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Identification of intact oxidation products of glycerophospholipids *in vitro* and *in vivo* using negative ion electrospray iontrap mass spectrometry

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Abstract

Free radical-induced oxidation products of polyunsaturated fatty acids esterified to phospholipids have been implicated in a number of human diseases including atherosclerosis and neurodegenerative diseases. Some of these phospholipid oxidation products have potent biological activities and likely contribute to human pathophysiological conditions. Oxidation products have also been used as markers of oxidative stress *in vivo*. Identification and quantification of phospholipid oxidation products are often performed by analyzing the oxidized free fatty acid moieties after hydrolysis from the phospholipids head groups by gas chromatography–mass spectrometry (GC–MS) or liquid chromatography–mass spectrometry (LC–MS). We now describe the definitive identification of intact oxidized products of glycerophospholipids including glycerophosphatidylcholine (GPC), glycerophosphatidylethanolamine (GPE), and glycerophosphatidylserine (GPS) *in vitro* and *in vivo* using iontrap MS. For these analyses, the negative ions of the oxidation products of phospholipids are fragmented to MSⁿ and unequivocal structural characterization is obtained based on collision-induced dissociation (CID) of the *sn*-2 carboxylate ion. This technique overcomes the need to hydrolyze fatty acids from phospholipids in the analysis. The method has been used to identify a number of oxidation products of glycerophospholipids including hydroxyeicosatetraenoates (HETEs) and isoprostanes (IsoPs) esterified to different classes of glycerophospholipids *in vitro* and *in vivo*. These studies thus provide a new approach to identify the intact oxidation products of glycerophospholipids.

Keywords

phospholipids; free radicals; oxidative stress; liquid chromatography–mass spectrometry; isoprostanes; iontrap MS

Introduction

Oxidative stress and free radical-induced oxidation of polyunsaturated fatty acids (PUFA)-containing lipids have been implicated in a number of human diseases including atherosclerosis

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and neurodegenerative diseases.[1–4] Phospholipids are the major components of cellular membrane and have important structural and functional properties. Glycerophospholipids are made up of several distinct classes with different polar head groups, including glycerophosphatidylcholine (GPC), glycerophosphatidylethanolamine (GPE), and glycerophosphatidylserine (GPS) (Fig. 1).[5] The temporal and spatial distribution of different classes of phospholipids is believed to have important biological significance. Each phospholipid class consists of a different combination of fatty acids that can vary in chain length and degree of unsaturations at *sn*-1 (R_1) and *sn*-2 (R_2) positions on the glycerol backbone. Mass spectrometry-based lipidomics approaches have been widely used to study the distribution of these different lipid species in biological systems and correlate the changes to biological response.[6–9]

PUFA-containing phospholipids are primary targets for free radical attack.[3,10–13] A number of oxidation products have been identified by a variety of MS methods and isoprostanes (IsoPs), isomers of prostaglandins, are one of the major oxidation products.[14] IsoPs and other oxidation products are believed to be formed via a unified free radical mechanism and a number of different types of oxidation products can be formed (Fig. 2).[12,15] Direct analysis of these oxidation products by MS has proven difficult; therefore, most structural characterizations and quantification are usually carried out on the free acid after base or enzymatic hydrolysis.[16–18] Measurement of the levels of F₂-IsoPs has evolved as the gold standard for assessing oxidative stress *in vivo*, but this method requires extensive derivatization including reduction, base hydrolysis, pentafluorobenzyl (PFB) esterification and trimethylsilyl (TMS) derivatizations before gas chromatography–mass spectrometry (GC–MS) analysis.[19,20] A number of liquid chromatography–mass spectrometry (LC–MS) methods have been developed to study the oxidation products with reduced sample preparation.[21] However, some of the oxidation products, such as peroxides, and D/E or A/J-IsoPs, are unstable under basic hydrolytic conditions and phospholipase hydrolysis often provides irreproducible results. We report here a LC–MSⁿ method using an iontrap MS to identify intact oxidation products of phospholipids based on fragmentation of MSⁿ. Unequivocal structural characterization of these intact phospholipids oxidation products is reported. This method has been applied to study the oxidation products *in vitro* and *in vivo*.

Experimental

Reagents

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. 1-Stearoyl-2-arachidonyl-phosphatidylcholine (SAPC), 1-stearoyl-2-arachidonyl-phosphatidylethanolamine (SAPE), 1-palmitoyl-2-linoleoyl-phosphatidylserine (PLPS), and 1-palmitoyl-2-linoleoylphosphatidylcholine (PLPC) were purchased as a chloroform solution. Arachidonic acid and linoleic acid were purchased from NuChek Prep (Elysian, MN, USA) and were of the highest purity (>99%). Methanol, water, and 2-propanol were of HPLC quality and purchased from either Fisher Chemical (Phillipsburg, NJ, USA) or EM Science (Gibbstown, NJ, USA). Hexanes were purchased from Burdick and Jackson (Muskegon, MI, USA). All other reagents were purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI, USA) and used without further purification. The free radical initiator 2, 2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeOAMVN) was generously donated by Wako Chemicals USA Inc. (Richmond, VA, USA).

Oxidation of lipids *in vitro*

Arachidonic acid (25 mg, 80.24 μ mol) was dissolved in 1.0 ml benzene. To the mixture was added 0.1 equivalent of MeOAMVN as radical initiator. The mixture was kept under atmospheric oxygen at 37 °C for 24 h. The reaction was quenched by adding 2 mg of butylated

hydroxytoluene (BHT). The reaction mixture was stored at -80°C until analysis. F₂-isoprostanes are generated by PPh₃ reduction *in situ*. D₂/E₂-IsoPs were generated by incubation of the oxidation mixture in aqueous media and reduced by PPh₃. Phospholipids were oxidized as a thin film under atmospheric oxygen at 37°C for 24 h using the free radical initiator MeOAMVN.[21]

Extraction of phospholipids from the rat liver

Sprague–Dawley rats from Charles Rivers (Wilmington, MA, USA) were administered CCl₄ (1 ml/kg) orogastrically to induce oxidative stress 4 h before killing. Livers were removed and immediately flash-frozen in liquid nitrogen and stored at -80°C . Analyzed tissue samples were homogenized in 5 ml of ice-cold chloroform : methanol (2 : 1, v/v) containing BHT (0.005%) and triphenolphosphine to prevent *ex vivo* auto-oxidation. The extraction of IsoPs from livers of rats treated with CCl₄ was performed as reported previously.[22] Phospholipids were separated from other lipids as follows. An inert II silica Sep-Pak (500 mg) was pre-washed with 5 ml hexanes followed by 5 ml chloroform. Each sample was applied to a column and initially extracted with 12 ml of hexanes/methyl *tert*-butyl ether (MTBE) (200/3, v/v). Unoxidized cholesterol esters and triglycerides elute in this fraction. Subsequently, the column was extracted with 12 ml of methanol/MTBE (5/95, v/v) to remove nonpolar lipids and oxidized cholesterol. A final extraction using 15 ml of MTBE/methanol/ammonium acetate (0.01 M) (5/8/2, v/v/v) eluted oxidized and unoxidized phospholipids. Samples were dried under N₂ and reconstituted in methanol/water (2/1).

Liquid chromatography–mass spectrometry

Oxidized phospholipids were analyzed by ultra performance liquid chromatography (UPLC)/ESI-Ion Trap–MS using a Waters Acquity UPLC (Waters Corp., Milford, MA, USA) with a Phenomenex Luna 3- μm C-8 column (150 $\times$ 4.6 mm) at a flow rate of 1.0 ml/min and a flow splitter which sent approximately 35% of the mobile phase to the mass spectrometer. A linear gradient started with 75% solvent A (methanol : water = 1 : 1, 20 mM ammonium acetate) to 0% in 1–10 min, held at 0% from 10 to 20 min, and returned to 75% at 25 min. Mobile phase B consisted of 20 mM ammonium acetate in methanol. MS was carried out on a Thermo Finnigan LTQ Iontrap mass spectrometer or Thermo Quantum Ultra triple quadrupole mass spectrometer. The electrospray ionization (ESI) source was fitted with a stainless steel capillary (100 μm inner diameter), and the mass spectrometer was operated in the negative ion mode. Nitrogen was used as the sheath gas at 38 psi. The capillary temperature was 350°C . The spray voltage was 4.5 kV, and the tube lens voltage was 100 V. Spectra were displayed by averaging scans across chromatographic peaks. Data acquisition and analysis were performed using Xcaliber software, version 2.0.

Results and Discussion

Analyses of phospholipids by MS methods have been primarily focused on the unoxidized lipids and have been widely used to study the distribution of different species of phospholipids in biological systems. Triple quadrupole, iontrap and time-of-flight (TOF) instruments have been used for this purpose in both negative and positive mode. In recent years, the oxidation products of phospholipids have attracted intensive research interest due to their diverse biological functions in human pathophysiology. These functions include a role in atherosclerosis, hyperlipidemia, and acute lung injury.[3,5,23,24] It should be noted that analysis of the oxidation products of phospholipids is a more challenging task than the analysis of corresponding parent phospholipids due to the complexity of the oxidation products, the sensitivity of the methods, and instability of some of the oxidation products. ESI and matrix-assisted laser desorption ionization (MALDI) have been used to study the oxidation products

of phospholipids and an excellent review on the progress of this field is published recently. [25]

Free radical-induced oxidation of PUFA-containing phospholipids generates a complex mixture of oxidation products (Fig. 2). The reaction proceeds from the hydrogen atom abstraction of the bis-allylic hydrogen atoms and initially forms peroxy radicals, such as **1b**, that can generate hydroperoxide **1c** and hydroxyl products **1d**, known as hydroxyeicosatetraenoates (HETEs). Cyclization of the peroxy radical **1b** gives rise to bicyclic endoperoxides **1f** that can be reduced to form F₂-IsoPs (**1g**) or rearrange to D₂/E₂-IsoPs (**1h**). The latter can dehydrate to form cyclopentenones A₂-IsoP (**1i**). Some of the product hydroperoxides are unstable and will decompose to reactive intermediate such as **1e**. The formation of these complex oxidation products has been shown to have potent biological consequences. The conventional methods to identify and quantify the levels of these oxidation products involve hydrolysis of these compounds from the polar head group. The free acid then can be analyzed in the negative mode using ESI-MS or atmospheric pressure chemical ionization mass spectrometry (APCI-MS) and important structural information has been obtained from the analysis of the oxidation products of free acids.[21,26] These methods have limitations because some of the oxidation products, such as peroxides, D₂/E₂-IsoPs and A₂/J₂-IsoPs, are not stable under the basic hydrolytic conditions. Enzymatic hydrolysis using phospholipase A₂ (PLA₂) often results in poorly reproducible results. It is therefore of great importance to develop a method that can characterize the intact oxidation products of phospholipids.[25,27] Iontrap MS has been widely used to study biological compounds such as proteins and lipids based on MSⁿ, [28] and we report here the use of this method to study the oxidation products of phospholipids *in vitro* and in rat livers after treatment with CCl₄ to induce oxidative stress.

Figure 3 shows the mass spectra of HETE SAPC products obtained from oxidation of SAPC *in vitro* under free radical conditions. The structure of 9-HETE SAPC is shown as an example. The MS² in the negative mode on the acetate adduct of HETE SAPC *m/z* 884 resulted in a peak at *m/z* 810 that is indicative of methyl group loss from HETE SAPC. MS³ on *m/z* 810 shows the *sn*-1 carboxylate of *m/z* 283 (stearoyl) and *sn*-2 *m/z* 319 (HETE). It is noteworthy that assignment of *sn*-1 and *sn*-2 carboxylates is based on the fact that *sn*-1 normally contains saturated fatty acid, whereas *sn*-2 contains unsaturated fatty acid. A peak at *m/z* 508 resulted from the loss of the *sn*-2 acyl chain. Further fragmentation of the *m/z* 319 in MS⁴ resulted in the fragmentation of HETEs. Free radical oxidation of arachidonate generated six regioisomers of HETEs, namely 5-, 8-, 9-, 11-, 12-, and 15-HETEs. The structures and their characteristic collision-induced dissociation (CID) fragmentation of these HETEs incorporated into SAPC are shown in Fig. 3(d). For example, in MS⁴ spectrum, the fragment ion at *m/z* 115 is characteristic of 5-HETE. Other HETE regioisomers can be readily identified in the MS⁴ spectrum as follows: *m/z* 155, 8-HETE; *m/z* 167, 9, or 11 HETE; *m/z* 179, 12-HETE; *m/z* 219, 15-HETE. Overall the MS⁴ fragmentation pattern of the HETE-SAPC mimics that of the HETEs of arachidonic acid (free acid) in the negative ion mode using a triple quadrupole instrument.[29,30] It is noteworthy that ions with *m/z* 301 and 275 resulted from the dehydration and loss of carbon dioxide from the *sn*-2 carboxylate ion, respectively.

This iontrap technique has been further extended to study the oxidation products of other glycerol phospholipids that can generate negative ions in the MS. GPS can readily form negative ion in the ESI without formation of an acetate adduct. Oxidation of PLPS gives rise to two regioisomeric hydroperoxides at 9- and 13-positions and the hydroperoxides can be reduced to form hydroxyoctadecadienoates (HODEs)-PLPS. PLPS and HODE-PLPS can be identified in the oxidation mixture with *m/z* 758 and 774, respectively (Fig. 4(a)). MS² of *m/z* 774 showed a fragment ion with *m/z* 255 that corresponds to the palmitate at *sn*-1 position, whereas *m/z* 295 is indicative of HODEs at the *sn*-2 position. Further fragmentation of *m/z* 295

resulted in two distinct fragments at m/z 171 and 195 that are characteristic fragments of the 9- and 13-HODEs. The structures and fragmentations of standard 9-HODE and 13-HODE of free fatty acid using a triple quadrupole instrument are shown in Fig. 4(b). It is obvious that MS^3 of HODEs-PLPS mimics the CID spectrum of 9- and 13-HODEs free acid, but this iontrap MS method also provided head group information for the glycerophospholipids.

Based on the free radical mechanism illustrated in Fig. 2, other highly oxidized products from PUFA-containing phospholipids, such as D_2/E_2 or F_2 -IsoPs, can be formed. Some of the isomers of D_2/E_2 -IsoPs have shown vasoconstriction by interaction with thromboxane receptors.[31] Identification of these compounds is often based on the free fatty acid even though these compounds are initially formed esterified to phospholipids. A few preliminary studies were carried out to identify the intact isoprostane phospholipids using fast atom bombardment (FAB)-MS after extensive sample purification.[32–34] Using our iontrap MS technique coupled with online LC separation, D_2/E_2 -IsoPs of SAPE are identified in the oxidation mixture of SAPE under free radical conditions (Fig. 5(a)). MS^2 on the parent molecular ion of m/z 814 produced m/z 283 and 351 of stearate and D_2/E_2 -IsoP on respectively. A fragment of m/z 480 represents the loss of *sn*-2 acyl chain. MS^3 of m/z 351 generates a CID spectrum almost identical to that derived from oxidation products of arachidonic acid using a triple quadrupole instrument (Fig. 5(b)). The formation of these oxidation products in rat liver treated with CCl_4 to induce oxidative stress are identified by this technique (Fig. 5(c)). The MS^2 and MS^3 of D_2/E_2 -IsoP SAPE *in vivo* are almost identical to those from the oxidation mixture *in vitro*.

As already mentioned, measurement of F_2 -IsoPs by GC-MS has been evolved as the gold standard to assess oxidative stress *in vivo*. These compounds are also formed initially as phospholipids and then hydrolyzed by platelet-activating factor (PAF) acetyl hydrolases *in vivo*. [35] Based on the mechanisms shown in Fig. 2, four series of regioisomeric F_2 -IsoPs can be formed based on the initial hydrogen atom abstraction. We applied this iontrap MS method to identify the intact F_2 -IsoP esterified to PE head group *in vitro* and *in vivo* (Fig. 6(a), (b)). MS^2 of m/z 816 gave rise to fragments of m/z 283 (*sn*-1, stearate), and m/z 353 (F_2 -IsoP on *sn*-2). Further fragmentation of the m/z 353 in MS^3 produced the product ions of all the four regioisomers of F_2 -IsoPs: m/z 115, 5-series F_2 -IsoPs; m/z 127, 12-series F_2 -IsoPs; m/z 151, 8-series F_2 -IsoPs; m/z 193, 15-series F_2 -IsoPs. Structures of the four regioisomers of F_2 -IsoP SAPE and their characteristic fragmentation are shown in Fig. 6(c). These characteristic fragment ions have been identified from the oxidation of arachidonic acid and extensively studied synthetic standards and isotope labeling.[36,37] MS^3 resembles the CID of F_2 -IsoPs in free acid but the iontrap MS gives additional information on the head group.

We then attempted to identify these intact oxidation products in liver tissues of rats after treatment with carbon tetrachloride to induce oxidative stress (Fig. 6(b)). MS^2 on m/z 816 and MS^3 on m/z 353 showed identical patterns of fragments to those from the *in vitro* oxidation mixture. The four regioisomers of F_2 -IsoPs can be readily identified. Analysis of the oxidation products esterified to other glycerophospholipids head groups, such as GPC, gives similar results (data not shown). Other oxidation products, such as A_2/J_2 -IsoPs, esterified to different head groups can also be identified using this technique.[38]

In summary, identification of intact oxidation products esterified to glycerol phospholipids has been achieved using negative iontrap MS. The structure elucidation is based on well-established fragmentation mechanisms of nonesterified fatty acid oxidation products using a triple quadrupole MS. Even though the positive ion mode can be used for structural characterization of oxidized glycerophospholipids (GPLs), predominant products ions resulted from the loss of the head group. In the case of GPC, acetate adducts of phospholipids were fragmented following MS^4 to give structural information of the *sn*-2 acyl chain as well as the

head group information; however, sensitivity can be an issue in some cases. Negative ion can be formed readily from the molecular ions of GPE and GPS, which were then further fragmented to give structural information. Overall, this technique has been applied to identify an array of oxidation products resulting from free radical-induced oxidation of glycerol phospholipids without the hydrolysis of the head group. It overcomes the lack of stability of some oxidation products and also provides head group information. This technique has been applied to study the oxidation products of phospholipids *in vivo*.

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Glycerophospholipids (GPL)

- $R_3 = -^+N(CH_3)_3$, $R_4 = H$, glycerophosphatidylcholine (GPC)
 $R_3 = -^+NH_3$, $R_4 = H$, glycerophosphatidylethanolamine (GPE)
 $R_3 = -(^+NH_3)$, $R_4 = COOH$, glycerophosphatidylserine (GPS)

Figure 1.

Chemical structure of glycerophospholipids. R_1 , acyl chain on *sn*-1 position; R_2 , acyl chain on *sn*-2 position. R_3 , R_4 , polar head groups.

Figure 2.

Free radical mechanisms that lead to the formation of oxidation products of arachidonic acid containing glycerophospholipids. IsoPs, isoprostanes; GPL, glycerophospholipids.

Figure 3.

Identification of HETEs SAPC by LC-iontrap MS method. (a) MS² of *m/z* 884, acetate (Ac) adduct of HETE–SAPC. 9-HETE SAPC is illustrated as an example. (b) MS³ of *m/z* 884–810 and major fragments. (c) MS⁴ of *m/z* 884 to 810 to 319. Bold numbers indicate position of the hydroxyl group on the arachidonyl chain. (d) Chemical structure of the six HETE SAPC regioisomers and their characteristic fragments that can be used to locate the hydroxyl group on the side chain.

Figure 4. Identification of HODEs PLPS by LC-iontrap MS method. (a) MS² of *m/z* 774, HODEs PLPS; MS³ of *m/z* 774–295 (b) MS² spectra of standard 9-HODE and 13-HODEs and their characteristic fragmentation on a triple quadrupole instrument. (c) Chemical structure of the two major isomers of HODEs PLPS and their characteristic fragments.

Figure 5.

Identification of D₂/E₂-IsoPs SAPE formed *in vitro* and *in vivo* by LC-iontrap MS method. (a) D₂/E₂-IsoP SAPE from *in vitro*. MS² of m/z 814.5, D₂/E₂-IsoP SAPE; MS³ of m/z 814.5–351.2. (b) CID spectrum of D₂/E₂-IsoP from arachidonic acid oxidation using a triple quadrupole instrument. (c) D₂/E₂-IsoP SAPE *in vivo* from rat liver after CCl₄ treatment. MS² of m/z 814.5, D₂/E₂-IsoP SAPE; MS³ of m/z 814.5–351.2.

Figure 6.

Identification of F₂-IsoPs SAPE formed *in vitro* and *in vivo* by LC-iontrap MS method. (a) F₂-IsoP SAPE *in vitro*. MS² of m/z 816.5, F₂-IsoP SAPE; MS³ of m/z 816.5–353.2. (b) F₂-IsoP SAPE *in vivo* from rat liver after CCl₄ treatment. MS² of m/z 816.5, F₂-IsoP SAPE; MS³ of m/z 816.5–353.2. Bold numbers indicate position of the hydroxyl group on the arachidonyl chain. (c) Chemical structures of four region-isomeric F₂-IsoP SAPE and the characteristic fragmentation for each regioisomers. These fragmentations are based on the standards of F₂-IsoPs from arachidonic acid oxidation products.