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Stable Isotope Labeled 4-(Dimethylamino)benzoic Acid Derivatives of Glycerophosphoethanolamine Lipids

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A set of four (D_0, D_4, D_6, and D_{10}) deuterium enriched 4-(dimethylamino)benzoic acid (DMABA) *N*-hydroxysuccinimide (NHS) ester reagents was developed that react with the primary amine group of glycerophosphoethanolamine (PE) lipids to create derivatives where all subclasses of DMABA labeled PE are detected by a common precursor ion scan. The positive ion collision induced dissociation data from (D_0, D_4, D_6, and D_{10})-DMABA labeled PE standards indicated that a precursor ion scan of *m/z* 191.1, 195.1, 197.1, and 201.1 could be used to selectively detect (D_0, D_4, D_6, and D_{10})-DMABA modified PE, respectively, in a complex biological mixture. The PE lipids from a time course (0, 30, 60, and 300 min) of 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH) treatment of liposomes made of RAW 264.7 cell phospholipids were each labeled with the D_0-, D_4-, D_{10}-, and D_6-DMABA NHS ester reagents, respectively. The DMABA derivatives revealed loss of endogenous PE lipids and an increase in oxidized PE lipid throughout the time course of AAPH treatment. These DMABA NHS ester reagents provide a universal scan for diacyl, ether, and plasmalogen PE lipids that cannot be readily observed otherwise, enable differential labeling, and provide an internal standard for each PE lipid.

Phospholipids are the main component of cellular membranes that have important structural and functional properties. In addition to forming the physical boundary of cells, phospholipids are involved in key regulatory cellular functions. Phospholipids are precursors for biologically active molecules, such as platelet activating factor (PAF)[1] and arachidonate derived lipid mediators,[2] and signaling lipids, such as diacylglycerol.[3] Additionally, free radical oxidation of polyunsaturated fatty acids (PUFA) containing phospholipids has been implicated in numerous diseases including atherosclerosis and neurodegenerative diseases.[4−7] These findings have demonstrated the importance of obtaining a detailed analysis of phospholipid species present in certain tissues and cells.

One very powerful technique used to analyze phospholipids employs electrospray tandem mass spectrometry. Tandem mass spectrometry of the $[M + H]^+$ of phospholipids yields unique product ions and neutral losses that are indicative of the polar headgroup.[8] For diacyl and ether glycerophosphoethanolamine (PE) lipids, collision induced dissociation (CID) of the $[M + H]^+$ results in the neutral loss of phosphoethanolamine (141 amu).[9] However, the collision induced dissociation behavior of the $[M + H]^+$ of plasmalogen PE lipids, which contain a vinyl ether substituent at the sn-1 position of the glycerol backbone, results in two prominent fragment ions; one that was characteristic of the sn-1 position and one that was characteristic of the sn-2 position with a very minor ion that results from the neutral loss of 141 amu (NL141).[10] Since all subclasses of PE lipids do not fragment similarly in positive ion mode, there is not a universal scan for PE lipids that allows for selective detection of all PE subclasses in a biological sample.

Few studies have used tandem mass spectrometry to detect oxidized PE in samples. In most of these studies the oxidation products were first predicted and then detected either in the negative ion mode using a precursor ion scan of a predicted oxidized fatty acid[11] or in the positive ion mode using multiple reaction monitoring (MRM) of the NL141 of predicted oxidized PE species.[12] The detection of oxidized PE in both of these previous studies depended on correctly predicting the oxidized PE products that would most likely be observed; however, it is possible to inadvertently exclude many different oxidized species in a complex biological sample. Additionally, the MRM method

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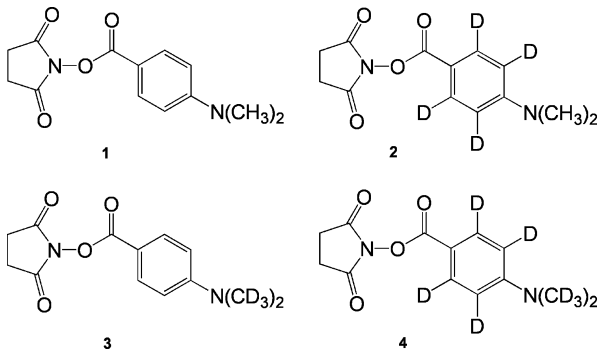
Scheme 1



using the NL141 of the predicted oxidized PE species would completely discriminate against any of the oxidized products that are plasmalogen PE lipids. Typically, plasmalogen PE contain high amounts of esterified PUFA.[13,14] Since PUFA are highly susceptible to oxidation, many oxidized products would be absent using the above MRM method. Therefore, a selective precursor ion scan that universally detects all subclasses of PE and potential oxidized products would be of significant value.

In the current study, a set of four (D_0, D_4, D_6, and D_{10}) deuterium enriched 4-(dimethylamino)benzoic acid (DMABA) *N*-hydroxysuccinimide (NHS) ester reagents (Scheme 1) were developed that react with the primary amine group of PE lipids. These reagents were developed in order to create DMABA PE derivatives where all subclasses of DMABA labeled PE could be detected by a common precursor ion scan. In addition, this set of reagents permitted differential labeling of PE and thereby generate stable isotope labeled variants of all PE molecular species by comparison of control lipids with treated lipids. The tandem mass spectrometric behavior in both the positive and negative ion mode of DMABA labeled PE lipids was determined using (D_0, D_4, D_6, and D_{10})-DMABA labeled 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18:1*a*/18:1-PE) standards. (Abbreviations for individual PE molecular species used in this paper: *n*:*jk*/*s*:*t*-PE, where *n* is the number of carbon atoms in the sn-1 substituent and *j* is the number of double bonds in the sn-1 hydrocarbon chain; *k* 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; *s* is the number of carbons and *t* is the number of double bonds in the sn-2 substituent.) Additionally, the DMABA NHS ester reagents were used to assess the changes that occurred in the distribution of PE lipids after exposure of liposomes made from phospholipids extracted from RAW 264.7 cells to 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH).

EXPERIMENTAL SECTION

Materials. 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18:1*a*/18:1-PE), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (16:0*a*/20:4-PE) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (14:0*a*/14:0-PE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Hanks' balanced salt solution (1×) (HBSS) was obtained from Invitrogen (Carlsbad, CA). 2,2′-Azobis-(2-amidinopropane) hydrochloride (AAPH), triethylammonium bicarbonate buffer, and methoxyamine hydrochloride (MOX) were purchased from Sigma Chemical Company (St. Louis, MO). Sodium hydroxide (1 N) was purchased from J. T. Baker (Phillipsburg, NJ). HPLC solvents were purchased from Fisher Scientific (Fair Lawn, NJ) and used for HPLC and extraction.

Synthesis of DMABA-NHS Ester Reagents. The details of the synthesis, purification, and characterization of D_4-4-(dimethylamino)benzoic acid, D_6-4-(dimethylamino)benzoic acid, and D_{10}-4-(dimethylamino)benzoic acid are provided in the Supporting Information. Additionally the synthesis, purification, and characterization of the (D_0, D_4, D_6, and D_{10})-DMABA NHS ester reagents using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) coupling chemistry are also provided in the Supporting Information.

Synthesis of DMABA Amide Tagged PE Lipids. A solution of 18:1*a*/18:1-PE (20 μg) in chloroform was dried down under a stream of nitrogen and resuspended in ethanol (65 μL) and 0.25 M triethylammonium bicarbonate buffer (15 μL). The (D_0, D_4, D_6, or D_{10})-DMABA NHS ester reagent in methylene chloride (20 μL of a 10 mg/mL solution) was then added to 18:1*a*/18:1-PE and allowed to incubate at 60 °C for 1 h. Water (0.4 mL) was added to hydrolyze any unreacted DMABA NHS ester reagent for 30 min. The phospholipids were then extracted by addition of chloroform−methanol according to the method of Bligh and Dyer.[15] The extracted DMABA labeled PE was resuspended in 60:20:20 (v/v/v) methanol/acetonitrile/water with 1 mM ammonium acetate at a concentration of 1 ng/μL for mass spectrometric analysis.

Electrospray Ionization Tandem Quadrupole Mass Spectrometry. The (D_0, D_4, D_6, or D_{10})-DMABA labeled 18:1*a*/18:1-PE lipids were infused into a Sciex API 2000 QTRAP mass spectrometer (PE Sciex, Toronto, Canada) at a flow rate of 5 μL/min. Unless specifically indicated, enhanced product ion spectra utilizing the terminal linear ion trap of this triple quadruple are reported as product ion scans. In the positive ion mode, an electrospray voltage of 5000 V, a declustering potential of 50 V, and a collision energy of 30 V was used to obtain the product ion spectra. In the negative ion mode, the experimental parameters used to obtain the product ion spectra were an electrospray voltage of −4500 V, a declustering potential of −50 V, and a collision energy of −50 V. For some experiments, the terminal quadrupole was not operated as an ion trap and product ions were obtained under normal triple quadrupole operation. A NL141 scan was used to detect unlabeled PE lipids from RAW 264.7 cells in the positive ion mode at a collision energy of 25 V.

RAW 264.7 Cells. The cells used in these experiments were the RAW 264.7 macrophage cell line that was obtained from ATCC laboratories (Manassas, VA). In brief, the cells were grown in an incubator with a 5% CO_2 humidified atmosphere maintained at 37 °C. The cells were grown until 80% confluent 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 (40×10^6) by a Bligh−Dyer extraction. The phospholipid extract was split equally into four

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different test tubes, taken to dryness under a stream of nitrogen, and resuspended in HBSS (0.4 mL). Small, unilamellar vesicles were prepared by vortexing the phospholipid suspensions for 30 min followed by sonication for 10 min. The liposomes were then subjected to AAPH treatment (10 mM final concentration), and a time course was performed. The liposomes were incubated with AAPH for 0, 30, 60, and 300 min at 37 °C and the phospholipids were extracted using a Bligh−Dyer extraction after the internal standard (14:0*a*/14:0-PE, 1 μg) was added. Methoxime derivatives of ketone or aldehyde groups present on the phospholipids were prepared using a gas phase MOX procedure.[16]

The PE lipids in the control sample (0 min) were labeled with the D_0-DMABA NHS ester reagent, while the PE lipids in the AAPH treated samples (30, 60, and 300 min) were labeled with the D_4-, D_{10}-, and D_6-DMABA NHS ester reagents, respectively, according to the above protocol that was followed for the 18:1*a*/18:1-PE standard. After the reaction of the DMABA NHS ester reagent with PE lipids was complete, the control and oxidized samples were all combined together and extracted by the method of Bligh and Dyer.[15] The combined DMABA labeled control and oxidized samples were then introduced onto an aminopropyl SepPak column (Supelco, Bellefonte, PA) that was conditioned with hexane in order to separate phospholipids by classes.[17,18] 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 glycerophosphocholine (PC). The DMABA labeled PE lipids were eluted using methanol/chloroform/3.6 M aqueous ammonium acetate (60:30:8) and collected. Chloroform (3 mL) and water (1 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.

DMABA Labeled PE Separation by RP-HPLC and Analysis by Electrospray Ionization Mass Spectrometry. The D_0-, D_4-, D_6- and D_{10}-DMABA labeled PE species were separated according to lipophilicity by reverse phase HPLC with a Gemini 5 μm C_{18} (2.0 mm × 150 mm) column (Phenomenex, Torrance, CA) coupled to a Sciex API 2000 QTRAP mass spectrometer (PE Sciex, Toronto, Canada). The HPLC was operated at a flow rate of 0.2 mL/min with a mobile phase of methanol/acetonitrile/water 60:20:20 (v/v/v) with 2 mM ammonium acetate (solvent A) and 2 mM methanolic ammonium acetate (solvent B). The gradient was 25% solvent B to 100% solvent B in 20 min, followed by isocratic elution at 100% solvent B for 20 min. The D_0-, D_4-, D_6-, and D_{10}-DMABA labeled PE lipids from 1×10^6 cells were detected during one chromatographic run by alternating between precursors of *m/z* 191.1 (P191), *m/z* 195.1 (P195), *m/z* 197.1 (P197), and *m/z* 201.1 (P201) scans, respectively, every 1.5 s with a collision energy of 35 V.

Oxidation of 16:0*a*/20:4-PE Standard. Standard 16:0*a*/20:4-PE (10 μg) in chloroform was taken to dryness under a stream of nitrogen and resuspended in HBSS (0.4 mL). Small, unilamellar vesicles were prepared by vortexing the phospholipid suspensions for 30 min followed by sonication for 10 min. The liposomes were then subjected to AAPH treatment (10 mM final concentration) for 1 h at 37 °C. The reaction was terminated by immersion in an ice bath and the addition of chloroform−methanol according to the method of Bligh and Dyer.[15] At this point, half of the oxidized 16:0*a*/20:4-PE sample was saved and half was labeled with the D_4-DMABA NHS ester reagent according to the above protocol. The oxidized 16:0*a*/20:4-PE and the D_4-DMABA labeled 16:0*a*/20:4-PE were resuspended in 60:20:20 (v/v/v) methanol/acetonitrile/water with 1 mM ammonium acetate at a concentration of 1 ng/μL for mass spectrometric analysis.

RESULTS

Mass spectrometric analysis was used to elucidate the products formed after the reaction of 18:1*a*/18:1-PE standard with the (D_0, D_4, D_6, and D_{10})-DMABA NHS ester reagents. Prior to labeling 18:1*a*/18:1-PE with the (D_0, D_4, D_6, and D_{10})-DMABA NHS ester reagents, the $[M + H]^+$ ion observed in the positive ion mass spectrum was at *m/z* 744.7. Upon reaction of the D_0-DMABA NHS ester reagent with 18:1*a*/18:1-PE, the $[M + H]^+$ ion shifted to *m/z* 891.7 (data not shown), which corresponded to the addition of 147 amu onto the $[M + H]^+$ of 18:1*a*/18:1-PE. The $[M + H]^+$ ions at *m/z* 895.7, 897.7, and 901.7 were observed upon reaction of 18:1*a*/18:1-PE with each of the (D_4, D_6, and D_{10})-DMABA NHS ester reagents, respectively, which corresponded to an addition of 151, 153, and 157 amu onto the $[M + H]^+$ of 18:1*a*/18:1-PE. The yield of the DMABA NHS ester reagents when labeling the 18:1*a*/18:1-PE standard was determined using the $[M + H]^+$ peak height of 18:1*a*/18:1-PE and the DMABA labeled 18:1*a*/18:1-PE found in the positive ion mass spectrum. It was found for each of the (D_0, D_4, D_6, and D_{10})-DMABA NHS ester reagents that >92% of the 18:1*a*/18:1-PE standard was converted to (D_0, D_4, D_6, and D_{10})-DMABA labeled 18:1*a*/18:1-PE using the described labeling conditions.

The collision induced dissociation behavior of the $[M + H]^+$ of the (D_0, D_4, D_6, and D_{10})-DMABA labeled 18:1*a*/18:1-PE was examined. One major fragmentation ion present in the positive ion CID spectrum of the (D_0, D_4, D_6, and D_{10})-DMABA labeled 18:1*a*/18:1-PE standards was at *m/z* 603.6 (Figure 1), which was also observed upon CID of 18:1*a*/18:1-PE $[M + H - 141]^+$ in the positive ion mode.[9] The other major fragmentation ion present in the positive ion CID spectra of (D_0, D_4, D_6, and D_{10})-DMABA labeled 18:1*a*/18:1-PE occurred at *m/z* 191.1 for D_0-DMABA labeled 18:1*a*/18:1-PE, *m/z* 195.1 for D_4-DMABA labeled 18:1*a*/18:1-PE, *m/z* 197.1 for D_6-DMABA labeled 18:1*a*/18:1-PE, and *m/z* 201.1 for D_{10}-DMABA labeled 18:1*a*/18:1-PE (Figure 1), which resulted from the cleavage at the phosphate−ethanolamine bond. Equivalents of these fragmentation ions were not observed in the CID spectrum of 18:1*a*/18:1-PE. There were also minor fragmentation ions in the positive ion CID spectra of (D_0, D_4, D_6, and D_{10})-DMABA-18:1*a*/18:1 labeled PE at *m/z* 148.1, 152.1, 154.1, and 158.1, respectively, that were due to cleavage of the amide bond that linked ethanolamine to DMABA. This positive ion CID data suggested that the (D_0, D_4, D_6, and D_{10})-DMABA PE specific ions at *m/z* 191.1, 195.1, 197.1, and 201.1, respectively, could be used to selectively detect (D_0, D_4, D_6, and D_{10})-DMABA modified PE in a complex biological mixture. The D_0-DMABA labeled 18:1*a*/18:1-PE standard was injected onto a reverse phase column and used

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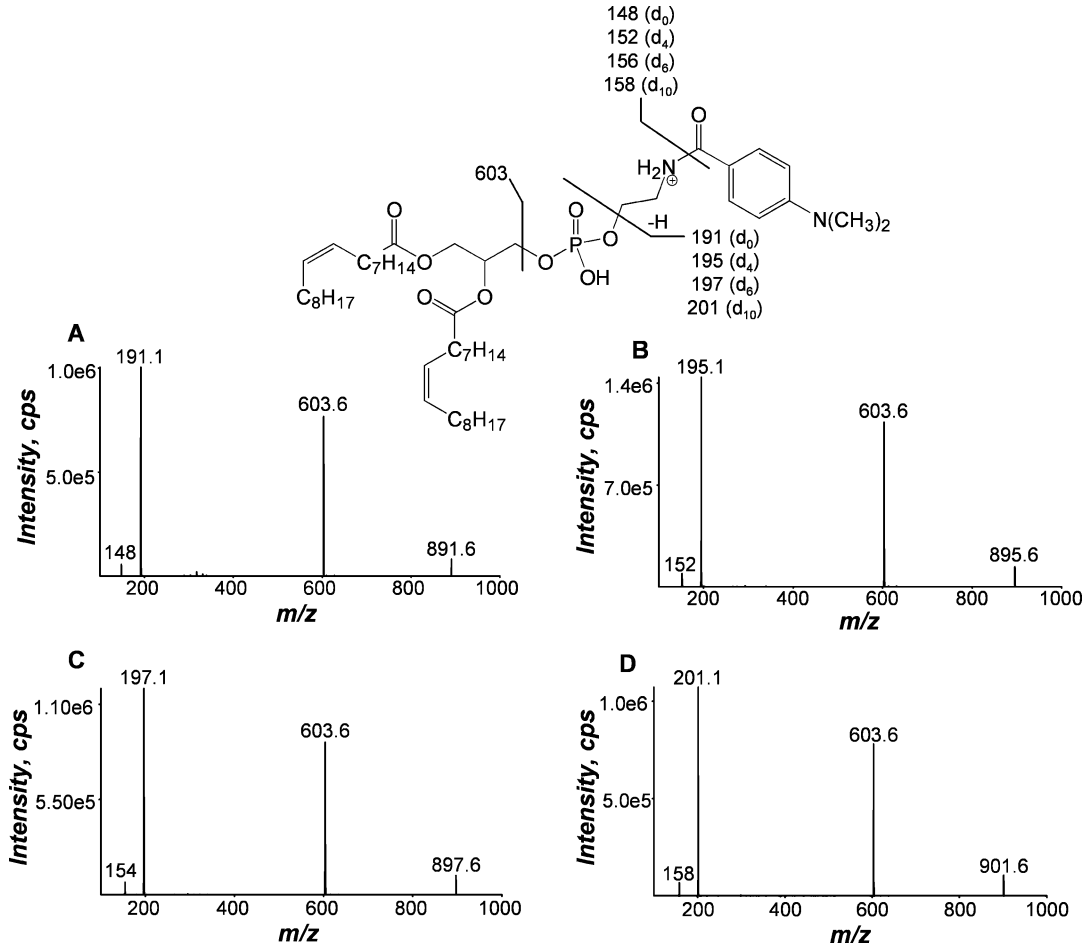

Figure 1. Positive ion CID spectra of the $[M + H]^+$ of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine labeled with (a) D_0-DMABA NHS ester reagent, (b) D_4-DMABA NHS ester reagent, (c) D_6-DMABA NHS ester reagent, and (d) D_{10}-DMABA NHS ester reagent at a collision energy of 30 V. The origins of the ions that resulted from collisional activation are indicated in the inset structure.

to determine the limits of detection using a P191 scan. It was found that 50 pg could be easily detected by a P191 scan and additional sensitivity could be achieved using an MRM method.

One major product ion at m/z 281.1 was observed in the negative ion CID spectrum of the $[M - H]^-$ at m/z 889.7, 893.7, 895.7, and 899.7 of the (D_0, D_4, D_6, and D_{10})-DMABA labeled 18:1a/18:1-PE (Figure 2). This ion was also present in the negative ion CID spectrum of 18:1a/18:1-PE and corresponded to the oleic acid carboxylate ion.[9] There were also minor product ions in the negative ion CID spectra of (D_0, D_4, D_6, and D_{10})-DMABA labeled 18:1a/18:1-PE at m/z 625.4, 629.4, 631.4, and 635.4, respectively, that corresponded to the loss of oleic acid present at the sn-2 position as a neutral ketene. Both the fatty acid ions and the neutral loss of the sn-2 position as a neutral ketene have been observed in the CID of unlabeled 18:1a/18:1-PE.[9] The negative ion CID behavior of DMABA labeled PE was largely unaltered in comparison to the unlabeled PE, which is important because negative ion tandem mass spectrometry can be used to characterize the fatty acyl substituents esterified to the glycerol backbone of DMABA labeled PE lipids.

The PE lipids extracted from RAW 264.7 cells were labeled with D_0-DMABA NHS ester reagent in order to determine if these reagents would work well for a complex mixture of PE lipids from biological samples. The $[M + H]^+$ for PE lipids extracted from RAW 264.7 cells were observed in the positive ion mass spectrum from m/z 690 to m/z 792 (Figure 3a). The CID spectra in the negative ion mode of the $[M - H]^-$ of all PE lipids in RAW 264.7 cells were obtained, and the fatty acids at the sn-1 and sn-2 position of the glycerol backbone were determined (Table S-1 in the Supporting Information). From this data, the presence of abundant plasmalogen PE lipids as well as acylated PUFAs in RAW 264.7 cells was established. A NL141 scan selectively detected diacyl and ether PE lipids, but plasmalogen PE were very poorly detected using a NL141 scan (Figure 3b). Once the PE lipids from RAW 264.7 cells were labeled with the D_0-DMABA NHS ester reagent, a positive ion mass spectrum scan revealed a distribution of PE lipids similar to the positive ion mass spectrum of PE present in the RAW 264.7 cells (Figure 3a) but shifted in mass +147 amu (Figure 3c). Because of the positive ion CID behavior of the D_0-DMABA labeled standards (Figure 1a), a P191 scan was used to selectively detect the D_0-DMABA labeled PE from RAW 264.7 cells (Figure 3d). The D_0-DMABA labeled PE were easily detected using this precursor ion scan, and the plasmalogen PE lipids were not discriminated against with this scan. The PE from RAW 264.7 cells was also labeled with (D_4, D_6, or D_{10})-DMABA NHS ester reagent, and the distribution of PE lipids similar to the mass spectrum of nonlabeled PE from RAW 264.7 cells, but shifted in mass 151, 153, or 157 amu, was observed using a P195, P197, or P201 scan, respectively (data not shown). It was concluded

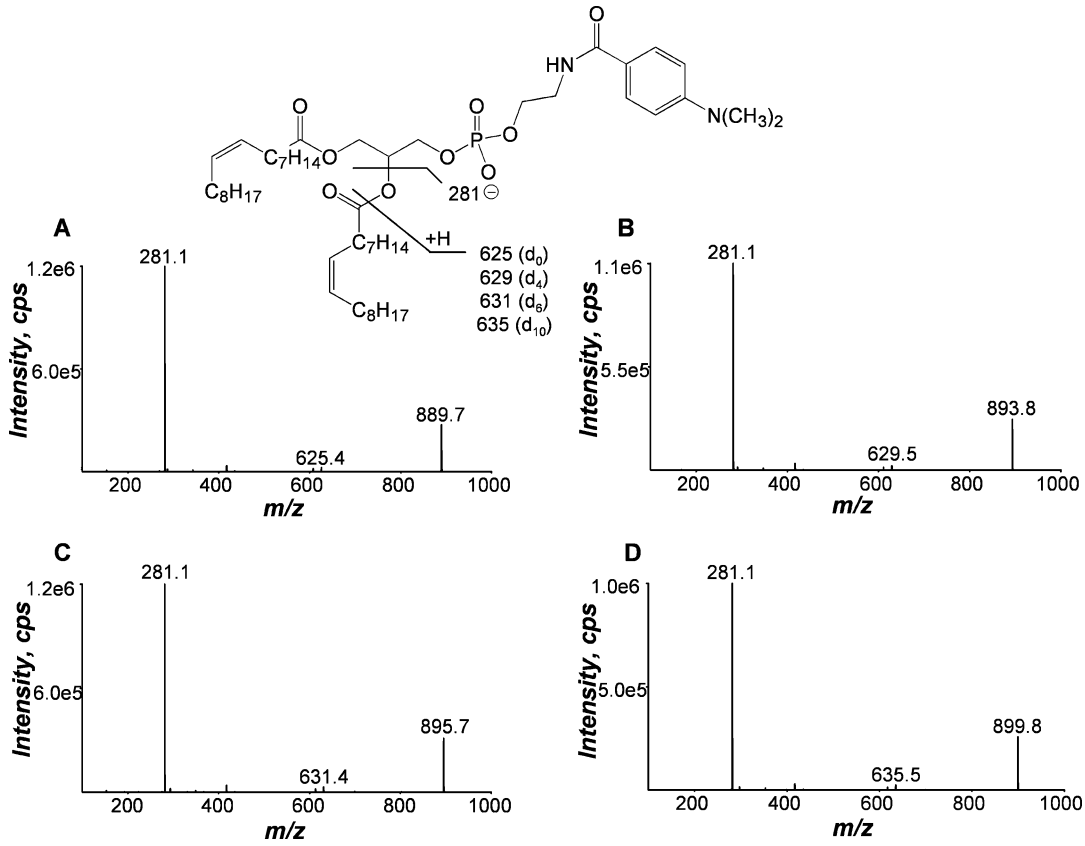

Figure 2. Negative ion CID spectra of the $[M - H]^-$ of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine labeled with (a) D_0-DMABA NHS ester reagent, (b) D_4-DMABA NHS ester reagent, (c) D_6-DMABA NHS ester reagent, and (d) D_{10}-DMABA NHS ester reagent at a collision energy of −50 V. The origins of the ions that resulted from collisional activation are indicated in the inset structure.

from this data that all subclasses of (D_0, D_4, D_6, or D_{10})-DMABA labeled PE species, including plasmalogens, could be detected using a P191, P195, P197, or P201 scan, respectively.

Control, 30 min AAPH treated, 60 min AAPH treated, and 300 min AAPH treated liposomes generated from RAW 264.7 phospholipids were labeled with the D_0-DMABA, D_4-DMABA, D_{10}-DMABA, and D_6-DMABA NHS ester reagents, respectively, and then combined. The sample was then subjected to RP-HPLC and the control D_0-DMABA labeled PE, 30 min AAPH treated D_4-DMABA labeled PE, 60 min AAPH treated D_{10}-DMABA labeled PE, and 300 min AAPH treated D_6-DMABA labeled PE were detected by a P191, P195, P201, and P197 scan, respectively (Figure 4). A scan rate of 1.5 s per precursor ion scan was used which fit with our chromatographic separation; however, faster scan rates are now available on newer instruments and may remove this limitation. The area of the D_0-, D_4-, D_{10}-, and D_6-DMABA internal standards were quite similar (Table 1), and therefore the chromatograms could be visually compared and changes in the PE distribution could be readily observed (Figure 4). Lipophilic compounds (23−36 min) that eluted from the column after the internal standard in the P191, P195, P201, and P197 chromatograms (Figure 4) were the endogenous, nonoxidized PE, which was confirmed by negative ion CID (data not shown). The chromatograms indicate that the endogenous PE decreased during the time course of AAPH treatment, which can be explained in part by loss of endogenous PE species to newly formed oxidation products during the time course of AAPH exposure.

The less lipophilic compounds (7−21 min) that eluted from the column before the internal standard in the P191, P195, P201, and P197 chromatograms (Figure 4) were the oxidized PE products formed upon AAPH treatment. The negative ion CID spectrum of one of the D_6-DMABA labeled PE species with a $[M - H]^-$ at m/z 760.6 that increased after 300 min of AAPH treatment and eluted from the reversed phase column at 15.41 min was obtained (Figure 5). The CID spectrum revealed fragmentation ions at m/z 633.5, 283.1, 144.1, and 112.1. The fragmentation ion at m/z 283.1 corresponded to the carboxylate ion of stearic acid, and the ion at m/z 633.5 corresponded to the loss of the sn-2 substituent as a neutral ketene.[9] Additionally, the m/z 144.1 product ion corresponded to the MOX derivative of 5-oxopentanoic acid and m/z 112.1 was consistent with the loss of neutral methanol (32 amu) from the MOX derivative of 5-oxopentanoic acid.[19] This data was consistent with proposed identification of this product with an $[M + H]^+$ at m/z 760.6 as the MOX derivative of D_6-DMABA labeled 1-stearoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphoethanolamine (18:0*a*/5*a*l-PE). The negative ion CID spectra of other species in this chromatographic region of interest were also obtained (data not shown) and permitted identification of several different types of oxidized PE lipids, including lyso PE, chain shortened ω-aldehydes at the sn-2 position, and direct oxidation of polyunsaturated fatty acids at the sn-2 position, which have been

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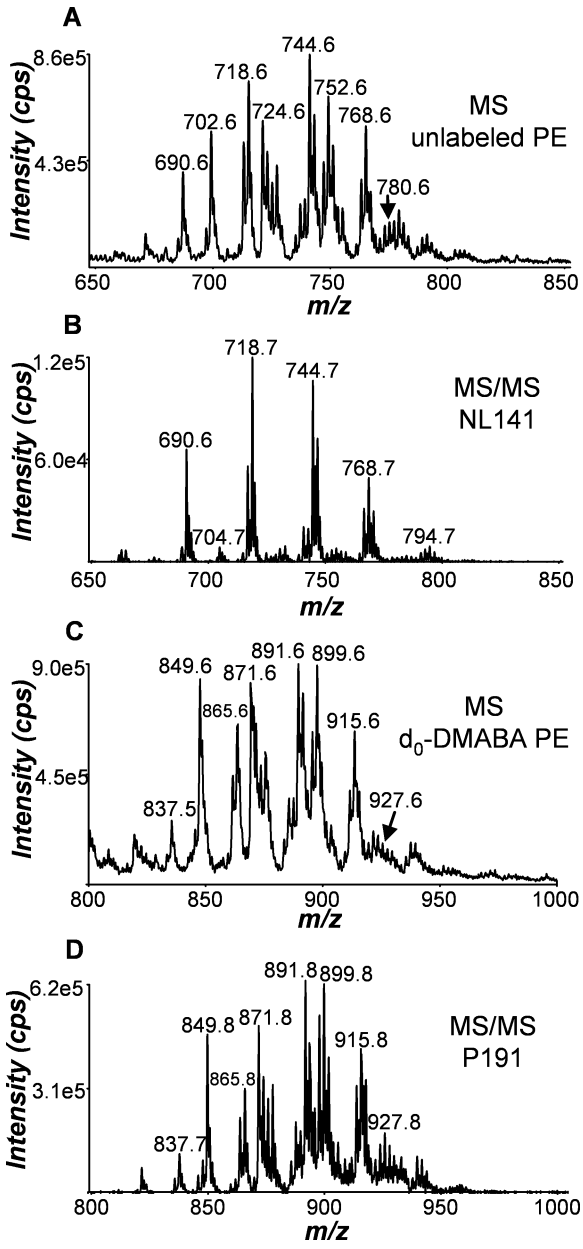

Figure 3. (a) Positive ion electrospray mass spectrum of the PE lipids present in RAW 264.7 cells. The ions correspond to $[M + H]^+$ of each PE present. (b) Neutral loss of 141 amu (NL141) scan to analyze PE present in RAW 264.7 cells. (c) Positive ion electrospray mass spectrum of the D_0-DMABA labeled PE lipids present in RAW 264.7 cells. Note the shift in the $[M + H]^+$ in the D_0-DMABA labeled PE lipids by 147 amu. The conversion of PE to DMABA labeled PE in RAW 264.7 cells was determined to be 89% based on the ratio of DMABA labeled ions (*m/z* 891) to the sum of DMABA labeled and corresponding unlabeled species (*m/z* 744, not shown in the figure). (d) Precursor ion scan of *m/z* 191.1 (P191) allows for specific detection of D_0-DMABA labeled PE lipids in RAW 264.7 cells.

previously observed.[20,21] These chromatograms indicate that the oxidized PE products increased during the time course of AAPH treatment, which can be explained by formation of new oxidized PE lipids.

The data above indicate PE oxidation products are detected using the DMABA NHS ester reagents; however, it was not clear if all oxidized species present before DMABA labeling could be detected after DMABA labeling. In order to examine this, 16:0*a*/20:4-PE was oxidized with AAPH for 1 h and half of the sample was derivatized with D_4-DMABA NHS ester reagent. The $[M + H]^+$ of the 16:0*a*/20:4-PE starting material is at *m/z* 740.7, and the $[M + H]^+$ of the D_4-DMABA labeled 16:0*a*/20:4-PE starting material is at *m/z* 891.7. The positive ion mass spectrum of oxidized 16:0*a*/20:4-PE (Figure 6a) and D_4-DMABA labeled oxidized 16:0*a*/20:4-PE (Figure 6b) included molecules that had molecular ions higher in mass than 16:0*a*/20:4-PE, presumably due to the addition of oxygen atoms to the arachidonoyl moiety of 16:0*a*/20:4-PE, and molecular ions lower in mass than 16:0*a*/20:4-PE, presumably due to oxidative fragmentation of 16:0*a*/20:4-PE. The product distribution of oxidized 16:0*a*/20:4-PE (Figure 6a) to D_4-DMABA labeled oxidized 16:0*a*/20:4-PE are very similar upon comparison, except that the D_4-DMABA labeled oxidized 16:0*a*/20:4-PE products are shifted in mass by 151 amu. From this data it was concluded that the oxidized PE species that are observable before labeling are still observable after DMABA labeling and that PE oxidation can be successfully monitored using the DMABA NHS ester reagents.

DISCUSSION

One of the most common ways to detect PE lipids using tandem mass spectrometry is a neutral loss of 141 amu scan. However, the disadvantage in using NL141 in order to determine PE content in complex lipid mixtures is that only diacyl and ether PE species undergo efficient neutral loss of phosphoethanolamine and that this selective scan discriminates against the detection of plasmalogen PE species. This difference in the CID behavior displayed by the various subclasses of PE lipids has complicated the detection of PE lipid because there is not a sensitive universal scan that permits selective detection of all PE lipids. In this study a set of four (D_0, D_4, D_6, and D_{10}) deuterium enriched DMABA NHS ester reagents was developed that react with the primary amine group of PE lipids in order to create derivatives where all subclasses of DMABA labeled PE could be detected by a common precursor ion scan. Indeed, the positive ion CID data from the (D_0, D_4, D_6, and D_{10})-DMABA labeled 18:1*a*/18:1-PE suggested that a single, common precursor ion scan could be used to selectively detect (D_0, D_4, D_6, and D_{10})-DMABA modified PE in a complex biological mixture. In fact, upon reaction of PE species extracted from RAW 264.7 cells with (D_0, D_4, D_6, and D_{10})-DMABA NHS ester reagents, all subclasses of PE lipids were easily detected and the analysis of all PE subclasses in a complex mixture became straightforward. Additionally, the negative ion CID behavior of DMABA labeled PE lipids was retained when compared to the unlabeled PE species, facilitating the identification of the radyl groups present at the sn-1 and sn-2 positions on the glycerol backbone, which is particularly valuable in structural studies of lipids.

While selective precursor ion scans allow for specific detection of certain classes of phospholipids, a major challenge still encountered has been the identification of those less abundant phospholipid species formed by free radical reactions initiated in certain disease states among an array of molecular species that do not change. The use of mass spectrometry and the stable isotope dilution strategy has been a powerful approach to address changes in biomolecules, but there has been a lack of available

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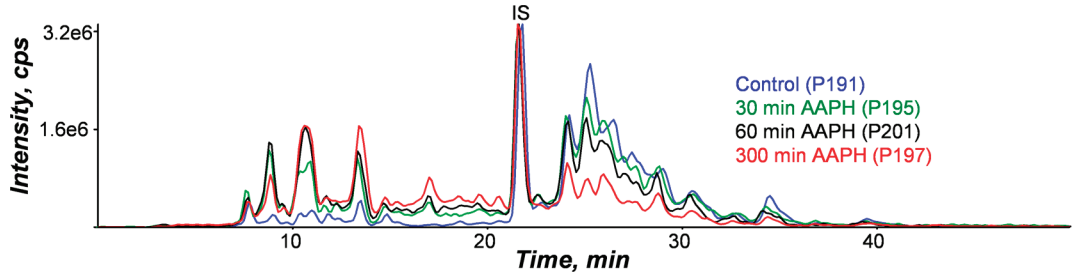

Figure 4. Reversed-phase HPLC separation and tandem mass spectrometry analysis (LC−MS/MS) of D_0-DMABA labeled PE from the control liposomes (blue), D_4-DMABA labeled PE from 30 min AAPH treated liposomes (green), D_{10}-DMABA labeled PE from 60 min AAPH treated liposomes (black), and D_6-DMABA labeled PE from 300 min AAPH treated liposomes (red). The elution of the D_0-DMABA labeled PE from the control liposomes from the reversed phase HPLC column was monitored by a precursor ion scan of *m*/*z* 191.1 (P191), while the elution of the D_4, D_{10}, and D_6-DMABA labeled PE from the AAPH treated liposomes from the reversed-phase HPLC column was monitored by a precursor ion scan of *m*/*z* 195.1, 201.1, and 197.1 (P195, P201, and P197), respectively. Note that the internal standard (IS) is recovered equally in each of the samples.

Table 1. Chromatographic Peak Area of the 14:0*a*/14:0-PE Internal Standard Used for the Time Course of Liposomes Exposed to AAPH

sample	$[M + H]^+$ of DMABA labeled 14:0*a*/14:0-PE (*m*/*z*)	retention time (min)	area of extracted chromatographic peak
control (D_0-DMABA)	783.7	21.73	5.58×10^7
30 min AAPH (D_4-DMABA)	787.7	21.63	5.45×10^7
60 min AAPH (D_{10}-DMABA)	793.7	21.49	5.39×10^7
300 min AAPH (D_6-DMABA)	789.7	21.57	5.62×10^7

phospholipid stable isotope internal standards to facilitate 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 or in certain disease states. These deuterium enriched DMABA NHS reagents address these issues because they enable differential labeling of PE lipids and thereby generate an internal standard for every PE lipid of interest. The deuterium enriched DMABA reagents allow for a direct comparison of control PE lipids to treated PE lipids and in addition direct comparison of the changes in abundance of PE lipids.

While labeling phospholipid extracts from cells or tissues does add another step to sample preparation, the synthesis of the DMABA NHS ester reagents and PE labeling procedures are straightforward. In addition, there are specific advantages to labeling PE lipids with DMABA NHS ester reagents. First, all subclasses of (D_0, D_4, D_6, and D_{10})-DMABA labeled PE lipids in a complex mixture can be selectively detected using a P191, P195, P197, and P201 scan, respectively. Additionally, modification of the primary amine group of the PE lipid upon reaction with (D_0, D_4, D_6, and D_{10})-DMABA NHS ester reagents, permitted the (D_0, D_4, D_6, and D_{10})-DMABA labeled PE to be separated from PC lipids in complex biological samples with a facile aminopropyl solid phase extraction (SPE) procedure. This aminopropyl separation of (D_0, D_4, D_6, and D_{10})-DMABA labeled PE lipids from PC lipids has the benefit of removing PC from the sample, and therefore the detection of (D_0, D_4, D_6, and D_{10})-

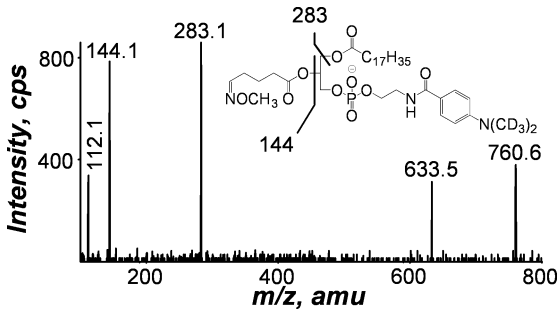

Figure 5. Negative ion CID of the $[M - H]^-$ at *m*/*z* 760.6 using a triple quadrupole mass spectrometer at a collision energy of −50 V with a retention time of 15.41 min. The origins of the ions that resulted from collisional activation are indicated in the structures of these molecules. This data indicates that the $[M + H]^+$ that eluted from the column at 15.41 min is MOX derivatized D_6-DMABA labeled 18:0*a*/5*a*l-PE.

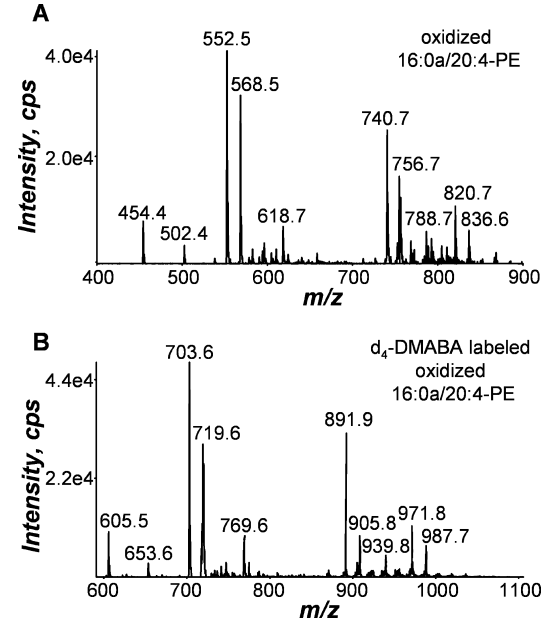

Figure 6. Positive ion mass spectrum of (a) oxidized 16:0*a*/20:4-PE and (b) D_4-DMABA labeled oxidized 16:0*a*/20:4-PE. Note the shift in the $[M + H]^+$ in the D_4-DMABA labeled oxidized PE lipids by 151 amu.

DMABA labeled PE is not suppressed in positive ion mode. Finally only one chromatographic run would be needed to analyze up to four different experimental conditions because of the differential display capability afforded by four different DMABA NHS ester reagents. Once labeled with the (D_0, D_4, D_6, and D_{10})-DMABA NHS ester reagents, the samples from four experimental variants could be mixed together and the products could be detected in one single chromatographic run by cycling through the P191, P195, P197, and P201 scans.

CONCLUSION

It has been found that the (D_0, D_4, D_6, or D_{10})-DMABA NHS ester reagents provide a novel and facile way to observe all subclasses of PE lipids by tandem mass spectrometry. Additionally, DMABA labeling is a novel and facile means to observe changes in the distribution of PE lipids and appearance of novel PE lipid products in a myriad of biological events (e.g., oxidation and apoptosis). This DMABA labeling method can also compensate for the lack of PE internal standards currently available.

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SUPPORTING INFORMATION AVAILABLE

Synthesis of DMABA NHS ester reagents and a table showing the PE present in RAW 264.7 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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