.1 or 65.1 plus the masses of naturally occurring FA for DMPE or MMPE (Figure 3) as aforementioned and by matching the numbers of their total carbon atoms and the total double bonds deduced from the last step. For example, an ion peak at m/z 767.5 was observed in all of NLS62.1, 186.1, and 192.1 mass spectra (indicated with a broken line), which was deduced as a 34:2 DMPE species. The NLS spectra of 62.1 plus the masses of naturally occurring FA showed that this ion peak at m/z 767.5 was also present in the spectra of NLS316.2 (i.e., corresponding to 16:1 FA), NLS318.2 (i.e., to 16:0 FA), NLS342.2 (i.e., to 18:2 FA), and NLS344.2 (i.e., to 18:1 FA). After baseline correction [30] and relative comparison of the intensity of the ion peak at m/z 767.5 among each of the NLS spectra, we determined that this 34:2 DMPE ion peak contains isomeric 18:2-16:0 and 16:1-18:1 DMPE species in a ratio of 35:65.

After quantification of MMPE and DMPE by using the established method and of PC and PE amount by reported methods [21, 25, 31], we found that the levels of PC species had the trends to decrease and the amounts of PE species tended to increase in the liver of STZ-induced diabetic mice comparing to their controls, but not significantly at the examined stage (Table 1). The DMPE and MMPE levels in diabetic mouse liver was 13.13 ± 0.59 and 8.26 ± 0.6 nmol/mg protein comparing to 13.66 ± 0.56 and 8.54 ± 0.17 nmol/mg protein in the control, respectively, which also did not show significant changes of DMPE and MMPE between these two groups of mice. The reason is probably that the determined liver tissues came from the very early stage of diabetes (one week after STZ induction), which is consistent with that the liver had not shown any damage signs since it is believed that the impaired PC biosynthesis was related to the liver damage [32-34]. We also found that the fatty acyl chain levels were altered in the liver tissues of diabetic mice. Specifically, the amounts of 16:1 FA of PC, DMPE, MMPE, and PE species were reduced and the levels of 18:2, 18:1, and 18:0 FA of PC, DMPE, MMPE, PE species were elevated in the liver of STZ-induced diabetic mice comparing to their controls. By using 16:1 and 18:1 FA in DMPE species as examples (Figure 4), we could see that the relative intensities of ion peaks at m/z 765.5, 767.5, 789.5, 791.5, and 813.5 in NLS316.2 (corresponding to 16:1 FA) were decreased significantly after normalization to the internal standard, and that the relative intensities of ion peaks at m/z 793.5, 817.5, and 841.5 in NLS344.2 (corresponding to 18:1 FA) were significantly increased as well in the lipid extracts from the diabetic mouse tissues. The observation was consistent with those early reported [29, 35], likely due to a

combination of synthesis, desaturation, degradation, and oxidation of fatty acids [36]. Interestingly, the remodeling of 16:0 FA of PC, DMPE, MMPE, and PE species were different with its increase in PE, no difference in MMPE, and decrease in DMPE and PC in the liver tissues of diabetic mice in comparison to their controls (spectra not shown). This result might show an early sign of the effects of STZ-induced diabetics on the PC *de novo* biosynthesis through the PE methylation pathway in diabetic mouse liver.

Finally, we could extend three points from the current study. First, this “mass-tag” strategy could also identify and quantify lyso-DMPE, -MMPE, and -PE species contemporarily with lysoPC species in biological samples in spite of their extremely low content. Second, with minor modification, this method could be useful for determination of any lipids containing amine groups in biological samples, such as phosphatidylserine, ceramide phosphoethanolamine, or possible discovery of the intermediates between ceramide phosphoethanolamine and sphingomyelin species. Third, this identification and quantification strategy by using different NLS by a mass offset of 3, 6 and 9 Da could also interfered by their isotopologues with three carbon-13 atoms of the corresponding species. For example, NLS62.1, 186.1, and 192.1 for determination of DMPE could also detect the corresponding $^{13}\text{C}_3$ -PC species. However, in order to detect those isotopologues of PC species by the proposed NLS, all of the three carbon-13 atoms have to be at the head group. This amount of $^{13}\text{C}_3$ -PC species is extremely low, and should not affect the quantification of DMPE. The same reason is also applied to the quantification of MMPE which is possibly interfered from the isotopologues of DMPE species.

In summary, on the basis of the principles of MDMS-SL technology and the reported “mass-tag” strategy, DMPE and MMPE species including their fatty acyl chains and regioisomeric structures in the crude lipid extracts of cellular samples could be identified and quantified simultaneously. By using this method, we investigated alternations of PC, DMPE, MMPE, and PE species in the liver of STZ-induced diabetic mice at the very early stage (one week after STZ injection) with comparison to their controls. The total amounts of the measured PC, DMPE, MMPE, and PE lipid species did not show significant changes between the diabetic and control groups although PC species looked like to decrease and PE species likely increased in the diabetic liver samples. However, we observed the remodeling of fatty acyl chains in these phosphocholine lipids with a reduction of the level of 16:1 FA and increases in the amounts of 18:2, 18:1, and 18:0 fatty acyl chains in diabetic mouse liver. We believed that this improved “mass-tag” method would serve as a powerful tool to understand deeply about the PC *de novo* biosynthesis pathway through methylation of PE species and provide new insight into the pathogenesis of liver diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Representative product ion ESI-MS analyses of lithiated methylated DMPE, MMPE and PE species. The MS/MS analyses of lithiated *d3*-methylated di16:0 DMPE (A), di16:0 MMPE (B), di16:0 PE (C), and 16:0-22:6 PE (D) were performed with collision energy of 35 eV and collision gas pressure of 1.0 mTorr.

Figure 2.

Correlation of the determined concentrations by the established method with the theoretical concentrations of DMPE, MMPE, and PE species. Di14:1 PC used as an internal standard was titrated into a series of equimolar mixture of di16:0 and di18:1 DMPE, di16:0 and di18:1 MMPE, and di16:0, di18:3, and 16:0-22:6 PE at different concentrations varied from 0.02 to 50 times of di14:1 PC. The x- and y-axes represented the prepared concentration (theoretical concentration) and calculated concentration of mixed DMPE, MMPE, and PE after methylation with CD₃I by comparing to the intensity of di14:1 PC after de-isotope (measured concentration).

Figure 3.

Representative 2D-MS analysis of lithiated methylated DMPE species demonstrating how to identify all of individual DMPE species present in lipid extracts of mouse liver samples.

NLS62.1, NLS186.1 and 192.1, and all NLS of FA chains plus trimethylamine were performed at collision energy of 26, 33, and 40 eV, respectively.

Figure 4. Comparison of fatty acyl chain levels of DMPE from lipid extracts of liver tissues of STZ-induced diabetic mice and their controls. Representative MS/MS spectra of NLS316.2 (i.e., corresponding to 16:1 FA, A and B) and NLS344.2 (i.e., corresponding to 18:1 FA, C and D) were acquired from lipid extracts of controls (A and C) and STZ-induced diabetic (B and D) mouse liver samples with addition of LiOH at collision energy of 40 eV. All of the spectra were separately normalized to the internal standard of each sample and then to the base peak in Panel A.

Table 1

Comparison of the amount of PC, DMPE, MMPE, and PE species in lipid extracts of liver samples between STZ-induced diabetic mice and their controls (nmol/mg protein, n = 4). “A”, “D”, and “P” denote alkyl-acyl (i.e., plasmalyl), diacyl (i.e., phosphatidyl), and alkenyl (i.e., vinyl ether-linked aliphatic chain)-acyl (i.e., plasmenyl) species, respectively.

PC			
	PC species	Control	Diabetic
	D16:1-16:0	1.24±0.30	0.59±0.41
	D16:0-16:0	1.24±0.16	1.12±0.22
	P16:0-18:1/P18:1-16:0	0.00±0.00	0.01±0.02
	P16:0-18:0/P18:0-16:0	0.21±0.07	0.17±0.08
	D16:1-18:2	2.65±0.39	2.25±0.26
	D16:0-18:2	26.02±4.70	23.33±3.63
	D16:0-18:1	12.44±1.73	11.66±2.89
	D16:0-18:0	0.10±0.07	0.12±0.09
	P18:0-18:1/P18:1-18:0	1.06±0.32	0.95±0.14
	A18:0-18:1/P18:0-18:0	7.85±1.75	6.75±0.86
	A16:0-20:0	3.29±0.40	3.08±0.84
	D18:2-18:3/D16:1-20:4	1.17±0.17	1.08±0.10
	D18:2-18:2/D16:0-20:4	16.46±1.45	14.35±1.60
	D18:1-18:2/D16:0-20:3	10.04±1.28	8.49±0.91
	D18:0-18:2/D18:1-18:1	12.71±1.43	14.05±2.43
	D18:0-18:1	2.35±0.51	2.28±0.61
	D18:0-18:0	0.24±0.17	0.40±0.41
	D18:2-20:5	1.14±0.21	0.93±0.26
	D16:0-22:6/D18:2-20:4	15.64±1.46	13.71±1.56
	D18:1-20:4/D16:0-22:5	5.77±0.46	5.52±0.25
	D18:2-20:2/D18:0-20:4	8.92±1.58	11.76±2.58
	D18:0-20:3	2.55±0.78	2.42±0.63
	D18:0-20:2/P18:2-22:6	0.77±0.30	0.84±0.44
	D18:0-20:1/P18:1-22:6	0.33±0.27	0.26±0.20
	D18:2-22:6	1.06±0.15	0.77±0.15
	D18:1-22:6/D18:2-22:5	2.46±0.37	2.26±0.48
	D18:0-22:6	3.42±0.40	4.36±0.89
	D18:0-22:5	0.55±0.24	0.55±0.09
	D18:0-22:4/D20:0-20:4/D20:2-20:2	0.20±0.23	0.28±0.27
	D18:0-22:3/D20:0-20:3/D18:2-22:1	0.07±0.08	0.09±0.15
	Total	141.94±3.36	134.41±6.57
DMPE			
	DMPE species	Control	Diabetic

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D16:1-16:0	0.12±0.06	0.08±0.05
D16:0-16:0	0.19±0.02	0.11±0.03
D16:1-18:2	0.23±0.02	0.22±0.05
D18:2-16:0/D16:1-18:1	2.62±0.37	2.37±0.16
D16:0-18:1	1.45±0.29	1.30±0.33
D16:0-18:0	0.05±0.05	0.01±0.01
D18:2-18:3/D16:1-20:4	0.15±0.10	0.10±0.06
D18:2-18:2/D16:0-20:4/D16:1-20:3	1.66±0.26	1.56±0.07
D18:1-18:2/D16:0-20:3	1.06±0.29	0.91±0.19
D18:0-18:2/D18:1-18:1/D16:0-20:2	1.47±0.08	1.53±0.32
D18:0-18:1	0.33±0.19	0.32±0.06
D18:0-18:0	0.06±0.04	0.08±0.11
D16:1-22:6	0.12±0.07	0.07±0.05
D16:0-22:6/D18:2-20:4	1.51±0.14	1.30±0.04
D18:1-20:4/D18:2-20:3/D16:0-22:5	0.56±0.08	0.58±0.15
D18:0-20:4/D18:1-20:3	1.11±0.19	1.41±0.25
D18:0-20:3	0.22±0.08	0.24±0.08
D18:0-20:2	0.08±0.04	0.11±0.11
D18:0-20:1	0.01±0.02	0.03±0.03
D18:2-22:6	0.06±0.03	0.07±0.03
D18:1-22:6	0.24±0.04	0.24±0.05
D18:0-22:6	0.30±0.08	0.45±0.10
D18:0-22:5	0.05±0.04	0.06±0.06
Total	13.66±0.56	13.13±0.59

MMPE

MMPE species	Control	Diabetic
D16:1-16:0	0.08±0.02	0.06±0.01
D16:0-16:0	0.18±0.02	0.13±0.06
D16:1-18:2	0.19±0.02	0.14±0.04
D18:2-16:0/D16:1-18:1	1.56±0.22	1.43±0.16
D16:0-18:1	0.94±0.10	0.82±0.19
D16:0-18:0	0.01±0.03	0.04±0.01
D18:2-18:3/D16:1-20:4	0.09±0.04	0.09±0.03
D18:2-18:2/D16:0-20:4/D16:1-20:3	1.07±0.13	0.91±0.07
D18:1-18:2/D16:0-20:3	0.68±0.17	0.57±0.14
D18:0-18:2/D18:1-18:1/D16:0-20:2	0.85±0.09	0.94±0.11
D18:0-18:1	0.17±0.08	0.18±0.09
D18:0-18:0	0.00±0.01	0.04±0.05
D16:1-22:6	0.08±0.06	0.04±0.01
D16:0-22:6/D18:2-20:4	0.90±0.07	0.90±0.16
D18:1-20:4/D18:2-20:3/D16:0-22:5	0.36±0.08	0.39±0.05

D18:0-20:4/D18:1-20:3	0.61±0.17	0.78±0.14
D18:0-20:3	0.17±0.04	0.21±0.07
D18:0-20:2	0.06±0.04	0.06±0.09
D18:0-20:1	0.07±0.10	0.02±0.02
D18:2-22:6	0.04±0.02	0.05±0.04
D18:1-22:6	0.17±0.07	0.12±0.02
D18:0-22:6	0.23±0.12	0.30±0.02
D18:0-22:5	0.02±0.02	0.04±0.03
Total	8.54±0.17	8.26±0.6

PE

PE species	Control	Diabetic
P18:1-16:0/P16:0-18:1	0.05±0.04	0.02±0.02
D16:1-18:2	0.30±0.12	0.10±0.07
D16:0-18:2/D16:1-18:1	2.78±0.56	2.48±0.31
D16:0-18:1	0.93±0.08	0.74±0.26
P14:0-22:6	0.00±0.01	0.01±0.02
P16:0-20:4	1.38±0.39	1.57±0.17
P16:0-20:3/P18:1-18:2	0.10±0.06	0.13±0.06
P18:1-18:1/P18:0-18:2/P16:0-20:2	0.04±0.03	0.05±0.03
P18:0-18:1/P16:0-20:1	0.19±0.07	0.18±0.06
D16:1-20:4	0.61±0.17	0.41±0.08
D16:0-20:4/D18:2-18:2	5.24±0.52	5.27±0.43
D18:1-18:2/D16:0-20:3	2.14±0.28	2.21±0.23
D18:0-18:2/D18:1-18:1/D16:0-20:2	1.95±0.27	2.50±0.82
D18:0-18:1/D16:0-20:1	0.24±0.04	0.27±0.11
D18:0-18:0/P16:0-22:6/P18:2-20:4	0.65±0.13	0.74±0.12
P18:1-20:4/P16:0-22:5	0.73±0.28	0.80±0.09
P18:0-20:4/P16:0-22:4/P18:1-20:3	1.00±0.27	1.18±0.02
P18:0-20:3	0.45±0.06	0.45±0.03
P20:0-18:0/P18:0-20:0	0.45±0.08	0.78±0.28
D16:1-22:6	0.55±0.13	0.26±0.05
D16:0-22:6	10.30±1.14	10.72±1.50
D18:1-20:4/D16:0-22:5	4.80±0.52	5.25±0.41
D18:0-20:4/D16:0-22:4	11.13±0.95	13.93±2.45
D18:0-20:3/D18:1-20:2/D16:0-22:3	0.72±0.09	1.02±0.21
D18:1-20:1/P18:2-22:6	0.20±0.04	0.27±0.23
D18:0-20:1/P18:1-22:6	0.24±0.06	0.23±0.11
D18:0-20:0/P18:0-22:6/P18:1-22:5	0.30±0.10	0.37±0.04
P18:0-22:5/P18:1-22:4	0.45±0.14	0.46±0.06
D18:2-22:6	0.22±0.07	0.30±0.17
D18:1-22:6	2.44±0.47	2.78±0.37

D18:0-22:6/D18:1-22:5	2.54±0.47	3.64±1.19
D18:0-22:5/D18:1-22:4	0.53±0.11	0.66±0.17
D20:0-20:4/D18:0-22:4	0.31±0.10	0.50±0.24
D20:0-20:3/D18:0-22:3	0.18±0.01	0.15±0.15
D20:0-20:2/D18:0-22:2	0.07±0.04	0.34±0.46
Total	54.21±5.96	60.79±6.67