Analysis of cell membrane aminophospholipids as isotope-tagged derivatives

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Abstract **Glycerophosphoethanolamine (GPEtn) and glycerophosphoserine (GPSer) lipids were reacted with a multiplexed set of differentially isotopically enriched *N*-methylpiperazine acetic acid *N*-hydroxysuccinimide ester reagents, which place isobaric mass labels at a primary amino group. The resulting derivatized aminophospholipids were isobaric and chromatographically indistinguishable but yielded positive reporter ions (*m/z* 114 or 117) after collisional activation that could be used to identify and quantify individual members of the multiplex set. The chromatographic and mass spectrometric response of *N*-methylpiperazine amide-tagged aminophospholipids was probed using glycerophosphoethanolamine and glycerophosphoserine lipid standards. The $[M+H]^+$ of each tagged aminophospholipid shifted 144 Da, and during collision-induced dissociation the major fragmentation ion was either *m/z* 114 or 117. This mode of detecting aminophospholipids was useful for an unbiased analysis of plasmalogen GPEtn lipids. Molecular species information on the esterified fatty acyl substituents was obtained by collisional activation of the $[M-H]^-$ ions. The isotope-tagged reagents were used to assess changes in the distribution of GPEtn lipids after exposure of liposomes made from phospholipids extracted from RAW 264.7 cells to Cu^{2+}/H_2O_2 to illustrate the ability of these reagents to aid in the mass spectrometric identification of aminophospholipid changes that occur during biological stimuli.**—Zemski Berry, K. A., and R. C. Murphy. **Analysis of cell membrane aminophospholipids as isotope-tagged derivatives. *J. Lipid Res.* 2005.** 46: **1038–1046.**

Supplementary key words glycerophosphoethanolamine lipids • glycerophosphoserine lipids • electrospray • tandem mass spectrometry • lipid oxidation

Phospholipids are building blocks of cellular membranes, but their role in biochemistry is only partially understood. Glycerophospholipids form the lipid bilayer of cells and are in a critical position to protect the intracellular contents from chemically reactive oxygen/nitrogen free radical species that are formed in a wide variety of biological reactions. The oxidation of phospholipids in biological membranes has been implicated in a variety of human diseases, such as atherosclerosis (1), ischemia (2), carcinogenesis (3), and Alzheimer's disease (4), and the lipid peroxidation products formed have been used to assess the severity of each of these disease states. In fact, some of these phospholipid oxidation products are potent, biologically active compounds (5, 6).

Electrospray tandem mass spectrometry is a sensitive means to achieve detailed structural analysis of glycerophospholipids at a physiologically relevant concentration range. Collision-induced dissociation (CID) of the $[M+H]^+$ or $[M-H]^-$ of glycerophospholipids results in fragmentation ions that are related to the polar head group or the fatty acyl substituents esterified to the glycerol backbone, respectively (7). This characteristic CID behavior of phospholipids in the positive ion mode coupled with reversed-phase (RP) HPLC separation allows for the specific detection of a certain class of phospholipids of interest and can be used as a tool to analyze changes in phospholipids after various biological stimuli by comparison of the HPLC separation and tandem mass spectrometry (LC/MS/MS) traces of experimental and control samples. Although this is a useful technique, the samples are complex and the data are usually overwhelmed by the phospholipid species that do not change during the reaction, which makes it difficult to assess minor phospholipid changes that do occur. However, this information is vital in terms of understanding the fate of phospholipids during biological reactions.

Recently, four different isotopically enriched *N*-methylpiperazine acetic acid NHS (*N*-hydroxysuccinimide) ester reagents were developed that place isobaric mass labels at the N terminus and lysine side chains of peptides in a digest mixture and allow for protein quantification (8). The resulting derivatized peptides were isobaric and chromatographically indistinguishable but yielded reporter ions during CID in the positive ion mode that could be used to identify and quantify individual members of the multiplex set. One of the advantages of these reagents was that multiple peptides were labeled per protein, because the NHS ester readily reacted with any primary amino group.

Manuscript received 12 January 2005 and in revised form 14 February 2005.

Published, JLR Papers in Press, February 16, 2005.
DOI 10.1194/jlr.M500014-JLR200

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Because these *N*-methylpiperazine acetic acid NHS ester reagents react with primary amines, it was thought that phospholipids that contain primary amino groups, such as glycerophosphoethanolamine (GPEtn) and glycerophosphoserine (GPSer) lipids, could be modified using these reagents and that this modification could aid in the mass spectrometric identification of phospholipid changes that occur during biological stimuli. In the current study, *N*-methylpiperazine amide-tagged 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine standards were synthesized and used to study the chromatographic and mass spectrometric responses of *N*-methylpiperazine amide-tagged primary amine-containing phospholipids. Additionally, the *N*-methylpiperazine acetyl amide derivative was used to assess changes that occurred in the distribution of GPEtn lipids after exposure of liposomes made from phospholipids extracted from RAW 264.7 cells to Cu^{2+}/H_2O_2 to determine the feasibility of these reagents for primary amine-containing phospholipids.

EXPERIMENTAL PROCEDURES

Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (16:0a/18:1-GPEtn), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (16:0a/18:1-GPSer), and 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine (17:0a/17:0-GPEtn) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The 114 and 117 *N*-methylpiperazine acetic acid NHS ester reagent (iTRAQ) development kit was obtained from Applied Biosystems (Foster City, CA). HBSS (1×) was obtained from Invitrogen (Carlsbad, CA). Copper (II) chloride, 30% H_2O_2, and sodium borohydride were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC solvents were purchased from Fisher Scientific (Fair Lawn, NJ) and used for HPLC and extraction.

Synthesis of *N*-methylpiperazine amide-tagged GPEtn and GPSer phospholipids

16:0a/18:1-GPEtn (20 μg) and 16:0a/18:1-GPSer (20 μg) in chloroform were taken to dryness under a stream of nitrogen. The glycerophospholipids were resuspended in 50:50 (v/v) ethanol/0.5 M triethylammonium bicarbonate buffer (30 μl). One vial of the 114 (117) *N*-methylpiperazine acetic acid NHS ester reagent in ethanol (70 μl) was then added to 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer and allowed to incubate at room temperature for 1 h. Any remaining 114 (117) *N*-methylpiperazine acetic acid NHS ester reagent was hydrolyzed over 30 min by the addition of water (1 ml). The glycerophospholipid mixture was introduced onto a conditioned and rinsed C_{18} solid-phase extraction column (Supelco, Bellefonte, PA) to remove the excess 114 (117) *N*-methylpiperazine acetic acid NHS ester reagent and salts. The *N*-methylpiperazine amide-tagged glycerophospholipids were eluted with 2:1 (v/v) methanol-chloroform, taken to dryness under a stream of nitrogen, and resuspended in 60:20:20 (v/v/v) methanol-acetonitrile-water with 1 mM ammonium acetate for mass spectrometric analysis.

Electrospray ionization tandem quadrupole mass spectrometry

The 114 (117) *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer phospholipids were infused into a Sciex API 2000 triple quadrupole mass spectrometer (PE Sciex, Toronto, Canada) at a flow rate of 5 μl/min. The relevant experimental parameters in the positive ion mode for both full-scan and CID experiments were an electrospray voltage of 5,000 V, a declustering potential of 50 V, and a focusing potential of 350 V. In the negative ion mode, the experimental parameters for CID experiments were an electrospray voltage of −3,900 V, a declustering potential of −40 V, and a focusing potential of −350 V. The full mass and precursors of *m/z* 114 (117) spectra were obtained by scanning from *m/z* 550 to 1,200. The collisional offset used in the precursors of *m/z* 114 (117) scans was 88 V. The CID mass spectra of 114 (117) *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer were acquired in the positive and negative ion modes at 3 s/scan over the mass range from *m/z* 50 to 950 with the collisional offset reported in the figure legends. Additionally, the CAD gas setting was 7 for all of the CID experiments reported here.

Chromatography

The different lipid classes present in the *N*-methylpiperazine amide-tagged standard mixture were separated using normal-phase (NP) HPLC with an Ultremex 5μ Si (4.6 × 250 mm) column (Phenomenex, Torrance, CA). The NP solvents used for the separation of phospholipid classes were 30:40 hexane-2-propanol (solvent A) and 30:40:7 hexane-2-propanol-water with a final concentration of 1 mM ammonium acetate (solvent B). The initial mobile phase was 47% solvent B at a flow rate of 1 ml/min. This initial mobile phase was held for 6 min, and then a linear gradient was started to 100% solvent B in 20 min. This was followed by isocratic elution at 100% solvent B for 34 min. Detection of the 114 (117) *N*-methylpiperazine amide-tagged phospholipids was determined using a precursors of *m/z* 114 (117) scan, and a postcolumn split was used to yield 50 μl/min into the mass spectrometer and 950 μl/min into a fraction collector, which collected one fraction per minute. The NP fractions of interest that contained *N*-methylpiperazine amide-tagged GPEtn and GPSer species were separated according to lipophilicity by RP-HPLC with a Columbus 5μ C_{18} (2.0 × 150 mm) column (Phenomenex). The RP solvents used for the separation of *N*-methylpiperazine amide-tagged GPEtn or GPSer species according to lipophilicity were 60:20:20 methanol-acetonitrile-water with 1 mM ammonium acetate (solvent A) and 1 mM methanolic ammonium acetate (solvent B). The initial mobile phase was 0% solvent B at a flow rate of 0.2 ml/min. This initial mobile phase was held for 2 min, and then a linear gradient was started to 100% solvent B in 40 min. This was followed by isocratic elution at 100% solvent B for 28 min. The *N*-methylpiperazine amide-tagged phospholipids were detected during the chromatography run using a precursors of *m/z* 114 (117) scan.

RAW 264.7 cells

The cells used in these experiments were the RAW 264.7 macrophage cell line that was obtained from the American Type Culture Collection. In brief, the cells were grown in an incubator with a 5% CO_2 humidified atmosphere maintained at 37°C. The cells were grown to 80% confluence, at which time they were harvested and used in the experiments described below.

Preparation of liposomes from lipids extracted from RAW 264.7 cells and oxidation procedure

Lipids were extracted from RAW 264.7 cells (120×10^6) by the addition of chloroform-methanol according to the method of Bligh and Dyer (9). The phospholipids were extracted twice with chloroform, and the organic layer was taken to dryness under a stream of nitrogen and resuspended in 30:40 (v/v) hexane-2-propanol (500 μl). The phospholipids extracted from RAW 264.7 cells

were then split equally into two different test tubes, taken to dryness under a stream of nitrogen, and resuspended in HBSS (1 ml). Small, unilamellar vesicles were prepared by vortexing the phospholipid suspension for 30 min followed by sonication for 20 min. Copper (II) chloride and 30% H_2O_2 were added to the phospholipid suspension in one of the test tubes for final concentrations of 100 μM and 70 mM, respectively. The control and Cu^{2+}/H_2O_2-treated liposomes were incubated at 37°C for 1 h, and the phospholipids were extracted from each sample by the method of Bligh and Dyer (9). The samples were dried down under a stream of nitrogen, and a saturated solution of $NaBH_4$ in ethanol (1 ml) was added to both the control and Cu^{2+}/H_2O_2-treated samples and allowed to incubate on ice for 30 min to reduce the aldehydes present in the sample (10). After this incubation, 17:0a/17:0-GPEtn (10 μg) was added as an internal standard to each test tube, and the control and Cu^{2+}/H_2O_2-treated samples were loaded onto separate C_{18} SepPak columns that were preconditioned with methanol and then rinsed with water. The excess Cu^{2+}, H_2O_2, and $NaBH_4$ were rinsed from the columns with water, and the phospholipids were eluted with 2:1 (v/v) methanol-chloroform. These fractions were taken to dryness under a stream of nitrogen and resuspended in chloroform (200 μl). The control and Cu^{2+}/H_2O_2-treated samples were then introduced onto separate aminopropyl SepPak columns (Supelco) that were conditioned with hexane to separate phospholipids by classes by a method proposed by Kim and Salem (11). The neutral lipids were eluted using 2:1 (v/v) chloroform-2-propanol (4 ml), and methanol (4 ml) was then added to the column to elute the GPEtn and glycerophosphocholine (GPCho) lipids, which were collected. The GPEtn lipids in the control sample were then labeled with the 114 *N*-methylpiperazine acetic acid NHS ester reagent, whereas the GPEtn lipids in the Cu^{2+}/H_2O_2-treated sample were labeled with the 117 *N*-methylpiperazine acetic acid NHS ester reagent, according to the protocol described above for GPEtn and GPSer standards. After the reaction of *N*-methylpiperazine acetic acid NHS ester reagent with GPEtn lipids was complete, the *N*-methylpiperazine amide-tagged control and Cu^{2+}/H_2O_2-treated samples were combined and then added onto a C_{18} SepPak column to remove excess reagent. To separate the GPCho and *N*-methylpiperazine amide-labeled GPEtn lipids, NP-HPLC was performed as described above and the *N*-methylpiperazine amide-tagged GPEtn species were detected by a precursors of *m/z* 117 scan. The NP fractions of interest that contained *N*-methylpiperazine amide-tagged GPEtn were subjected to RP-HPLC as described above to establish the difference in the GPEtn phospholipids present in the control compared with the Cu^{2+}/H_2O_2-treated liposomes. Specifically, two separate RP-HPLC runs were performed in which a precursors of *m/z* 114 scan was used to detect the GPEtn in the control sample and a precursors of *m/z* 117 scan was used to detect the GPEtn in the Cu^{2+}/H_2O_2 sample. After the data from these two separate chromatographic runs were obtained, the two traces were compared to assess the changes that occurred in GPEtn distribution upon exposure to Cu^{2+}/H_2O_2.

RESULTS

The products formed after the incubation of 16:0a/18:1-GPEtn (717 Da) and 16:0a/18:1-GPSer (761 Da) standards with the 114 *N*-methylpiperazine acetic acid NHS ester reagent were assessed by mass spectrometric analysis. Before 114 *N*-methylpiperazine amide tagging of the phospholipid standards, the abundant $[M+H]^+$ ions observed in the Q3 full-scan positive ion mass spectrum were at *m/z* 718 and 762 for 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer, respectively.[2] After the reaction of these phospholipid standards with the 114 *N*-methylpiperazine acetic acid NHS ester reagent, the abundant $[M+H]^+$ ions shifted to *m/z* 862 and 906 (data not shown), which corresponded to the addition of 145 Da onto the neutral phospholipid species. These $[M+H]^+$ ions at *m/z* 862 and 906 were also observed upon reaction of 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer standards with the 117 *N*-methylpiperazine acetic acid NHS ester reagent. Additionally, the $[M+H]^+$ at *m/z* 718 and 762 were no longer observable in the full-scan positive ion Q3 mass spectrum. From these data, it was determined that quantitative conversion of the primary amine phospholipid standards to 114 (117) *N*-methylpiperazine amide-tagged species was achieved.

Once new ions were observed that corresponded to 114 *N*-methylpiperazine amide-tagged GPEtn and GPSer lipids, the CID behavior of these ions in the positive ion mode was probed. At lower collision energies (50 V), the 114 reporter ion was not the major ion in the positive ion CID spectra of 114 *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer. Instead, the major fragmentation ions present in the positive ion CID spectrum of 114 *N*-methylpiperazine-tagged 16:0a/18:1-GPEtn were at *m/z* 286 and 577, whereas for 114 *N*-methylpiperazine amide-tagged 16:0a/18:1-GPSer the major fragmentation ions were observed at *m/z* 330 and 577. The *m/z* 577 ion was also observed during low-energy CID of nontagged 16:0a/18:1-GPEtn $[M+H-141]^+$ and 16:0a/18:1-GPSer $[M+H-185]^+$ in the positive ion mode (12). In both cases, this ion was likely generated from cleavage of the phosphate-glycerol bond with the site of protonation at the *sn*-2 ester, which resulted in the elimination of the polar head group as a neutral species (13). The ions present at *m/z* 286 and 330 in the CID spectrum of 114 *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer at 50 V corresponded to cleavage of the phosphate-glycerol bond with the site of protonation on the secondary amine at the *N*-methylpiperazine moiety. The equivalents of these fragmentation ions were not observed in the CID of nontagged 16:0a/18:1-GPEtn or 16:0a/18:1-GPSer. When the collision energy was increased to 88 V, the major fragmentation ions present in the positive ion CID spectra of both 114 *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer occurred at *m/z* 114 (**Fig. 1**). Additionally, the CID behavior of the $[M+H]^+$ of 117 *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer standards was also investigated. In this case, the same fragmentation ions were observed under low-energy CID conditions (50 V), but the major ion observed during CID at 88 V occurred at *m/z* 117.

Before 114 *N*-methylpiperazine amide tagging of the phospholipid standards, the abundant $[M-H]^-$ ions ob-

[2] The exact masses of naturally occurring aminophospholipids typically have mass defects ranging from 0.5 to 0.7 amu as a result of the total number of hydrogen atoms. Here, we refer to the observed mass-to-charge ratios as the nominal mass rather than the exact mass measured.

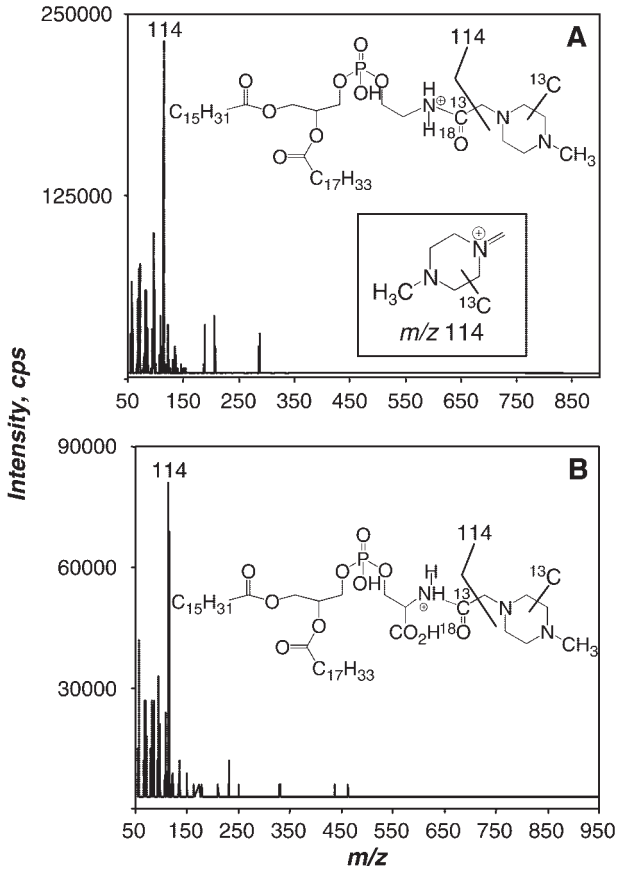

Fig. 1. Positive ion collision-induced dissociation (CID) spectra of the $[M+H]^+$ of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (A) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (B) 114 *N*-methylpiperazine amide-tagged standards at a collision energy of 88 V. The origins of the ions that resulted from collisional activation are indicated in the structures of these molecules. The positions of isotopes in the amide are labeled in the structures, but the location of the carbon-13 in the piperazine ring has not been disclosed; it is indicated as somewhere in this portion of the molecule for simplicity. The inset shows the suggested structure of the product ion *m/z* 114, which contains a single carbon-13 atom.

served in the Q3 full-scan negative ion mass spectrum were at *m/z* 716 and 760 for 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer, respectively. After the reaction of these phospholipid standards with the 114 *N*-methylpiperazine acetic acid NHS ester reagent, the abundant $[M-H]^-$ ions shifted to *m/z* 860 and 904 (data not shown). Additionally, these same $[M-H]^-$ ions at *m/z* 860 and 904 were also observed upon reaction of 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer standards with the 117 *N*-methylpiperazine acetic acid NHS ester reagent. Tandem mass spectrometry of diacyl phospholipids in the negative ion mode provides information on the fatty acids esterified to the *sn*-1 and *sn*-2 positions of the glycerol backbone and is typically used for detailed structural identification of phospholipids. Therefore, the negative ion CID behavior of these 114 *N*-methylpiperazine amide-tagged primary amine-containing phospholipid standards was probed.

The negative ion CID spectrum of the $[M-H]^-$ at *m/z* 860 of the 114 *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn had two major fragmentation ions at *m/z* 255 and 281 (**Fig. 2A**), which are present in the CID spectrum of nontagged 16:0a/18:1-GPEtn (12) and correspond to the carboxylate ions of palmitic acid and oleic acid, respectively. Additionally, the negative ion CID spectrum of 114 *N*-methylpiperazine amide-tagged 16:0a/18:1-GPSer had major fragmentation ions at *m/z* 255, 281, 391, and 673 (Fig. 2B), which are present in the CID spectrum of nontagged 16:0a/18:1-GPSer (12). The fragmentation ions that occur at *m/z* 673 and 391 correspond to the neutral loss of serine and the 114 *N*-methylpiperazine amide tag and the $[M\text{-}C_{17}H_{33}COOH\text{-}88\text{-}N\text{-methylpiperazine amide tag}]^-$, respectively. Additionally, the CID behavior of the $[M-H]^-$ of 117 *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer standards was also investigated. In this case, the same fragmentation ions were observed in the CID spectra of the $[M-H]^-$ of both the 114 and 117 *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer standards. Therefore, the negative ion CID behavior of both the 114 and 117 *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer was unaltered in comparison with the nontagged CID spectra, which would be beneficial in terms of being able

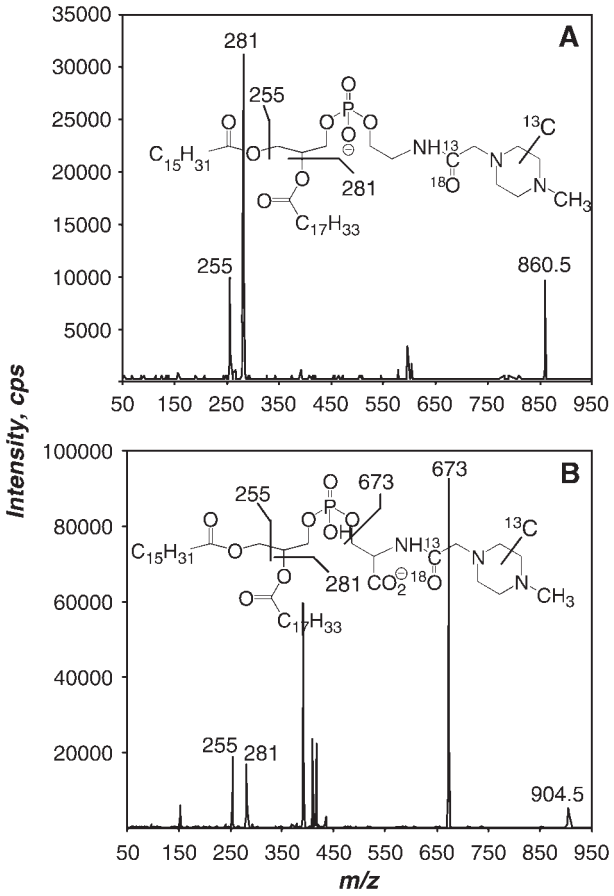

Fig. 2. Negative ion CID spectra of the $[M-H]^-$ of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (A) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (B) 114 *N*-methylpiperazine amide-tagged standards at a collision energy of 40 V. The origins of the ions that resulted from collisional activation are indicated in the structures of these molecules.

to structurally identify novel *N*-methylpiperazine amide-tagged phospholipids.

NP-HPLC separates phospholipids by class on the basis of head group polarity, and for the NP-HPLC solvents used in this study, the neutral lipids elute first, followed by GPEtn (11 min), glycerophosphoinositol lipids (GPIns) (13 min), GPSer (19 min), and GPCho (30 min) (14). The NP behavior of 114 (117) *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer was studied using mass spectrometric detection by a precursors of *m/z* 114 (117) scan. The elution of 114 (117) *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer occurred at 38 and 47 min, respectively. It was clear that upon addition of the *N*-methylpiperazine amide tag to primary amine-containing phospholipids, the NP retention times were extended dramatically compared with those of the nontagged primary amine phospholipids because the head group now contained a *N*-methylpiperazine moiety.

Even though NP-HPLC does separate a phospholipid mixture into the different phospholipid classes, the molecular species present in each phospholipid class are still rather complex. Therefore, RP-HPLC is typically used after NP-HPLC to separate molecular species from a class of phospholipids based on lipophilicity. The RP behavior of 114 (117) *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer was studied using mass spectrometric detection by a precursors of *m/z* 114 (117) scan. The elution of 114 (117) *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer occurred at 47 and 42 min, respectively. The retention times of nontagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer compared with 114 (117) *N*-methylpiperazine amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer did not differ because the elution sequence in RP-HPLC was dominated by the lipophilicity of the fatty acyl chains esterified to the glycerol backbone and not the head group of the phospholipid.

Once the mass spectrometric, CID, and chromatographic behavior of 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer standards tagged with the 114 (117) *N*-methylpiperazine acetic acid NHS ester reagent was established, the Q3 full-scan spectra of GPEtn phospholipids extracted from RAW 264.7 cells with and without the 114 (117) *N*-methylpiperazine amide tag were obtained to determine whether these reagents would work well for complex GPEtn mixtures from biological samples (**Fig. 3**). The $[M+H]^+$ for nontagged GPEtn phospholipids extracted from RAW 264.7 cells were observed in the positive ion Q3 mass spectrum from *m/z* 690 (32a:1-GPEtn) to *m/z* 792 (40a:6-GPEtn) (Fig. 3A). After tagging GPEtn phospholipids extracted from RAW 264.7 cells with the 114 (117) *N*-methylpiperazine acetic acid NHS ester reagent, the $[M+H]^+$ of the tagged GPEtn phospholipids shifted by 144 Da without altering the ion distribution profile. The $[M+H]^+$ observed after tagging with the 114 (117) *N*-methylpiperazine acetic acid NHS ester reagent ranged from *m/z* 834 to *m/z* 936 (Fig. 3B).

RAW 264.7 cells have abundant ether GPEtn phospholipids with either a 1-*O*-alkyl group or a vinyl ether group (plasmalogen species) at the *sn*-1 position. Importantly, the positive ion CID behavior of nontagged 1-*O*-alk-1′-enyl-2-acyl-GPEtn is significantly different from that of 1-*O*-alkyl-2-acyl- or diacyl-GPEtn species, making detection of the plasmalogen species somewhat problematic (15). A neutral loss of 141 Da scan can be used to detect 1-*O*-alkyl-2-acyl-

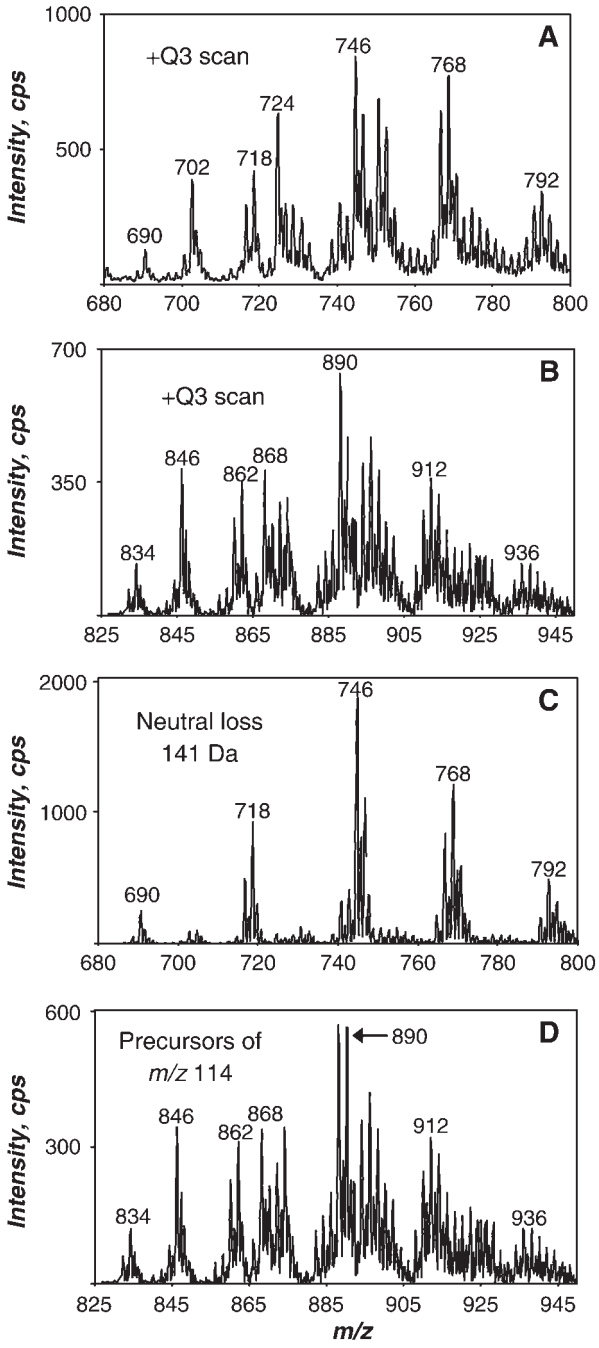

Fig. 3. A: Positive ion electrospray mass spectrum of the glycerophosphatidylethanolamine (GPEtn) species present in RAW 264.7 cells. The ions correspond to $[M+H]^+$ of each GPEtn present. B: Positive ion electrospray mass spectrum of the 114 *N*-methylpiperazine amide-tagged GPEtn species present in RAW 264.7 cells. Note the shift in the $[M+H]^+$ in the *N*-methylpiperazine amide-tagged GPEtn species by 144 Da. C: Neutral loss of 141 Da scan to analyze GPEtn present in RAW 264.7 cells. D: Precursor ion scan of *m/z* 114 allows for specific detection of *N*-methylpiperazine amide-tagged GPEtn species in RAW 264.7 cells.

or diacyl-GPEtn species (Fig. 3C). This neutral loss of 141 Da scan cannot be used to detect 1-*O*-alk-1′-enyl-2-acyl-GPEtn, which is apparent upon comparison of Fig. 3A, C. For example, when positive ion electrospray ionization was performed with the partially purified GPEtn lipid fraction, an abundant species was detected at *m/z* 702 (Fig. 3A). However, the neutral loss of 141 Da scan did not suggest that *m/z* 702 was an abundant GPEtn molecular species (Fig. 3C). Collisional activation of the corresponding negative ion $[M-H]^-$ at *m/z* 700 for this GPEtn species (**Table 1**) did reveal a single carboxylate anion at *m/z* 281 (18:1), consistent with 1-*O*-hexadec-1′-enyl-2-oleoyl-glycerophosphoethanolamine (16:0p/18:1-GPEtn) having a molecular mass of 701 Da. Direct evidence for the presence of this GPEtn species could be obtained from the *N*-methylpiperazine amide-tagged GPEtn molecular species at *m/z* 846 (Fig. 3B), which could also be detected as a precursor ion of *m/z* 114 (Fig. 3D). The same can be observed for *m/z* 724, which is an arachidonate-containing plasmalogen GPEtn that is best detected as the derivative at *m/z* 868 (Fig. 3D). All subclasses (1-*O*-alk-1′-enyl-2-acyl-, 1-*O*-alkyl-2-acyl-, or diacyl-) of *N*-methylpiperazine amide GPEtn species can be detected using a precursors of *m/z* 114 (117) scan (Fig. 3D).

The efficiency of tagging of a complex mixture of cellular lipids was also probed using the GPEtn extracted from RAW 264.7 cells. We found by mass spectrometric analysis that all GPEtn lipids were converted into 114 (117) *N*-methylpiperazine amide-tagged species (data not shown). CID in the negative ion mode was performed on the corresponding $[M-H]^-$ of the $[M+H]^+$ ions indicated in Fig. 3A, B (Table 1). The ions observed in the negative ion CID spectra of nontagged and *N*-methylpiperazine amide GPEtn species aided in identifying the radyl groups present at the *sn*-1 and *sn*-2 positions of the glycerol backbone. Therefore, it was determined that tagging of primary amine-containing phospholipids with the 114 (117) *N*-methylpiperazine acetic acid NHS ester reagents works well for complex mixtures extracted from biological samples and also permitted the structural identification of these phospholipids by CID in the negative ion mode.

TABLE 1. Summary of the CID fragmentation in the negative ion mode of the $[M-H]^-$ of nontagged and *N*-methylpiperazine amide-tagged GPEtn-labeled ions in Fig. 5A, B

Nontagged $[M-H]^-$	*N*-Methylpiperazine Amide-Tagged $[M-H]^-$	CID Product Ions	Identity of Phospholipid
	m/z		
688	832	253 255	16:0a/16:1-GPEtn
700	844	281	16:0p/18:1-GPEtn
716	860	255 281	16:0a/18:1-GPEtn
722	866	303	16:0p/20:4-GPEtn
744	888	281 283	18:0a/18:1-GPEtn
766	910	283 303	18:0a/20:4-GPEtn
790	934	283 327	18:0a/22:6-GPEtn

Nominal mass is presented, not the exact mass. CID, collision-induced dissociation; GPEtn, glycerophosphatidylethanolamine lipids.

Liposomes generated from phospholipids extracted from RAW 264.7 cells (120×10^6) were used to determine whether these 114 (117) *N*-methylpiperazine acetic acid NHS ester reagents would facilitate the detection of changes in GPEtn distribution after oxidation. In this particular set of experiments, the control and Cu^{2+}/H_2O_2-treated samples were treated identically except for the addition of the Fenton chemistry reagents to the oxidized samples. After incubation of the control and Cu^{2+}/H_2O_2-treated liposomes at 37°C for 1 h, the phospholipids were extracted and a saturated solution of sodium borohydride in ethanol was added to the control and oxidized samples and allowed to incubate on ice for 30 min to reduce all of the aldehydes present. At this time, an internal standard (17:0a/17:0-GPEtn) was added to each sample. The neutral glycerophospholipids (GPEtn and GPCho) were then separated from the acidic glycerophospholipids (GPSer and GPIns) in the control and Cu^{2+}/H_2O_2-treated samples using an aminopropyl solid-phase extraction column. The GPEtn lipids in the control sample were then labeled with the 114 *N*-methylpiperazine acetic acid NHS ester reagent, whereas the GPEtn lipids in the Cu^{2+}/H_2O_2-treated sample were labeled with the 117 *N*-methylpiperazine acetic acid NHS ester reagent. After formation of the *N*-methylpiperazine amide GPEtn species, the control and Cu^{2+}/H_2O_2-treated samples were mixed together and subjected to NP-LC/MS/MS to isolate the *N*-methylpiperazine amide-tagged GPEtn. The *N*-methylpiperazine amide-tagged GPEtn-oxidized species were detected using a precursors of *m/z* 117 scan while fractions were collected. The NP fractions of interest that contained *N*-methylpiperazine amide-tagged GPEtn were subjected to RP-HPLC to separate GPEtn phospholipid molecular species present in the control liposomes compared with Cu^{2+}/H_2O_2-treated liposomes. This was achieved by performing two separate RP-HPLC experiments in which a precursors of *m/z* 114 scan was used to detect the *N*-methylpiperazine amide-tagged GPEtn in the control sample and a precursors of *m/z* 117 scan was used to detect the *N*-methylpiperazine amide-tagged GPEtn in the Cu^{2+}/H_2O_2-treated sample. After the data from these two separate RP-HPLC experiments were obtained, the two traces were compared to assess the changes that occurred in GPEtn distribution upon exposure to Cu^{2+}/H_2O_2. It is understood that comparing the intensity of chromatographic peaks in two separate runs is less than optimal, but the internal standard was used to normalize the data.

Upon comparison of the two separate precursors of *m/z* 114 and 117 RP-HPLC traces, it was clear that there were two areas of interest in the chromatogram where the *N*-methylpiperazine amide GPEtn species showed changes. The first area of interest was the region where the lipophilic, intact *N*-methylpiperazine amide-tagged GPEtn lipids eluted from the RP column at 44–54 min (**Fig. 4A**, **B**). The amount of 117 *N*-methylpiperazine amide-tagged GPEtn species present in the oxidized sample (Fig. 4B) was drastically decreased compared with those 114 *N*-methylpiperazine amide-tagged GPEtn species present in the control sample (Fig. 4A).

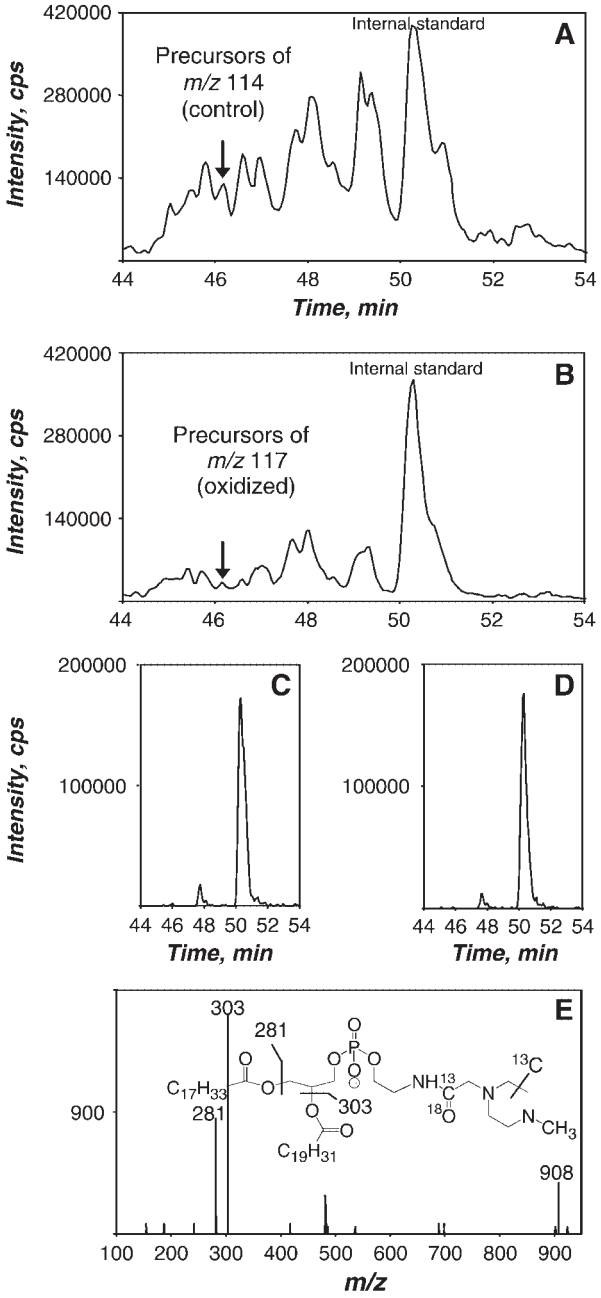

Fig. 4. Reversed-phase (RP) HPLC separation and tandem mass spectrometry analysis (LC/MS/MS) of 114 *N*-methylpiperazine amide-tagged GPEtn from the control liposomes and 117 *N*-methylpiperazine amide-tagged GPEtn from the oxidized liposomes from 44 to 54 min. A: The elution of the 114 *N*-methylpiperazine amide-tagged GPEtn in the control liposomes from the RP-HPLC column was monitored by precursor ion scans of *m/z* 114. B: The elution of the 117 *N*-methylpiperazine amide-tagged GPEtn in the oxidized liposomes from the RP-HPLC column was monitored by precursor ion scans of *m/z* 117. C: Extraction of 114 *N*-methylpiperazine amide-tagged 17:0a/17:0-GPEtn (*m/z* 864) from the RP-HPLC trace detected by a precursors of *m/z* 114 scan. D: Extraction of 117 *N*-methylpiperazine amide-tagged 17:0a/17:0-GPEtn (*m/z* 864) from the RP-HPLC trace detected by a precursors of *m/z* 117 scan. Note that the internal standard is recovered equally in both samples. E: Negative ion CID spectrum of the $[M-H]^-$ that elutes from the RP column at 46 min (arrows in A and B) at *m/z* 908 at a collision energy of 40 V. The origins of the ions that resulted from collisional activation are indicated in the structures of these molecules.

Additionally, the 17:0a/17:0-GPEtn internal standard in both samples had the same mass spectrometric response (Fig. 4C, D). The negative ion CID spectrum of one of the *N*-methylpiperazine amide-tagged GPEtn species that decreased after oxidation and eluted from the RP column at 46 min was obtained (Fig. 4E). This spectrum had two major fragmentation ions at *m/z* 281 and 303, which correspond to the carboxylate ions of oleic acid and arachidonic acid. Therefore, this *N*-methylpiperazine amide-tagged GPEtn species was identified as 18:1a/20:4-GPEtn. The negative ion CID spectra of other species in this chromatographic region of interest were also obtained, which allowed the identification of the other intact *N*-methylpiperazine amide-tagged GPEtn species (data not shown). The decrease in the amount of 117 *N*-methylpiperazine amide-tagged GPEtn in this chromatographic region can be explained, in part, by the loss of intact GPEtn species to newly formed oxidation products. It was also likely that the formation of covalent adducts with phospholipid aldehydes produced during oxidation had occurred, but the fate of these products was not further investigated.

The second area of interest upon comparison of the two separate precursors of *m/z* 114 and 117 RP-HPLC traces was the less lipophilic region of the chromatogram, where lyso *N*-methylpiperazine amide-tagged GPEtn lipids eluted from the RP column at 12–27 min (**Fig. 5A**, **B**). The amount of 117 *N*-methylpiperazine amide-tagged GPEtn species present in the oxidized sample in this region (Fig. 5B) was increased compared with those 114 *N*-methylpiperazine amide-tagged GPEtn species present in the control sample (Fig. 5A). Again, these chromatograms could be compared directly because the internal standard in the control and oxidized samples had the same mass spectrometric response (Fig. 4C, D). The negative ion CID spectrum of one of the *N*-methylpiperazine amide-tagged GPEtn species that increased after oxidation and eluted from the RP column at 18 min was obtained (Fig. 5C). This spectrum had one major fragmentation ion at *m/z* 281, which corresponded to the carboxylate ion of oleic acid. Therefore, this species was identified as 117 *N*-methylpiperazine amide-tagged 18:1a/lyso-GPEtn. The negative ion CID spectra of other species in this chromatographic region of interest were also obtained, which permitted identification of the other lyso *N*-methylpiperazine amide-tagged GPEtn compounds (data not shown). The increase of the amount of 117 *N*-methylpiperazine amide-tagged GPEtn species in this chromatographic region can be explained by the formation of lyso compounds as a result of oxidation.

DISCUSSION

The *N*-methylpiperazine acetic acid NHS ester reagents used in this study consisted of a reporter group (*N*-methylpiperazine), a mass balance group (carbonyl), and a primary amine-reactive group (NHS ester) (8). The overall masses of the reporter and balance components were kept constant in each of the four reagents available using differ-

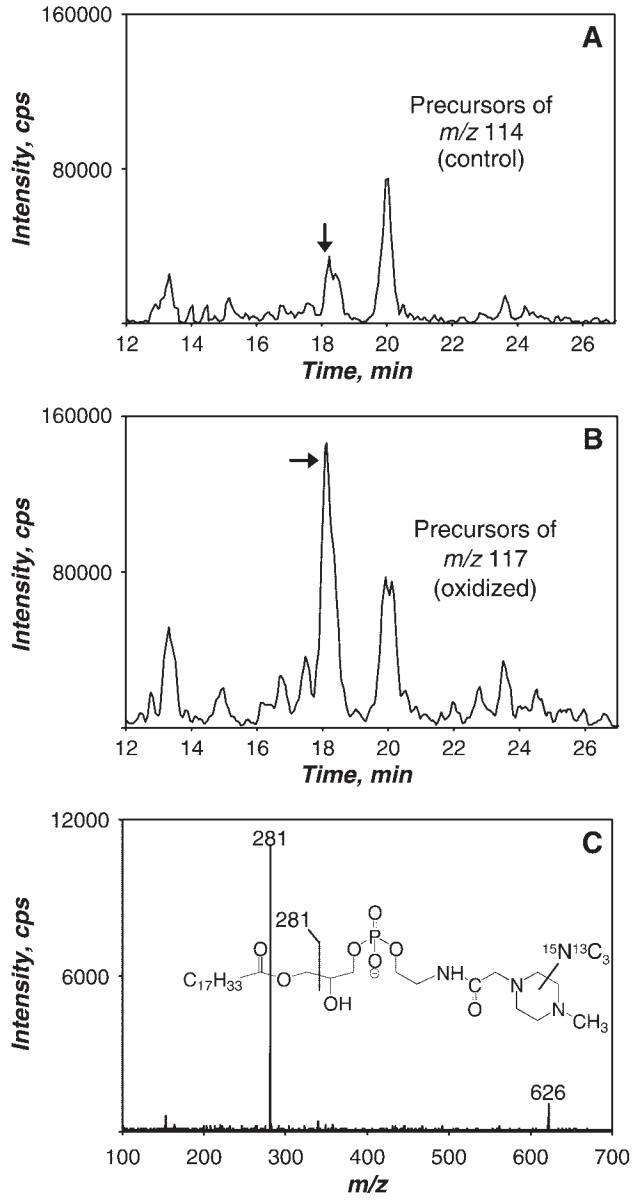

Fig. 5. RP-LC/MS/MS of 114 *N*-methylpiperazine amide-tagged GPEtn from the control liposomes and 117 *N*-methylpiperazine amide-tagged GPEtn from the oxidized liposomes from 12 to 27 min. A: The elution of the 114 *N*-methylpiperazine amide-tagged GPEtn in the control liposomes from the RP-HPLC column was monitored by precursor ion scans of *m/z* 114. B: The elution of the 117 *N*-methylpiperazine amide-tagged GPEtn in the oxidized liposomes from the RP-HPLC column was monitored by precursor ion scans of *m/z* 117. C: Negative ion CID spectrum of the $[M-H]^-$ that elutes from the RP column at 18 min (arrows in A and B) at *m/z* 626 at a collision energy of 40 V. The origins of the ions that resulted from collisional activation are indicated in the structures of these molecules.

ential isotopic enrichment, in which the reporter group ranges from 114 to 117 Da and the balance group ranges from 31 to 28 Da. This ensured that the mass of the tag that was covalently attached to the primary amine of interest by an amide linkage remained constant (145 Da) for each of the reagents. In peptide studies, it was found that these amide linkages fragment in a similar manner to the backbone of peptide bonds when subjected to CID in the positive ion mode (8). Typically, after fragmentation of the tag amide bond, the balance (carbonyl) moiety and attached peptide were lost, whereas charge was retained by the reporter group fragment, which resulted in ions at *m/z* 114, 115, 116, or 117, depending on which reagent was used. These reagents were also found to work well for complex mixtures of primary amine-containing phospholipids extracted from biological samples. *N*-Methylpiperazine amide-tagged aminophospholipids do not add complexity to the mixture and permit a more universal means by which aminophospholipid molecular species can be tracked. Additionally, the negative ion CID behavior of *N*-methylpiperazine amide-tagged GPEtn and GPSer was retained compared with the nontagged GPEtn and GPSer species, facilitating the identification of the radyl groups present at the *sn*-1 and *sn*-2 positions on the glycerol backbone (Table 1).

One of the unanticipated advantages of using the *N*-methylpiperazine acetic acid NHS ester reagents was the shift in mass of the $[M+H]^+$ by 144 Da that occurred for GPEtn and GPSer species. Typically, in a positive ion mass spectrum of glycerophospholipids present in the Bligh and Dyer extract from cells, the GPEtn and GPSer distribution of molecular ions in the positive ion mode overlaps with the distribution of GPCho molecular ions (16). However, upon derivatization with *N*-methylpiperazine acetic acid NHS ester reagents, the $[M+H]^+$ of each of the GPEtn or GPSer species increased by 144 Da, which shifted the distribution of *N*-methylpiperazine amide-tagged GPEtn and GPSer species out of the range of most of the GPCho lipids without adding increased complexity to the mixture. The only GPCho species that would overlap with the *N*-methylpiperazine amide-tagged GPEtn or GPSer after the 144 Da shift would be very long-chain, rare GPCho species. However, these overlapping GPCho species could be readily distinguished from the *N*-methylpiperazine amide-tagged GPEtn or GPSer upon CID in the positive ion mode. All GPCho species as $[M+H]^+$ collisionally activate into *m/z* 184 (17), and the *N*-methylpiperazine amide-tagged GPEtn or GPSer fragment into the reporter ion (*m/z* 114 or 117) specific to the *N*-methylpiperazine acetic acid NHS ester reagent used.

CID of the $[M+H]^+$ of diacyl-GPEtn typically results in the loss of the phosphoethanolamine head group (141 Da), and constant neutral loss of 141 Da has been used as a diagnostic tool for the mass spectrometric determination of GPEtn species in complex lipid mixtures (18). One disadvantage of using constant neutral loss of 141 Da to determine GPEtn content in lipid mixtures is that 1-*O*-alk-1′-enyl-2-acyl-GPEtn species do not undergo neutral loss of phosphoethanolamine to the same extent as diacyl-GPEtn (Fig. 3C). Instead, the CID of the $[M+H]^+$ of 1-*O*-alk-1′-enyl-2-acyl-GPEtn resulted in two prominent fragment ions: one that was characteristic of the *sn*-1 position and one that was characteristic of the *sn*-2 position (15). This difference in CID behavior displayed by the various subclasses of GPEtn species has complicated the detection of GPEtn phospholipid changes during biological reactions,

because there is no single common transition that permits all GPEtn species to be detected. However, upon reaction of GPEtn species with *N*-methylpiperazine acetic acid NHS ester reagents, the analysis of all GPEtn subclasses is straightforward, because there is a common reporter ion (*m/z* 114 or 117) at high collision energy (88 V).

These derivatives readily revealed that several lyso GPEtn species were formed upon exposure to Cu^{2+}/H_2O_2 (Fig. 5A, B) and that many of the control GPEtn species decreased after oxidation (Fig. 4A, B). One of the major challenges in determining changes in oxidized phospholipids in such an experiment is the lack of internal standards with chemical properties that can be copurified and behave identically in the mass spectrometric experiment so that quantitative assessment of changes in molecular species can be made. These derivatives enable a direct comparison of such oxidized aminophospholipids using stable isotope tagging to differentiate the oxidized species, permitting direct quantitation of the changes in abundance.

It has been found that the *N*-methylpiperazine amide isotope tag is a novel and facile means to observe changes in the distribution of GPEtn and GPSer species by mass spectrometry. In the experiments described here, oxidation was used to illustrate the power of differentially isotopically tagged GPEtn and GPSer species, although this technique can be applied to assessing the changes in GPEtn or GPSer species in myriad biological events. During CID in the positive ion mode, the reporter ions that are specific to the *N*-methylpiperazine acyl amide could be used in the development of sensitive mass spectrometric assays to determine changes that occurred in aminophospholipid distribution. Importantly, structural identification of the newly formed oxidation products with *N*-methylpiperazine amide tags could be determined by CID in the negative ion mode.

This work was supported in part by the Lipid Maps Large Scale Collaborative Grant from General Medical Sciences (GM069338) and by a grant from the Heart, Lung, and Blood Institute (HL-34303) of the National Institutes of Health.

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