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Analysis of polyunsaturated aminophospholipid molecular species using isotope-tagged derivatives and tandem mass spectrometry/mass spectrometry/mass spectrometry

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Abstract

When aminophospholipids with only saturated and monounsaturated fatty acids esterified to the glycerol backbone were labeled with isotopically enriched *N*-methylpiperazine acetic acid *N*-hydroxysuccinimide ester reagents, it was found that they could be readily detected as *N*-methylpiperazine-amide-tagged aminophospholipids using a precursor scan of the stable isotope reporter ion (*m*/*z* 114–117) formed by tandem mass spectrometry/mass spectrometry. However, it was found in the current study that these precursor ion scans are not useful in determining the changes of aminophospholipids with polyunsaturated fatty acids (PUFAs) esterified to the glycerol backbone due to the presence of interfering ions in the reporter ion region. Therefore, a method was developed using tandem mass spectrometry/mass spectrometry/mass spectrometry (MS^3) to obtain reporter ion ratios that were not distorted by interfering ions present in the collision-induced dissociation spectra of nontagged aminophospholipids with PUFAs. This new MS^3 method for *N*-methylpiperazine-amide-tagged aminophospholipids was used to examine the fate of diacyl, ether, or plasmalogen glycerophosphoethanolamine (GPEtn) species after exposure of human polymorphonuclear leukocytes to A23187 and granulocyte macrophage–colony-stimulating factor/formyl-methionyl-leucyl-phenylalanine stimuli, which can induce eicosanoid biosynthesis, to follow those GPEtn molecular species which were the source of arachidonic acid released. Upon stimulation of the human polymorphonuclear leukocyte, it was found that the abundant arachidonoyl GPEtn plasmalogen molecular species were uniquely reduced in relative content compared to ether or diacyl species and this subclass of GPEtn may be a source of the arachidonic acid converted to leukotrienes by the 5-lipoxygenase pathway activated in this cell.

Keywords: Aminophospholipids; Glycerophosphoethanolamine lipids; Glycerphosphoserine lipids; Mass spectrometry; Isotope tagged derivative; ITRAQ; Neutrophil; Human; MS^3

Phospholipids are building blocks of cellular membranes, but their roles in biochemistry are only partially understood. In addition to forming the physical boundary of cells and compartmentalizing the subcellular components, phospholipids are involved in key regulatory functions within mammalian cells. For example, membrane phospholipids are the precursors for biologically active species such as platelet-activating factor [1] and signaling molecules such as phosphatidylinositol triphosphate and diacylglycerol [2]. Additionally, membrane phospholipids are the source of arachidonic acid, which is the precursor of lipid mediators such as leukotrienes and prostaglandins [3]. Phosphatidylserine lipids are of particular interest during apoptosis due to their externalization from the inner phospholipid bilayer leaflet of the cell membrane, which is a recognition signal for the removal of apoptotic cells by phagocytes [4]. Furthermore, exposure of phosphatidylserine lipids on the outer surface of activated platelets initiates the blood clotting cascade [5]. In addition to the regulatory

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functions of phospholipids, they also serve as a protective barrier to the intracellular contents from chemically reactive species formed in biological reactions [6]. Given the importance of phospholipids, there is an interest in monitoring these species and being able to observe the changes that occur as a result of stimulation of a cell to initiate a biological response.

A powerful method to analyze these phospholipids present in a cell extract is to employ electrospray tandem mass spectrometry, which yields unique product ions and neutral losses in both the positive and the negative ion modes that are indicative of the polar headgroup and fatty acyl substituents [7]. While this is a useful technique, the phospholipid samples from biochemical sources are complex mixtures because there are many possible combinations of chain lengths, double bonds, linkages to the glycerol backbone, and headgroups that lead to the presence of several hundred phospholipid molecular species in the extract of cells. A major challenge has been to identify those less abundant phospholipid species that may be altered by initiation of biochemical reactions among an array of molecular species that do not change. While the use of mass spectrometry and the stable isotope dilution strategy has been a powerful qualitative and quantitative approach to address changes in biomolecules, there has been a lack of available phospholipid stable isotope internal standards for such studies. These factors have combined to make it problematic to monitor minor phospholipid molecular species changes that arise in the phospholipid molecular species mixture after cell stimulation.

Previously it was reported that aminophospholipids could be modified by four different isotopically enriched *N*-methylpiperazine acetic acid *N*-hydroxysuccinimide (NHS)[1] ester reagents that placed isobaric mass labels at the primary amine group and yield reporter ions (*m*/*z* 114, 115, 116, or 117) upon collisional activation in the positive ion mode. This led to the generation of stable-isotope-labeled variants of all aminophospholipid molecular species that could be used in a modified isotope dilution quantitative assay [8]. This previous study focused on aminophospholipids with saturated and monounsaturated fatty acids esterified to the glycerol backbone and detection of these species was performed using a precursor scan for the reporter ion. However, it was found in the current study that these precursor ion scans were not useful in determining the changes of aminophospholipids with polyunsaturated fatty acids (PUFAs) esterified to the glycerol backbone due to the presence of interfering ions in the reporter ion region. Therefore, this method was developed using MS^3 to obtain valuable reporter ion ratios that were not distorted by interfering ions present in the collision-induced dissociation (CID) spectra of aminophospholipids with PUFAs. This new MS^3 method for *N*-methylpiperazine-amide-tagged aminophospholipids was used to determine the changes that occurred in the distribution of GPEtn lipid molecular species after exposure of human polymorphonuclear leukocytes to A23187 (calcium ionophore) and GM-CSF/fMLP, which both induced the 5-lipoxygenase pathway of arachidonic acid metabolism, to determine the fate of GPEtn species containing arachidonic acid esterified to the glycerol backbone.

Experimental

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), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (14:0a/14:0-GPEtn), 1,2-di-*O*-phytanyl-*sn*-glycero-3-phosphoethanolamine, and brain glycerophosphoethanolamine, which contained numerous molecular species including 1-*O*-octadec-1′-enyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (18:0p/20:4-GPEtn), were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The internal standards d_4-LTB_4 and d_8-5-HETE were obtained from Cayman Chemical (Ann Arbor, MI). The 114, 115, 116, and 117 *N*-methylpiperazine acetic acid NHS ester reagent (iTRAQ) kit was obtained from Applied Biosystems (Foster City, CA). Hanks' balanced salt solution (1X) without Ca^{2+} and Mg^{2+} ($HBSS^{=}$) was obtained from Invitrogen (Carlsbad, CA). A23187, granulocyte macrophage–colony-stimulating factor (GM–CSF), formyl-methionyl-leucyl-phenylalanine (fMLP), palmitic acid, oleic acid, arachidonic acid, and docosahexaenoic acid were purchased from Sigma (St. Louis, MO). HPLC solvents were purchased from Fisher Scientific (Fair Lawn, NJ) and used for HPLC and extraction.

Analysis of nontagged aminophospholipids and N-methylpiperazine-amide-tagged aminophospholipids using nanoelectrospray ionization tandem mass spectrometry

The nontagged and *N*-methylpiperazine-amide-tagged aminophospholipids were infused using a NanoMate 100 (Advion BioSciences, Ithaca, NY) into a Sciex API 4000 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (PE Sciex, Toronto, Canada). Nanoelectrospray was

[1] *Abbreviations used:* 16:0a/18:1-GPEtn, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; 16:0a/18:1-GPSer, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; 14:0a/14:0-GPEtn, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; 18:0p/20:4-GPEtn, 1-*O*-octadec-1′-enyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine; A23187, calcium ionophore; CAD, collisionally activated dissociation; CID, collision-induced dissociation; GM-CSF/fMLP, granulocyte macrophage–colony-stimulating factor/formyl-methionyl-leucyl-phenylalanine; GPCho, glycerophosphocholine lipid; GPEtn, glycerophosphoethanolamine lipid; GPSer, glycerophosphoserine lipid; $HBSS^{=}$, Hanks' balanced salt solution (1X) without Ca^{2+} and Mg^{2+}; HETE, hydroxyeicosatetraenoic acid; IS, internal standard; LTB_4, leukotriene B_4; MS^3, tandem mass spectrometry/mass spectrometry/mass spectrometry where an initial product ion obtained from collisional activation of a precursor ion is itself collisionally activated to form a secondary product ion followed by mass analysis; NHS, *N*-hydroxysuccinimide; PLA_2, phospholipase A_2; PUFA, polyunsaturated fatty acid.

initiated using the NanoMate by applying a 1.32-kV spray voltage and a 0.15-psi nitrogen head pressure to the sample in the pipette tip. The typical flow rates achieved using these settings were 200 nl/min. The relevant mass spectrometric experimental parameters in the positive ion mode for both enhanced product ion and MS^3 experiments were a declustering potential of 120 V, a collisional offset of 40 V, and the CAD gas set at high. The pertinent MS^3 experimental parameters were excitation energy of 175 V, excitation time of 150 ms, q0 trapping, and fixed linear ion trap fill time of 150 ms. The concentration of the standards that were infused in these experiments was approximately 60 nM in methanol:acetonitrile:water (60:20:20) with 1 mM ammonium acetate.

Exact mass measurements

A QSTAR XL (PE Sciex) hybrid QqTOF mass spectrometer with an electrospray source was used to obtain high-resolution data. The sample was infused into the mass spectrometer at a flow rate of 5 μl/min in methanol:acetonitrile:water (60:20:20) with 1 mM ammonium acetate. The relevant experimental parameters used during operation of the QSTAR XL were declustering potential of 70 V and ion spray voltage of 5000 V. The collision energy was set to 55 V and the collision gas was nitrogen.

The exact mass data for the *m/z* 115 and 117 ions present in nontagged GPEtn species with PUFAs esterified to the glycerol backbone were obtained using a two-point mass calibration. Nontagged 18:0p/20:4-GPEtn and 114 *N*-methylpiperazine-amide-tagged 16:0a/18:1-GPEtn were mixed together and collision-induced dissociation of the $[M+H]^+$ at *m/z* 752 and 862 was performed. An experiment where alternating product ion scans of *m/z* 752 and 862 were obtained for 1 min was established. After this time the two averaged product ion spectra obtained were added together and the *m/z* 114 and 286 ions were used for the two-point calibration, where the exact masses of *m/z* 114.1112 (manufacturer information) and *m/z* 286.1328 (based on the elemental composition of this product ion as described below) were used. The product spectrum was then calibrated using the two above ions as reference and the exact masses of *m/z* 115 and 117 were determined.

Human polymorphonuclear leukocyte experiments with A23187 and GM-CSF/fMLP

Human polymorphonuclear leukocytes (neutrophils) were obtained from the whole blood of volunteers using the Percoll gradient centrifugation technique as previously described [9]. The neutrophils (60×10^6 cells) were spun down and resuspended in $HBSS^=$ at a concentration of 20×10^6 cells/ml and 1,2-di-*O*-phytanyl-*sn*-glycero-3-phosphoethanolamine (12 μg) was added in ethanol (6 μl) as an internal standard. The cell suspension (0.5 ml) was then aliquotted into separate tubes for the control, GM-CSF/fMLP, and A23187 experiments, which were performed in duplicate. For the GM-CSF/fMLP samples, GM-CSF was added to the cell suspension to achieve a final concentration of 1 nM and allowed to incubate at 37 °C for 30 min. After the 30-min incubation, $CaCl_2$ (final concentration 2 mM) and $MgCl_2$ (final concentration 500 μM) were added to the GM-CSF-treated sample, the control, and the A23187 sample so that the final volume in each tube was 1 ml. Additionally, A23187 was added to the A23187 sample at a final concentration of 2 μM and fMLP was added to the GM-CSF-treated sample for a final concentration of 100 nM. The samples were allowed to incubate at 37 °C for 15 min and the reaction was stopped by the addition of one volume of ice-cold methanol containing d_4-LTB_4 (4 ng) and d_8-5-HETE (4 ng). The cell membranes were pelleted by centrifugation and both the supernatants and the pellets were saved for mass spectrometric analysis.

Arachidonic acid metabolite separation by RP-HPLC and analysis by electrospray ionization–mass spectrometry

Water (2 ml) was added to the supernatants from the control and the GM-CSF/fMLP- and A23187-treated samples and solid-phase extraction was performed using Strata C18-E cartridges (Phenomenex, Torrance, CA). The analytes of interest were eluted from the solid-phase extraction column by methanol (2 ml), which was taken to dryness using a SpeedVac evaporating centrifuge (Savant Instruments, Farmingdale, NY). The arachidonic acid metabolites were reconstituted in 40 μl of HPLC mobile phase A (8.3 mM acetic acid buffered to pH 5.7 with ammonium hydroxide) and 20 μl of methanol and injected into a HPLC gradient pump system directly interfaced to the electrospray source of a Sciex API 2000 triple quadrupole mass spectrometer (PE Sciex). A Columbus 5μ C_{18} reversed-phase (1 × 150-mm) column (Phenomenex) was used to separate the arachidonic acid metabolites in the supernatant. The initial mobile phase was 25% solvent B (acetonitrile:methanol, 65:35) at a flow rate of 50 μl/min. A linear gradient was started to 85% solvent B in 24 min and followed by an increase to 98% solvent B in 2 min. This was followed by isocratic elution at 98% solvent B for 12 min. Mass spectrometric analyses were performed in the negative ion mode using multiple reaction monitoring of the specific transitions *m/z* 351 → 195 for 20-OH-LTB_4, *m/z* 335 → 195 for LTB_4, *m/z* 339 → 197 for d_4-LTB_4, *m/z* 319 → 115 for 5-HETE, *m/z* 327 → 116 for d_8-5-HETE, and *m/z* 303 → 205 for arachidonic acid eluting from the RP-HPLC column. Quantitation of LTB_4 and 5-HETE in the samples was performed using a standard isotope dilution curve as previously described [10].

Phospholipid extraction and synthesis of N-methylpiperazine-amide-tagged aminophospholipids

Lipids were extracted from the cell pellets of the control and the A23187- and GM-CSF/fMLP-treated samples by addition of chloroform–methanol–water according to the method of Bligh and Dyer [11]. Additionally, 14:0a/14:0-GPEtn (2 μg) was added as a second internal standard to

each sample. The procedure of tagging aminophospholipids with the *N*-methylpiperazine acetic acid NHS ester reagent has been described in detail previously [8]. Briefly, the extracted cell phospholipids were taken to dryness under a stream of nitrogen and resuspended in ethanol:0.5 M triethylammonium bicarbonate buffer (50:50) (30 μl). One vial of the *N*-methylpiperazine acetic acid NHS ester reagent (isotope tag 114, 115, or 116) was suspended in ethanol (70 μl), and the reagent (35 μl) was added to the phospholipid extraction and allowed to incubate at room temperature for 1 h. In these studies the control aminophospholipids were labeled with the 114 *N*-methylpiperazine acetic acid NHS ester reagent, the A23187 aminophospholipids were labeled with the 115 *N*-methylpiperazine acetic acid NHS ester reagent, and the GM-CSF/fMLP aminophospholipids were labeled with the 116 *N*-methylpiperazine acetic acid NHS ester reagent. Any remaining *N*-methylpiperazine acetic acid NHS ester reagent was hydrolyzed over 30 min by the addition of water (1 ml).

At this point the control and the A23187- and GM-CSF/fMLP-treated lipid extracts were mixed together and introduced onto a conditioned and rinsed C_{18} solid-phase extraction column (Supelco, Bellefonte, PA) to remove the excess *N*-methylpiperazine acetic acid NHS ester reagent and salts. The glycerophospholipids were eluted with methanol:chloroform (2:1), taken to dryness under a stream of nitrogen, and resuspended in chloroform (200 μl). The samples were then introduced onto aminopropyl SepPak columns (Supelco) that were conditioned with hexane to separate phospholipids by classes [12,13]. The neutral lipids were eluted using chloroform/2-propanol (2:1) (4 ml), and methanol (4 ml) was then added to the column to elute the unlabeled glycerophosphocholine (GPCho) lipids. The *N*-methylpiperazine-amide-tagged GPEtn and GPSer lipids were eluted using methanol:chloroform:3.6 M aqueous ammonium acetate (60:30:8) and collected. Chloroform (1.2 ml) and water (1.92 ml) were added to these fractions so that a Bligh–Dyer extraction could be performed to remove the large amount of ammonium acetate present in this solution. The *N*-methylpiperazine-amide-tagged GPEtn and GPSer lipids were resuspended in methanol:acetonitrile:water (60:20:20) with 1 mM ammonium acetate for mass spectrometric determination of the reporter ion ratios for each sample.

Determination of GPEtn relative abundance changes

Each $[M+H]^+$ of the major *N*-methylpiperazine-amide-tagged GPEtn molecular species was collisionally activated at 40 V to obtain the product ion at *m*/*z* 286, which contains the headgroup and isotope tag. This *m*/*z* 286 product ion that was common to all *N*-methylpiperazine-amide-tagged GPEtn species was collisionally activated (MS^3) to obtain reporter ions. The various reporter ion peak areas from the MS^3 spectra were used to calculate the relative changes of the individual GPEtn species in the A23187- or GM-CSF/fMLP-treated samples compared to the control samples using the formula (area treated/area control)/(area treated IS/area control IS), where areas correspond to the same reporter ion abundance area from treated samples or control samples and IS is internal standard reporter ion.

Statistical analysis

Statistical analysis was conducted by an unpaired *t* test with Welch correction using the GraphPad InStat version 3.06 statistical program (GraphPad Software, San Diego, CA).

Results

Previously, it was reported that GPEtn and GPSer lipids could be derivatized by *N*-methylpiperazine acetic acid NHS ester reagents through reaction with their respective primary amino group [8]. However, this study focused on aminophospholipids with saturated and monounsaturated fatty acids esterified to the glycerol backbone. The positive ion CID spectra of nontagged GPEtn or GPSer species that have only saturated or monounsaturated fatty acids esterified to the glycerol backbone did not contain any interfering ions in the *m*/*z* 114–117 region of the mass spectrum. This was evident in the product ion scan of the $[M+H]^+$ at *m*/*z* 718 of 16:0a/18:1-GPEtn (Fig. 1A), which contained product ions at *m*/*z* 119, 121, and 123. In the current study it was necessary to monitor the changes in aminophospholipids that contained PUFAs esterified to the glycerol backbone. Unfortunately, the positive product ion spectra of nontagged GPEtn or GPSer species that have PUFAs esterified to the glycerol backbone contained interfering ions in the *m*/*z* 114–117 region of the mass spectrum. This was exemplified in the product ion scan of the $[M+H]^+$ at *m*/*z* 752 of 18:0p/20:4-GPEtn (Fig. 1B) which had product ions at *m*/*z* 115, 117, 119, 121, 123, and 125.

High mass resolution data were acquired on a tandem quadrupole/time-of-flight mass spectrometer to determine the identity of the *m*/*z* 115 and 117 ions present in the product ion spectra (Fig. 1B) of aminophospholipids with PUFAs esterified to the glycerol backbone. The exact mass of the most abundant product ion in this region following collisional activation of $[M+H]^+$ of 18:0p/20:4-GPEtn was measured as *m*/*z* 117.0720. The possible elemental compositions for this product ion (Table 1) within a 10-mDa error are $C_5H_{12}NP$, C_9H_9, $C_5H_{11}NO_2$, and $CH_{11}NO_5$. The $C_5H_{11}NO_2$ and $CH_{11}NO_5$ elemental compositions were ruled out because their exact mass had a larger mDa error compared to the other two possibilities (Table 1) and because these possibilities did not correspond with known fragmentation mechanisms of aminophospholipids. Additionally, $C_5H_{12}NP$ was dismissed as a possibility because the mechanism of formation of this product would require breaking all oxygen phosphodiester bonds in the parent ion, while retaining the phosphorous and nitrogen atoms and no oxygen atoms in the product ion. This particular mechanism has not been observed during the CID of any

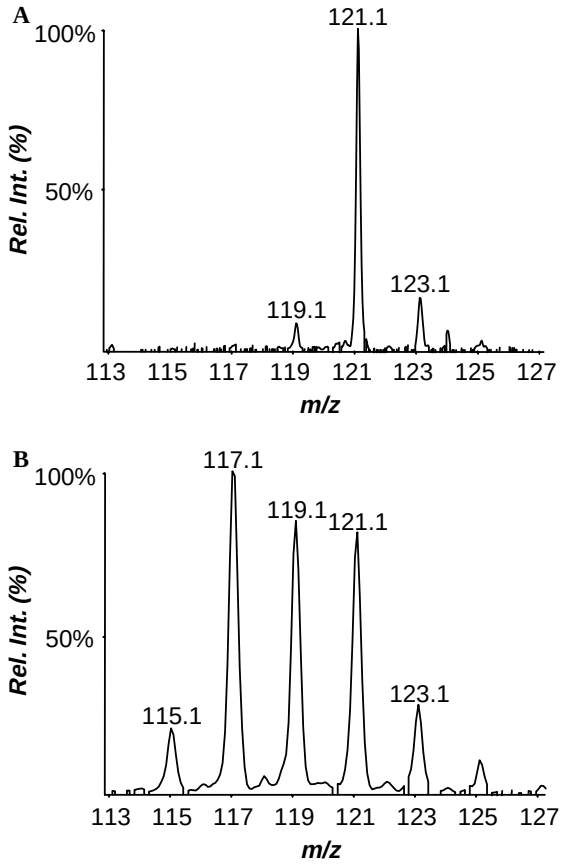

Fig. 1. Enhanced product ion spectra of the $[M+H]^+$ of (A) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (16:0a/18:1-GPEtn) and (B) 1-*O*-octadec-1′-enyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (18:0p/20:4-GPEtn) at a collision energy of 40 V. Note the interfering ions present in the reporter ion region (*m/z* 114–117) of the enhanced product ion spectrum of 18:0p/20:4-GPEtn.

Table 1
Possible elemental compositions of the *m/z* 117 ion, which was determined to have an exact mass of 117.0720 Da using a tandem quadrupole time-of-flight mass spectrometer, observed during the product ion scans of aminophospholipids with PUFAs esterified to the glycerol backbone

Formula	Calculated *m/z* (Da)	mDa error
$C_5H_{12}NP$	117.0707	1.2621
C_9H_9	117.0704	1.5746
$C_5H_{11}NO_2$	117.0789	−6.9787
$CH_{11}NO_5$	117.0637	8.2773

aminophospholipids and would require extensive rearrangements in the gas phase to be observed. Therefore, the identity of the product ion at *m/z* 117.0720 in the product ion spectrum of aminophospholipids with PUFAs esterified to the glycerol backbone was most likely C_9H_9 that could originate from the polyunsaturated fatty acyl substituent to make a highly unsaturated aromatic cation. An exact mass of the *m/z* 115.0569 ion was determined in a similar fashion for the other abundant ion in this region. From these data, the identity of the *m/z* 115 ion was deduced to be C_9H_7, which had an exact mass of *m/z* 115.0548. To verify the identification and origin of the *m/z* 115 and 117 product ions, the product ion spectra of the $[M+H]^+$ of palmitic, oleic, arachidonic, and docosahexaenoic acids were obtained (data not shown). The product ions observed in the collision-induced spectra of the $[M+H]^+$ of arachidonic and docosahexaenoic acids included *m/z* 115, 117, 119, 121, and 123. However, the only product ions observed for palmitic and oleic acids were at *m/z* 119, 121, and 123. This set of data further supported the fact that the product ions observed in the reporter ion region of nontagged aminophospholipids with PUFAs esterified to the glycerol backbone were derived from the polyunsaturated fatty acyl moiety. Due to the presence of these interfering ions in the positive ion CID spectra of GPEtn and GPSer species with PUFAs esterified to the glycerol backbone, simple product ion scans could not be used to obtain reporter ion ratios that accurately reflected the relative amounts of these species in a sample.

To develop a different method for obtaining accurate reporter ion ratios, the collisional activation of *N*-methylpiperazine-amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer standards was re-examined. The most abundant product ions present in the product ion mass spectrum in the positive ion mode at a collision energy of 40 V (laboratory frame of reference) of 114 *N*-methylpiperazine-tagged 16:0a/18:1-GPEtn were at *m/z* 286 and 577 (Fig. 2A), whereas, for 114 *N*-methylpiperazine-amide-tagged 16:0a/18:1-GPSer, the major product ions were observed at *m/z* 330 and 577 (Fig. 2B). The product ion at *m/z* 577 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 [7]. In separate experiments with different GPEtn and GPSer molecular species, this major ion shifted in mass, depending upon the fatty acyl component (data not shown). The ions present at *m/z* 286 and 330 in the product ion spectrum of 114 *N*-methylpiperazine-amide-tagged 16:0a/18:1-GPEtn and 16:0a/18:1-GPSer at 40 V corresponded to cleavage of the phosphate–glycerol bond with the site of protonation located on the *N*-methylpiperazine-amide-tagged headgroup region of the aminophospholipid. These product ions were always observed at *m/z* 286 and 330, respectively, for all *N*-methylpiperazine-amide-tagged GPEtn or GPSer species, no matter which of the four isotope tagging reagents was used and no matter which fatty acids were esterified to the glycerol backbone. Since the two product ions at *m/z* 286 and 330 contained the *N*-methylpiperazine-amide-tagged GPEtn or GPSer headgroup and not the fatty acyl moiety, MS^3 was performed in an attempt to yield reliable reporter ion information.

The MS^3 spectrum of *m/z* 286 that was a product ion of 114 *N*-methylpiperazine-amide-tagged 16:0a/18:1-GPEtn had four abundant product ions at *m/z* 206, 188, 145, and 114 (Fig. 3A). The product ion at *m/z* 206 resulted from the loss of HPO_3 (neutral loss of 80 Da from *m/z* 286) and *m/z* 188 was likely due to neutral loss of 98 Da (H_3PO_4) from *m/z* 286. Most importantly, the MS^3 spectrum contained a

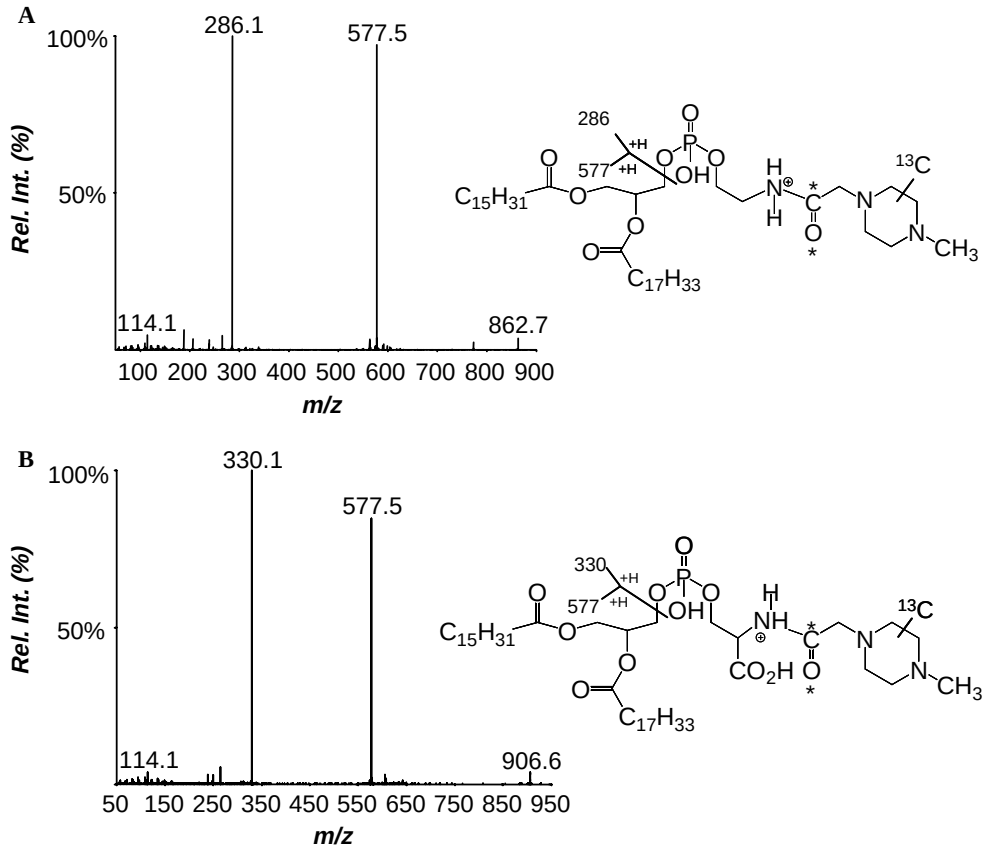

Fig. 2. Enhanced product ion spectra of the $[M+H]^+$ of (A) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and (B) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine 114 *N*-methylpiperazine-amide-tagged standards at a collision energy of 40 V. The origins of the ions that resulted from collisional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

product ion at *m*/*z* 114, which was the reporter ion expected from 114 *N*-methylpiperazine-amide-tagged 16:0a/18:1-GPEtn. Additionally, the MS^3 spectrum of *m*/*z* 330 that was a product ion of 114 *N*-methylpiperazine-amide-tagged 16:0a/18:1-GPSer had three abundant product ions at *m*/*z* 312, 232, and 114 (Fig. 3B). The product ion at *m*/*z* 312 resulted from dehydration of the precursor ion and the product ion at *m*/*z* 232 corresponded to loss of H_3PO_4 (neutral loss of 98 Da from *m*/*z* 330). Most importantly, one of the product ions present in the MS^3 spectrum occurs at *m*/*z* 114, which was the reporter ion that is expected from the 114 *N*-methylpiperazine-amide-tagged 16:0a/18:1-GPSer. The fragmentation of *m*/*z* 286 or 330 into the corresponding reporter ions during MS^3 was observed for all *N*-methylpiperazine-amide-tagged GPEtn or GPSer species and the only ions that appeared in the reporter ion region (*m*/*z* 114–117) corresponded to the *N*-methylpiperazine acetic acid NHS ester reagents used to label the aminophospholipids.

To establish the validity of using MS^3 rather than product ion scans for the determination of accurate reporter ion ratios, the MS^3 and product ion spectra of 114 and 115 *N*-methylpiperazine-amide-tagged 18:0p/20:4-GPEtn were obtained. The product ion spectrum of the $[M+H]^+$ at *m*/*z* 896 of 114 and 115 *N*-methylpiperazine-amide-tagged 18:0p/20:4-GPEtn (Fig. 4A) contained ions at *m*/*z* 114 and 115 that were attributed to reporter ions from the 114 and 115 *N*-methylpiperazine amide tags and ions at *m*/*z* 117 and 119, that were due to fragmentation of the PUFA as described above. Since there was clearly an ion at *m*/*z* 117 even though the 117 *N*-methylpiperazine acetic acid NHS ester reagent was not used to label 18:0p/20:4-GPEtn, product ion scans could not reveal accurate reporter ion ratios for *N*-methylpiperazine-amide-tagged aminophospholipids that contain PUFAs because these species produce interfering ions at *m*/*z* 115 (data not shown) and 117. Accurate reporter ion ratios of the 114 and 115 *N*-methylpiperazine-amide-tagged 18:0p/20:4-GPEtn could be determined using a MS^3 scan (Fig. 4B). The only ions present in this MS^3 spectrum from *m*/*z* 110–120 occurred at *m*/*z* 114 and 115, which were the reporter ions, and there were no interfering ions present at *m*/*z* 117 in this MS^3 spectrum.

The MS^3 method of obtaining reporter ions from *N*-methylpiperazine-amide-tagged aminophospholipids was used to investigate the relative changes in GPEtn species induced by treatment of human neutrophils with either A23187 or GM-CSF/fMLP as compared to untreated neutrophils. In this particular set of experiments the control GPEtn species were tagged with the 114 reagent, the A23187-treated GPEtn species were tagged with the 115 reagent, and the GM-CSF/fMLP-treated species were tagged with the 116 reagent. The GPEtn species for each condition examined in

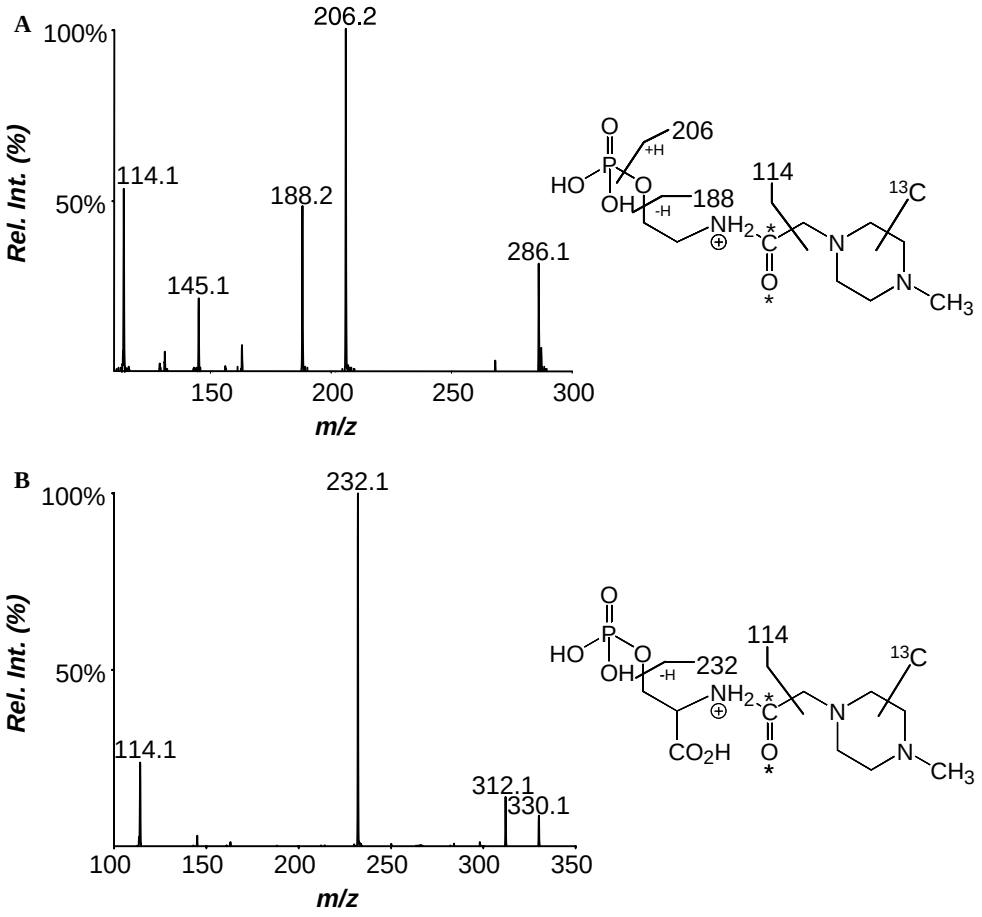

Fig. 3. Product ions (MS^3) in linear ion trap spectrometer following collisional activation of (A) *m/z* 286 generated from the $[M+H]^+$ at *m/z* 862 of 114 *N*-methylpiperazine-amide-tagged 16:0a/18:1-GPEtn and (B) *m/z* 330 generated from the $[M+H]^+$ at *m/z* 906 of 114 *N*-methylpiperazine-amide-tagged 16:0a/18:1-GPSer. The origins of the ions that resulted from collisional activation are indicated in the structures of these molecules. Note the presence of the *m/z* 114 reporter ions in both spectra.

this study were labeled with the *N*-methylpiperazine acetic acid NHS ester reagents efficiently and there were no untagged GPEtn species observed. The relative quantification of the GPEtn species present in the control and the A23187- and GM-CSF/fMLP-treated samples was based on the peak areas of the characteristic reporter ions derived from the *N*-methylpiperazine amide tags (*m/z* 114, 115, and 116) that were obtained from the MS^3 experiments.

Exogenous internal standards (1,2-di-*O*-phytanyl-GPEtn and 1,2-dimyristoyl-GPEtn) were used to account for sample preparation losses, to correct for the isotopic impurity of the *N*-methylpiperazine acetic acid NHS ester reagents, and to normalize the samples. The 1,2-di-*O*-phytanyl-GPEtn standard was added to the neutrophils at the beginning of the experiment before splitting the cells into the three treatment conditions. This internal standard was chosen because it had an ether linkage at both the sn-1 and the sn-2 positions of the glycerol backbone and would not be hydrolyzed upon activation of phospholipase A_2 enzymes induced by A23187 or GM-CSF/fMLP stimulation. This first exogenous internal standard was used so that any cell aliquoting errors could be tracked. A second internal standard, 14:0a/14:0-GPEtn, was added at the Bligh and Dyer extraction step. This diacyl-GPEtn internal standard could be added at this point in the sample preparation because the PLA_2 enzymes present would be inactivated by the extraction. The reporter ion areas of the two exogenous internal standards obtained from these experiments are shown in Table 2. It is apparent from the relative ratios found for both of the internal standards that the recovery of both internal standards was similar and that there was minimal variation in the splitting of the cells at the beginning of the experiment. In these experiments, the diacyl-GPEtn exogenous internal standard was chosen to normalize the data.

The relative changes in the GPEtn molecular species distribution of the A23187- or GM-CSF/fMLP-treated neutrophils compared to that of the control neutrophils were determined (Fig. 5). Only 18 GPEtn species were arbitrarily included in this figure since these are the major species present in the neutrophil [14]. Since both A23187 and GM-CSF/fMLP induced the release of arachidonic acid from phospholipids and activation of the 5-lipoxygenase pathway, arachidonic acid metabolites present in the supernatants from these experiments were quantified using stable isotope dilution techniques to determine the extent of

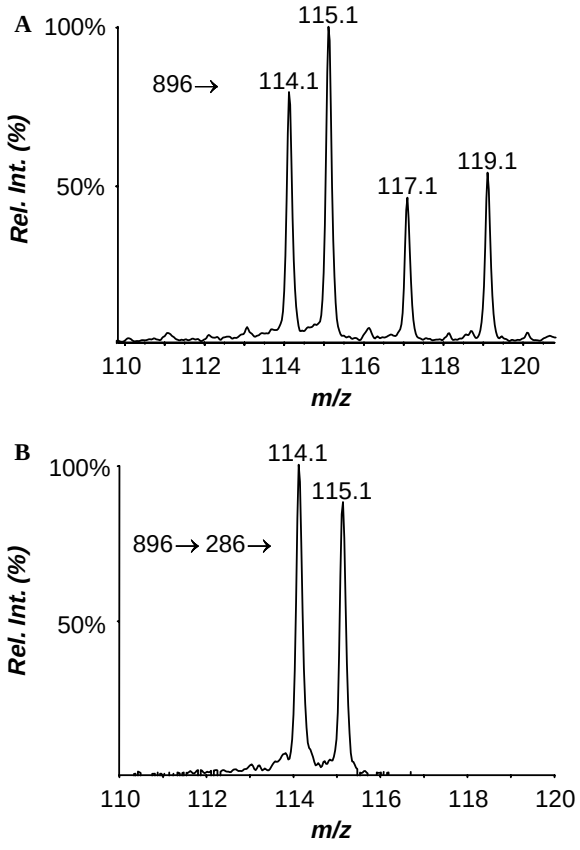

Fig. 4. (A) Enhanced product ion spectrum of the $[M+H]^+$ of 114 and 115 *N*-methylpiperazine-amide-tagged 1-*O*-octadec-1′-enyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (18:0p/20:4-GPEtn) at a collision energy of 40 V. Note the interfering ion present in the reporter ion region at *m/z* 117. (B) Product ions (MS^3) in linear ion trap spectrometer following collisional activation of *m/z* 286 generated from the $[M+H]^+$ of 114 and 115 *N*-methylpiperazine-amide-tagged 18:0p/20:4-GPEtn. Note that there are no interfering ions present in the MS^3 spectrum.

activation of these neutrophils in control and A23187- and GM-CSF/fMLP-treated samples (Table 3). It was apparent from these data that A23187 was a more robust stimulus with regard to arachidonic acid metabolite production than GM-CSF/fMLP and that no metabolites were detected in the control cell aliquot.

Upon stimulation of neutrophils with A23187, all of the major GPEtn species that did not contain arachidonoyl decreased to a normalized ratio value between 0.7 and 0.78 in the treated sample as compared to the control sample (Fig. 5A). The GPEtn species that contained arachidonic acid, including 36p:4[2], 38p:5, and 38p:4, decreased more significantly ($p < 0.001$) to a normalized ratio value between 0.5 and 0.65. In contrast, the stimulation of neutrophils with GM-CSF/fMLP caused less of a general change in the overall GPEtn distribution (Fig. 5B). Most of the GPEtn species did not show a change upon stimulation with normalized ratio values close to 1. However, the most abundant GPEtn species that contained arachidonic acid, including 36p:4, 36e:4, 38p:4, and 38a:4, decreased significantly to a normalized ratio value of between 0.8 and 0.9. It was clear that substantial changes in the GPEtn molecular species were observed upon stimulation with A23187 as compared to GM-CSF/fMLP stimulation as expected (which agreed with the arachidonic acid metabolite quantitation results in Table 3). Furthermore, both methods of stimulation showed decreases in the arachidonate-containing GPEtn molecular species compared to the other GPEtn species.

Discussion

The *N*-methylpiperazine acetic acid NHS ester reagents used in this study were originally developed for peptide quantitation and consisted of a reporter group (*N*-methylpiperazine), a mass balance group (carbonyl), and a primary amine reactive group (NHS ester) [14]. The overall masses of the reporter and balance components are kept constant in each of the four different reagents using differential isotopic enrichment, where the reporter group ranged from 114 to 117 Da while the balance group ranged from 31 to 28 Da. The resulting derivatized peptides were isobaric but yielded reporter ions (*m/z* 114, 115, 116, and 117) during MS/MS in the positive ion mode that could be used to provide relative quantitation information for individual members of the multiplex set. The MS/MS scans provided reliable reporter ion information because the *m/z* 114–117 region of the MS/MS spectra of peptides was free of interfering product ions [15]. Therefore, there was no question that the product ions that occurred in the reporter ion region of MS/MS spectra of *N*-methylpiperazine-amide-tagged peptides were derived from the *N*-methylpiperazine amide tag and the peak areas of these reporter ions reflected the relative abundance of a given peptide.

One of the major problems encountered in this study was that MS/MS of some *N*-methylpiperazine-amide-tagged aminophospholipids did not give accurate reporter ion ratios because the positive product ion spectra of nontagged aminophospholipids that have PUFAs esterified to the glycerol backbone contained interfering ions in the *m/z* 114–117 region of the mass spectrum (Fig. 1B), which led to inaccurate reporter ion ratios. Therefore, another method was developed using MS^3 scans of *m/z* 286 and 330 that were abundant products in the MS/MS of *N*-methylpiperazine-amide-tagged GPEtn and GPSer species containing only the *N*-methylpiperazine-amide-tagged headgroup region. This MS^3 technique was found to be valuable for obtaining accurate reporter ion areas that could reflect the relative amount of aminophospholipid in a comparative study.

In this study the relative changes of GPEtn species after A23187 or GM-CSF/fMLP stimulation compared to control was investigated using *N*-methylpiperazine amide isotope tags. The relative changes in the individual GPEtn

[2] Abbreviations for individual GPEtn molecular species used in this paper are ab:c, where a is the number of carbon atoms in the sn-1 and sn-2 substituents and c is the number of double bonds in the sn-1 and sn-2 hydrocarbon chains; b represents the type of sn-1 linkage, where p refers to plasmalogen (1-*O*-alk-1′-enyl), e refers to ether (1-*O*-alkyl), and a refers to acyl.

Table 2
MS^3 reporter ion area information from the two exogenous GPEtn internal standards used for the activation of human neutrophils by A23187 and GM-CSF/fMLP experiment

GPEtn internal standard	Reporter ion (*m*/*z*)	Reporter ion area	Relative area (114, 115 or 116/114)
1,2-dimyristoyl-GPEtn	114	$1.27 \times 10^7 \pm 8.5 \times 10^5$	1
	115	$1.14 \times 10^7 \pm 5.3 \times 10^5$	0.90 ± 0.02
	116	$1.18 \times 10^7 \pm 7.1 \times 10^5$	0.93 ± 0.01
1,2-di-*O*-phytanyl-GPEtn	114	$1.75 \times 10^4 \pm 1056$	1
	115	$1.54 \times 10^4 \pm 450$	0.88 ± 0.03
	116	$1.58 \times 10^4 \pm 695$	0.90 ± 0.01

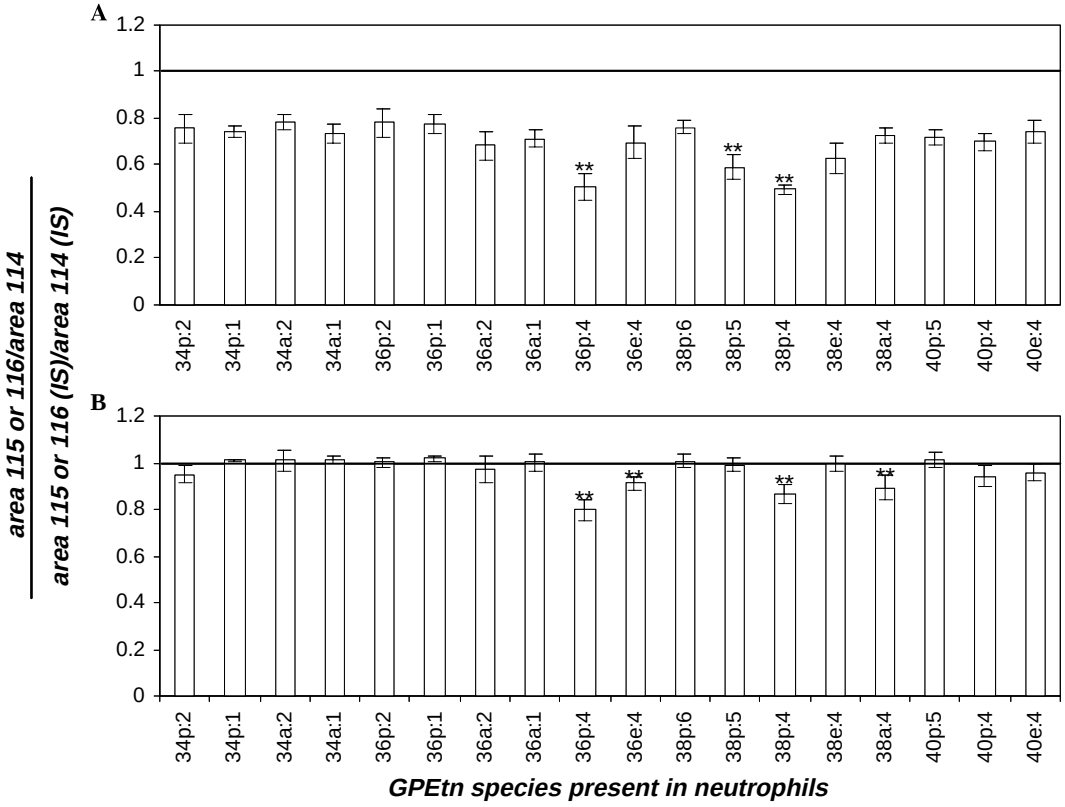

Fig. 5. Relative GPEtn abundance in (A) A23187- and (B) GM-CSF/fMLP-treated neutrophils compared to control neutrophils ($n = 6$). The abbreviations of the GPEtn molecular species on the abscissa are defined in Footnote 2 in the text. The control, A23187, and GM-CSF/fMLP samples were labeled with the 114, 115, and 116 *N*-methylpiperazine acetic acid NHS ester reagents, respectively. All of the GPEtn species shown in this graph are the major GPEtn species found in human neutrophils and the exogenous internal standard (14:0a/14:0-GPEtn) was used to normalize these data. Indicated by **, significant decrease compared to GPEtn molecular species that do not contain esterified arachidonic acid ($p < 0.001$).

Table 3
Amount of LTB_4 and 5-HETE produced upon activation of human neutrophils by A23187 (2 μM) and GM-CSF (1 nM)/fMLP (100 nM) for 15 min

Stimulus	Amount of LTB_4 (ng/5 × 10^6neutrophils)	Amount of 5-HETE (ng/5 × 10^6 neutrophils)
Control	0[a]	0[a]
A23187	23.2 ± 0.3	16.1 ± 0.1
GM-CSF/fMLP	0.10 ± 0.05	0.34 ± 0.09

[a] Below detection limit.

species in the A23187- or GM-CSF/fMLP-treated samples compared to the control samples were calculated and normalized to account for any losses during sample preparation and for the isotopic impurity of the *N*-methylpiperazine acetic acid NHS ester reagents. Typically, in protein studies the ratios are normalized using the overall ratios for all proteins in the sample, since relative abundances of most proteins are close to unity [16]. However, it was not known how the distribution of aminophospholipids would change after A23187 and GM-CSF/fMLP stimulation and for this reason two different exogenous internal standards were added before splitting the neutrophil suspension and before sample preparation. The relative areas of the reporter ion peaks for both internal standards were evaluated (Table 2) and it was determined that there was similar recovery of both internal standards. This meant that either internal standard could be used to normalize the data and it was decided to use the diacyl-GPEtn internal standard.

Biological stimulation by A23187 and GM-CSF/fMLP will activate phospholipase A_2 enzymes, which hydrolyze fatty acids from the sn-2 position of a phospholipid. Mammalian cells have several different forms of PLA_2 including secretory PLA_2 ($sPLA_2$), calcium-independent PLA_2 ($iPLA_2$), and cytosolic PLA_2 ($cPLA_2$) [17]. The only PLA_2 that preferentially hydrolyzes arachidonic acid from the sn-2 position of phospholipids is $cPLA_2$ [18]. Even though $sPLA_2$ and $iPLA_2$ do not exhibit acyl chain specificity they can also mediate arachidonic acid release depending on the cell type and stimulus. This release of arachidonic acid and eicosanoid production induced by A23187 and GM-CSF/fMLP in human neutrophils has been extensively examined [19,20], but much about these phospholipid molecular species that provide arachidonic acid for leukotriene production remains to be elucidated [21,22]. Therefore, *N*-methylpiperazine amide isotope tags were used to study the fate of diacyl, ether, or plasmalogen GPEtn species after stimulation by A23187 and GM-CSF/fMLP to determine the major source of the arachidonic acid released.

The two different biological stimuli used in this study activate phospholipase A_2 enzymes in human neutrophils by different mechanisms. The GM-CSF/fMLP stimulus employed a receptor-mediated response because the GM-CSF binds to a cytokine receptor, promotes up-regulation of the fMLP receptor [23], activates mitogen-activated protein kinases [24], and induces phosphorylation and translocation of $cPLA_2$ [25]. GM-CSF does not have the ability to increase intracellular calcium concentration; however, this is achieved by the addition of fMLP after initial priming by GM-CSF. This particular stimulus is considered to be close to a physiological event and rather specific for $cPLA_2$ activation. Leukotriene production was initiated by this stimulus (Table 3) and the *N*-methylpiperazine-amide-tagged GPEtn data revealed a significant reduction of two abundant plasmalogen GPEtns after stimulation with GM-CSF/fMLP that contained arachidonate (Fig. 5B). In contrast, activation of neutrophils using the calcium ionophore A23187 involves increase in intracellular calcium caused by the transport of Ca^{2+} into the cytosol by an electrochemical gradient. This stimulation was clearly more robust than the GM-CSF/fMLP stimulation, as evidenced by total leukotriene production (Table 3) and modification of all major GPEtn molecular species (Fig. 5A). An obvious reduction of plasmalogen GPEtn species that contained arachidonate was still observed. However, in contrast to the GM-CSF/fMLP data, GPEtn species from A23187-treated neutrophils that do not contain arachidonate also decreased relative to control. This was most likely due to increased intracellular calcium levels that led to activation of additional phospholipase A_2 enzymes, not just $cPLA_2$, and this robust hydrolysis could not be compensated by reacylation of free arachidonic acid. Additionally, it was interesting to note that phospholipase A_2 activation by GM-CSF/fMLP removed arachidonic acid from plasmalogen GPEtn molecular species to the largest extent. In the neutrophil, these molecular species are a major reservoir of esterified arachidonic acid [21,22], which argues for this class of phospholipids providing arachidonate metabolized by 5-lipoxygenase into the leukotrienes.

In summary, changes in the abundance of all GPEtn species present in the human neutrophil were examined using *N*-methylpiperazine amide isotope tags. It was found that plasmalogen GPEtn species containing esterified arachidonate uniquely decreased compared to other GPEtn phospholipids after stimulation of human neutrophils by A23187 or GM-CSF/fMLP. This method of using *N*-methylpiperazine amide stable isotope tags to comparatively assess all GPEtn molecular species individually was sufficiently sensitive to follow a cell activation event, even when a rather specific stimulus was used that resulted in only modest alteration of molecular species distribution. While the lack of available stable isotope internal standards has posed a problem in the quantitation of phospholipids by mass spectrometric strategies, the use of the *N*-methylpiperazine amide tags created an internal standard for each of the molecular species of aminophospholipids. These *N*-methylpiperazine acetic acid NHS ester reagents open a door for aminophospholipid analysis because of the ability to comparatively examine minor changes in aminophospholipid molecular species without the need for establishing standard curves or response factors.

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