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Characterization of Oxidized Phosphatidylethanolamine Derived from RAW 264.7 Cells using 4-(Dimethylamino)benzoic Acid Derivatives

Karin A. Zemski Berry, William W. Turner[†], Michael S. VanNieuwenhze[†], and Robert C. Murphy^{*}

Department of Pharmacology, University of Colorado Denver, Aurora, CO 80045

[†]Department of Chemistry, Indiana University, Bloomington, IN 47401

Abstract

Recently, a derivative of phosphatidylethanolamine (PE), namely the 4-(dimethylamino)benzoic acid derivative has been developed with various isotope labeled variants that provided a universal precursor ion scan for diacyl, ether, and plasmalogen PE lipids that can not be accomplished otherwise. This derivative was further investigated as a means to facilitate characterization of various oxidized phosphatidylethanolamine lipids by collision activation. Phospholipids derived from RAW 264.7 cells were treated with a free radical generating system to generate a complex mixture of oxidized and nonoxidized lipids that were separated by reversed phase HPLC and detected using a precursors of m/z 191 scan for the d_0 -DMABA labeled control sample and a precursors of m/z 197 scan for the d_6 -DMABA labeled oxidized sample. Collisional activation of the corresponding $[M-H]^-$ ions permitted the identification of several chained shortened ω -aldehydes, as well as direct oxygen addition products including isoprostane PE and monohydroxy PE oxidized phospholipids that were not easily detected without the use of the DMABA derivatives. The stable isotope labeled derivatives permitted assessment of relative quantitative changes in oxidized lipids based upon ion abundance.

Keywords

electrospray; tandem mass spectrometry; lipid oxidation; derivatization

Introduction

The oxidation of phospholipids (PL) in tissues is thought to be a central process taking place in numerous human diseases including emphysema (1), atherosclerosis (2), and Alzheimer's disease (3). Yet the detection and characterization of oxidized phospholipids remains a challenge. Most mass spectrometric studies of the oxidation of PL have focused on the oxidation of phosphatidylcholine lipids (PC) because they are reservoirs of abundant polyunsaturated fatty acyl (PUFA) groups, but also yield readily detectable product ions after collisional activation (4–6). Thus, precursors of m/z 184 readily identify all PC-lipids including oxidized PC.

Phosphatidylethanolamine lipids are also abundant PLs containing PUFA groups. One of the most common ways to detect PE lipids using tandem mass spectrometry is a neutral loss of

Correspondence: Robert C. Murphy, Ph.D., Department of Pharmacology, University of Colorado Denver, Mail Stop 8303, 12801 E. 17th Ave., Aurora, CO 80045-0511, Tel: 303-724-3352 / Fax: 303-724-3357 / Robert.Murphy@ucdenver.edu.

141 Da scan, however, this selective scan discriminates against the detection of plasmalogen PE species (7). 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. Recently we described DMABA N-hydroxysuccinimide (NHS) ester reagents 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 in the positive ion mode. The negative ion CID behavior was retained and readily revealed the radyl groups present at the sn-1 and sn-2 position of the glycerol backbone (8), which could be readily interpreted in terms of oxidized products within the structure of phosphatidylethanolamine lipids.

Experimental

Materials

1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (14:0a/14:0-PE)¹ was purchased from Avanti Polar Lipids, Inc (Alabaster, AL). 2,2'-Azobis-(2-amidinopropane) hydrochloride (AAPH) and methoxyamine hydrochloride (MOX) were purchased from Sigma Chemical Company (St. Louis, MO). HPLC solvents were purchased from Fisher Scientific (Fair Lawn, NJ) and used for HPLC and extraction.

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

Lipids were extracted from RAW 264.7 cells (20×10^6) by a Bligh-Dyer extraction (9). The phospholipid extract was split equally into two 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 suspension for 30 min followed by sonication for 10 min. AAPH was added to the phospholipid suspension in one of the test tubes for a final concentration of 10 mM. The control and the AAPH treated liposomes were incubated at 37°C for 5 h and the phospholipids were extracted using Bligh-Dyer extraction after the internal standard (14:0a/14:0-PE, 1 μ g) was added and methoxime derivatives of lipids were prepared using a gas phase MOX procedure (10).

DMABA labeled PE separation by RP-HPLC and analysis by electrospray ionization mass spectrometry

Synthesis of DMABA NHS ester reagents and the labeling procedure for PE lipids (Scheme 1) have been described previously (8). Briefly, the (d_0 or d_6)-DMABA NHS ester reagent (20 μ l of a 10 mg/ml solution) was added to the extracted lipids in 80/20 ethanol/0.25 M triethylammonium bicarbonate buffer (80 μ l) and allowed to incubate at 60°C for 1 h. The PE lipids in the control sample were labeled with the d_0 -DMABA NHS ester reagent, while the PE lipids in the AAPH treated sample were labeled with the d_6 -DMABA NHS ester reagent. 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 (9). The combined DMABA labeled control and oxidized PE lipids were then purified by solid phase extraction followed by reversed phase HPLC as previously described (8).

¹Abbreviations for individual PE molecular species used in this paper: n:j/k/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.

Briefly, the d_0 - and d_6 -DMABA labeled PE species were separated with a Gemini 5μ C₁₈ (2.0×150 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 - and d_6 -DMABA labeled PE lipids from 1×10^6 cells were detected during one chromatographic run by alternating between a precursors of m/z 191.1 (P191) and 197.1 (P197) scan, respectively, every 3 s with a collision energy of 35 eV. The negative ion CID spectra were obtained with an electro spray voltage of -4500 V, a declustering potential of -50 V, and collision energy of 50 eV.

Results and Discussion

The d_0 - and d_6 -DMABA labeled PE from the equivalent of 1×10^6 cells from the combined control and oxidized samples was subjected to RP-HPLC in order to separate the molecular species according to lipophilicity. This was achieved in one HPLC experiment where the d_0 -DMABA labeled PE from the control liposomes was detected using a P191 scan and the d_6 -DMABA labeled PE from the oxidized liposomes was detected by a P197 scan (Figure 1). The area of the d_0 -DMABA 14:0a/14:0-PE internal standard, which eluted at 20.96 min in the P191 chromatogram, was 6.63×10^7 and the area of the d_6 -DMABA 14:0a/14:0-PE internal standard, which eluted at 20.87 min in the P197 chromatogram, was 6.75×10^7 . The area of the d_0 - and d_6 -DMABA internal standards was very similar and therefore the control d_0 -DMABA labeled PE and the oxidized d_6 -DMABA labeled PE chromatograms could be visually compared and changes in the PE distribution in oxidized sample could be readily observed. The lipophilic compounds (23–36 min) that eluted from the column after the internal standard (Figure 1) were the endogenous, non-oxidized PE, which was confirmed by negative ion CID (data not shown). The chromatograms indicate that the endogenous PE decreased after AAPH treatment, which can be explained in part by loss of endogenous PE species to newly formed oxidation products.

The oxidized d_6 -DMABA labeled PE lipids were less lipophilic than the internal standard and eluted at 7–20 min (Figure 1). When all ions over the mass range of 570–700 Da which corresponded to the P191 scan (control d_0 -DMABA labeled PE) were superimposed on all the ions from the P197 scan (oxidized d_6 -DMABA labeled PE) in this chromatographic region (Figure 2a), the most abundant $[M+H]^+$ observed was at m/z 633.4 derived from the oxidized sample (P197). This d_6 -DMABA labeled PE $[M+H]^+$ at m/z 633.4 eluted at 10.17 min (Figure 2b) as single chromatographic peak and the corresponding d_0 -DMABA labeled PE $[M+H]^+$ at m/z 627.4 in the control sample at 10.22 min (Figure 2b). The CID spectrum of the corresponding negative ion at m/z 631.4 (Figure 2c) eluting at this retention time revealed an abundant ion at m/z 281.1, which corresponded to the intact carboxylate ion of oleic acid, and the product ion at m/z 153.1 corresponds to the 1,2-cyclic phosphodiester of glycerol (11). This was consistent with the identification of the $[M+H]^+$ at m/z 633.4 in the P197 total ion mass spectrum as d_6 -DMABA labeled 18:1a/OH-PE, a lyso PE. The negative ion CID spectra of all of the ions present in the d_6 -DMABA labeled lyso PE region total ion mass spectrum from the P197 (Figure 2a) were obtained and this data permitted identification of the other d_6 -DMABA labeled lyso PE compounds present in the oxidized sample (Table 1). It is important to note that these lyso PE species had acyl radical groups (sn-position undetermined) and no plasmalogen lyso PE species were detected. Previously it has been shown that AAPH induced free radical oxidation of plasmalogen phospholipids results in oxidation at the 1-O-alk-1'-enyl position to form lysophospholipids (17). It is thought that this mechanism was quite prevalent in the current studies due to the abundance of lyso PE generated that contained PUFA (Table 1).

Additional oxidation products were observed when the total ion spectrum over the mass range of 700–850 Da of the P191 chromatogram of control d₀-DMABA labeled PE and the P197 of oxidized d₆-DMABA labeled PE chromatogram from 7–20 min were overlaid (Figure 3a). All abundant ions found in this region had even mass [M+H]⁺, which suggested that these species contained either a ketone or aldehyde group that was derivatized by methoxylation. The second most abundant [M+H]⁺ in the total ion mass spectrum was at *m/z* 746.7 (Figure 3a) which eluted as a P197 at 14.03 and 15.68 min as two chromatographic peaks (Figure 3b). The corresponding d₀-DMABA labeled PE [M+H]⁺ at *m/z* 740.7 was not detected as a P191 in the control sample (Figure 3b). The negative ion CID spectrum of the corresponding [M–H][–] at *m/z* 744.7 revealed a product ion at *m/z* 617.6 (Figure 3c), which corresponded to loss of the sn-2 substituent as a neutral ketene. Product ions at *m/z* 144.1, which corresponded to the MOX derivative of 5-oxopentanoic acid, and 112.1, which was consistent with the loss of neutral methanol (32 Da) from the MOX derivative of 5-oxopentanoic acid, were also observed (Figure 3c). This data was consistent with identification of this product with an [M+H]⁺ at *m/z* 746.7 as the MOX derivative of d₆-DMABA labeled 1-O-octadec-1'-enyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphoethanolamine (18:0p/5al-PE). Other oxidized PE products similar to MOX derivatized d₆-DMABA labeled 18:0p/5al-PE were present in Figure 3a and a summary of these chain shortened ω-aldehyde PE products are listed in Table 2. The product ions in the CID spectra of [M–H][–] of the d₆-DMABA labeled ω-aldehyde PE products after MOX derivatization (Table 2) were consistent with the results obtained for 18:0p/5al-PE. Therefore, it was concluded that these other compounds in Table 2 and Figure 3a were also chain shortened ω-aldehyde PE products. It is thought that the chain-shortened ω-aldehydes esterified to the sn-2 position, including 5-oxopentanoic acid (5al), 7-oxoheptanoic acid (7al), and 9-oxononanoic acid (9al), result from oxidation of carbon-5, carbon-7, and carbon-9 of arachidonic acid, adrenic acid, and linoleic acid, respectively.

Additional oxidation products were observed when the total ion spectrum over the mass range of 850–1050 Da of the P191 chromatogram of control d₀-DMABA labeled PE and the P197 of oxidized d₆-DMABA labeled PE chromatogram from 7–20 min were overlaid (Figure 4a). An [M+H]⁺ at *m/z* 953.7 was one of the abundant ions present in the total ion mass spectrum (Figure 4a) and eluted from the P197 chromatogram between 15–21 minutes (Figure 4b) as a number of separable components. The corresponding d₀-DMABA labeled PE [M+H]⁺ at *m/z* 947.7 was not detected in P191 chromatogram of the control sample (Figure 4b). The negative ion CID spectrum of the corresponding [M–H][–] for this species at *m/z* 951.7 had some product ions at *m/z* 617.6, 351.1, 333.1, and 271.1 (Figure 4c). The product ion at *m/z* 617.6 resulted from loss of the sn-2 substituent as a neutral ketene, while the product ions at *m/z* 351.1, 333.1, and 271.1 suggested D₂/E₂-isoprostanes (D₂/E₂-isoP) at the sn-2 position of the glycerol backbone (12). This data was consistent with the identification of this product with an [M+H]⁺ at *m/z* 953.7 as d₆-DMABA labeled 18:0p/D₂/E₂-isoP-PE. The identification of the [M+H]⁺ at *m/z* 953.7 as containing D₂/E₂-isoPs at the sn-2 position of the glycerol backbone was also consistent with the large number of separable components observed in the chromatography (Figure 4b) and indicated a complex mixture of species. It should be mentioned that D₂/E₂-isoPs contain one ketone group and that these molecular species were also detected as the methoxime derivative. However, MOX derivatization was not completely efficient in converting the ketone group on D₂/E₂-isoP containing PE into a methoxime derivative.

Other oxidized PE products, similar to d₆-DMABA labeled 18:0p/D₂/E₂-isoP-PE, were present in Figure 4a and a summary of some of these direct oxidation products of PUFAs, such as regioisomers of hydroperoxy, hydroxyl and ketone fatty acyl groups, are shown in Table 3. All of the compounds identified in this table did not have one single chromatographic peak, but multiple components, which suggested a complex mixture of

regioisomers of any given $[M+H]^+$ as detected by a P197 scan. In addition to the presence of D_2/E_2 -isoPs esterified to the sn-2 position of the glycerol backbone, there was indication of F_2 -isoprostanes (F_2 -isoP) (12–14), hydroxyoctadecadenoic acid (HODE) or hydroxyeicosatetraenoic acid (HETE), respectively (12,15) esterified to the sn-2 position of the glycerol backbone. It should be noted that only a few CID spectra were obtained of the direct oxidation products of PUFAs found in the m/z range of 850–1050 Da. While there were many more oxidation products in this region that were found in the P197 scan, they were not identified in the current study.

Conclusion

The DMABA derivatization of oxidized PE lipids yielded structurally relevant positive and negative product ions following collisional activation of $[M+H]^+$ and $[M-H]^-$, respectively. Several different families of oxidized PE products could be identified when RAW 264.7 cells were exposed to a free radical generating system even though these PE lipids were present in a complex mixture of nonoxidized (not deuterated) as well as oxidized molecular species (deuterated). Several chain-shortened ω -aldehydes and direct oxidation products (D_2/E_2 -isoP, F_2 -isoP, HODE, and HETE) at the sn-2 position with both acyl and vinyl ether linkages at the sn-1 position. Since 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 co-purified and behave identically in the mass spectrometric experiment, the advantage of the deuterated DMABA derivatization is to enable an assessment of changes in molecular species by stable isotope tagging. Furthermore, collision induced dissociation of both positive and negative ions of the DMABA PE derivatives yielded valuable information as to the identity of the oxidized PE molecular species.

List of Abbreviations

DMABA	4-(dimethylamino)benzoic acid
NHS	N-hydroxysuccinimide
AAPH	2,2'-azobis-(2-amidinopropane) hydrochloride
NL141	neutral loss of 141 Da
PE	glycerophosphoethanolamine
PC	glycerophosphocholine
CID	collision induced dissociation
P191	precursors of m/z 191.1
P197	precursors of m/z 197.1
MOX	methoxyamine hydrochloride
PUFA	polyunsaturated fatty acyl
MRM	multiple reaction monitoring

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Figure 1.

Reversed phase HPLC separation and tandem mass spectrometry analysis (LC/MS/MS) of d_0 -DMABA labeled PE from the control liposomes (gray) and d_6 -DMABA labeled PE from the oxidized liposomes (black). 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_6 -DMABA labeled PE from the oxidized liposomes from the reversed phase HPLC column was monitored by a precursor ion scan of m/z 197.1 (P197). Note that the internal standard is recovered equally in both samples.

Figure 2.

(a) The total ion mass spectrum over the mass range of 570–700 Da of the P191 chromatogram of control d_0 -DMABA labeled PE and the P197 chromatogram of oxidized d_6 -DMABA labeled PE from 7–20 min in Figure 4 have been overlaid demonstrating increased oxidized lipid production in AAPH treated liposomes (black) compared to control (gray). (b) Extraction of the $[M+H]^+$ of d_0 -DMABA labeled PE at m/z 627.4 detected by a P191 scan (gray) and the $[M+H]^+$ of d_6 -DMABA labeled PE at m/z 633.4 detected by a P197 scan. Note how the ion present in the oxidized sample is much bigger than the corresponding ion in the control sample. (c) Negative ion CID spectrum of the $[M-H]^-$ at m/z 631.4 at a collision energy of 50 eV. The origins of the ions that resulted from

collisional activation are indicated in the structures of these molecules. This data indicates that the most abundant ion present in panel (a) is identified as d₆-DMABA labeled 18:1a/OH-PE.

Figure 3.

(a) The total ion mass spectrum over the mass range of 700–850 Da of the P191 chromatogram of control d_0 -DMABA labeled PE and the P197 chromatogram of oxidized d_6 -DMABA labeled PE from 7–20 min in Figure 4 have been overlaid demonstrating increased oxidized lipid production in AAPH treated liposomes (black) compared to control (gray). (b) The second most abundant $[M+H]^+$ in the total ion mass spectrum was at m/z 746.7 (panel a) and was extracted from the P197 chromatogram (black) and the corresponding d_0 -DMABA labeled PE $[M+H]^+$ at m/z 740.7 was not detected in P191 chromatogram of the control sample (gray). (c) Negative ion CID spectrum of the $[M-H]^-$ at m/z 744.7 at a collision energy of 50 eV with a retention time of 15.68 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 15.68 min is identified as MOX derivatized d_6 -DMABA labeled 18:0p/5al-PE.

Figure 4.

(a) The total ion mass spectrum over the mass range of 850–1050 Da of the P191 chromatogram of control d₀-DMABA labeled PE and the P197 chromatogram of oxidized d₆-DMABA labeled PE from 7–20 min in Figure 4 have been overlaid demonstrating increased oxidized lipid production in AAPH treated liposomes (black) compared to control (gray). (b) One of the abundant [M+H]⁺ in the total ion mass spectrum was at *m/z* 953.7 (panel a) and was extracted from the P197 chromatogram (black) and the corresponding d₀-DMABA labeled PE [M+H]⁺ at *m/z* 947.7 was not detected in P191 chromatogram of the control sample (gray). (c) Negative ion CID spectrum of the [M–H][–] at *m/z* 951.7 at a collision energy of 50 eV. The origins of the ions that resulted from collisional activation are

indicated in the structures of these molecules. This data indicates that the $[M+H]^+$ at m/z 953.7 in panel (a) is identified as d₆-DMABA labeled d₆-DMABA labeled 18:0p/D₂/E₂-isoP-PE.

Table 1

Summary of the fragment ions observed in the negative ion CID spectra of the $[M-H]^-$ of the d_6 -DMABA labeled lyso PE found in oxidized liposomes.

d_6 -DMABA tagged $[M-H]^-$	Retention time (min)	Product ions	PE phospholipid
603.4	7.97	253.1	16:1a/OH-PE
677.4	8.28	327.1	22:6a/OH-PE
653.4	8.47	303.1	20:4a/OH-PE
629.4	8.70	279.1	18:2a/OH-PE
679.4	9.07	329.1	22:5a/OH-PE
605.4	9.90	255.1	16:0a/OH-PE
655.4	10.06	305.1	20:3a/OH-PE
631.4	10.17	281.1	18:1a/OH-PE
681.4	10.48	331.1	22:4a/OH-PE
657.4	11.55	307.1	20:2a/OH-PE
683.4	12.43	333.1	22:3a/OH-PE
633.4	12.79	283.1	18:0a/OH-PE
659.4	13.12	309.1	20:1a/OH-PE
685.4	13.94	335.1	22:2a/OH-PE
687.4	16.18	337.1	22:1a/OH-PE

Table 2

Summary of the fragment ions observed in the product ion spectra in the negative ion mode of the $[M-H]^-$ of the MOX derivatized d_6 -DMABA labeled PE with ω -aldehydes in the sn-2 position found in oxidized liposomes.

d_6 -DMABA tagged $[M-H]^-$	Retention time (min)	Product ions	PE phospholipid
732.6	11.70	255 144 112	16:0a/5al-PE
758.6	12.29	281 144 112	18:1a/5al-PE
716.6	12.84	589 144 112	16:0p/5al-PE
786.6	13.09	281 172 140	18:1a/7al-PE
744.6	14.03	589 172 144	16:0p/7al-PE
760.6	14.65	633 283 144 112	18:0a/5al-PE
814.6	14.95	631 281 200 168	18:1a/9al-PE
744.6	15.68	617 144 112	18:0p/5al-PE
788.6	15.68	283 172 140	18:0a/7al-PE
772.6	16.85	617 172 140	18:0p/7al-PE
816.6	17.24	633 283 200 168	18:0a/9al-PE
800.6	18.30	617 200 168	18:0p/9al-PE

Table 3

Summary of the fragment ions observed in the negative ion CID spectra of the $[M-H]^-$ of the d_6 -DMABA labeled PE with direct oxidation of PUFA at the sn-2 position found in oxidized liposomes.

d_6 -DMABA tagged $[M-H]^-$	Retention time (min)	Product ions	PE phospholipid
883.7	17–21	255/295	16:0a/HODE-PE
909.7	17–21	281/295	18:1a/HODE-PE
923.7	15–20	589/351/333/271	16:0p/D ₂ /E ₂ -isoP-PE
933.7	17–20	281/319	18:1a/HETE-PE
951.7	15–21	617/351/333/271	18:0p/D ₂ /E ₂ -isoP-PE
965.7	13–19	631/281/351/333/271	18:1a/D ₂ /E ₂ -isoP-PE
967.7	14–20	633/283/351/333/271 631/281/353	18:0a/D ₂ /E ₂ -isoP-PE 18:1a/F ₂ -isoP-PE
969.7	15–20	633/283/353	18:0a/F ₂ -isoP-PE