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Structural Identification of a Novel Pro-inflammatory Epoxyisoprostane Phospholipid in Mildly Oxidized Low Density Lipoprotein*

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One of the earliest steps in the development of the atherosclerotic lesion is the accumulation of monocyte/macrophages within the vessel wall. Oxidized lipids present in minimally modified-low density lipoproteins (MM-LDL) contribute to this process by activating endothelial cells to express monocyte-specific adhesion molecules and chemoattractant factors. A major focus of our group has been the isolation and characterization of the biologically active oxidized lipids in MM-LDL. We have previously characterized three oxidized phospholipids present in MM-LDL, atherosclerotic lesions of fat fed rabbits, and autoxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (Ox-PAPC) that induced human aortic endothelial cells to adhere human monocytes *in vitro*. We have used sequential normal and reverse phase-high performance liquid chromatography to isolate various isomers of an oxidized phospholipid from autoxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine. The fatty acid in the *sn*-2 position of this biologically active isomer and its dehydration product was released by phospholipase A_2 and characterized. Hydrogenation with platinum(IV) oxide/hydrogen suggested a cyclic moiety, and reduction with sodium borohydride suggested two reducible oxygen-containing groups in the molecule. The fragmentation pattern produced by electrospray ionization-collision induced dissociation-tandem mass spectrometry was consistent with a molecule resembling an E-ring prostaglandin with an epoxide at the 5,6 position. The structure of this lipid was confirmed by proton nuclear magnetic resonance spectroscopy analysis of the free fatty acid isolated from the dehydration product of *m/z* 828.5. Based on these studies, we arrived at the structure of the biologically active oxidized phospholipids as 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine. The identification of this molecule adds epoxyisoprostanes to the growing list of biologically active isoprostanes.

Atherosclerosis is a devastating disease responsible for profound human morbidity and mortality (1, 2). The precursor of the atherosclerotic lesion, the fatty streak, begins to develop in the first decade of life and is characterized by the accumulation of monocyte/macrophages within the intimal layer of the blood vessel (3, 4). There is evidence that oxidized lipids, primarily derived from low density lipoproteins (LDL),[1] contribute to all stages of atherosclerotic development (5–8). Initially, they facilitate monocyte deposition within the subendothelial space by stimulating endothelial cells to express monocyte-specific adhesion molecules (9, 10) and secrete monocyte chemoattractants (11, 12). Later, highly oxidized lipids such as malondialdehyde and 4-hydroxynonenal modify the protein component of LDL so that it is recognized by the macrophage scavenger/oxidized LDL receptor rather than the native LDL receptor (13–15). Uptake of oxidized LDL by macrophages generates foam cells that reside in the subendothelial space. It is the lipid-laden foam cells that are the hallmark of the fatty streak lesion.

We have previously demonstrated that mildly oxidized LDL, which we have termed "minimally modified" (MM-LDL), stimulated human aortic endothelial cells to bind human monocytes *in vitro* (9). By separating the components of MM-LDL it was found that the phospholipid fraction contained nearly all of the biological activity (9). When the phospholipids from MM-LDL and native LDL were compared it was found that phospholipids containing arachidonic acid were preferentially oxidized compared to phospholipids containing other polyunsaturated fatty acids (16). This lead us to suspect that the biologically active phospholipids were oxidized derivatives of arachidonic acid-containing phospholipids. We then found that autoxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (Ox-PAPC) had identical biological properties as MM-LDL, and we began using Ox-PAPC as a surrogate for MM-LDL (17).

Recently, our group has described three compounds present in MM-LDL, Ox-PAPC, and rabbit atherosclerotic lesions that stimulated endothelial cells to bind monocytes *in vitro* (18). All were derived from the oxidation of arachidonic acid-containing phospholipids in LDL (16). Interestingly, we found that antibodies to these lipids were spontaneously produced *in vivo* by

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This manuscript is dedicated to the memory of George J. Popják, M.D., D.Sc.

§ These authors contributed equally to this work.

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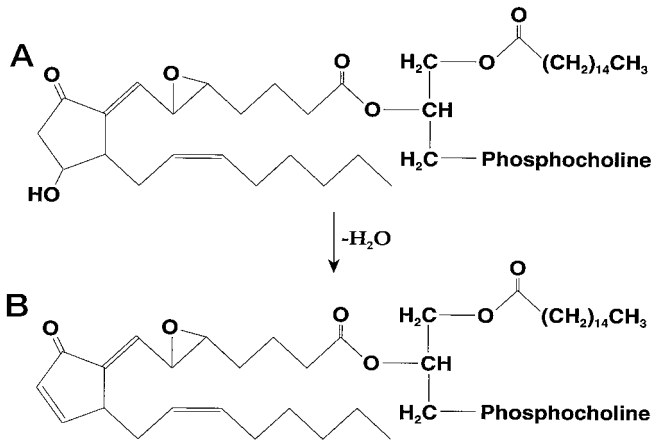
[1] The abbreviations used are: LDL, low density lipoprotein; MM-LDL, minimally modified-LDL; Ox-PAPC, autoxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; POVPC, 1-palmitoyl-2-(5)oxovaleryl-*sn*-glycero-3-phosphocholine; PGPC, 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine; ESI-MS, electrospray ionization-mass spectrometry; LC/MS, liquid chromatography/MS; HPLC, high performance liquid chromatography; RP, reverse phase; NP, normal phase; BHT, butylated hydroxytoluene; FIA, flow injection analysis; PEIPC, 1-palmitoyl-2-(5,6)-epoxyisoprostane E_2-*sn*-glycero-3-phosphocholine.



SCHEME 1.

apolipoprotein E knockout mice that were genetically predisposed to develop atherosclerosis (18). Two of the biologically active compounds were produced by oxidative fragmentation of the arachidonic acid moiety in the *sn*-2 position of PAPC and were identified as 1-palmitoyl-2-(5)oxovaleryl-*sn*-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC). The molecular structure of the third molecule, which gave a signal at *m/z* 828.5 by electrospray ionization-mass spectrometry (ESI-MS), was not determined at that time. In this study we provide evidence that this molecule contained an epoxyisoprostane in the *sn*-2 position of the phospholipid (Scheme 1*A*) and this molecule undergoes dehydration to form a structurally similar molecule with a mass of 810.5 [M + H^+] (Scheme 1*B*).

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media, serum, and supplements were obtained from Irvine Scientific and Hyclone Laboratories, Inc. Acetonitrile, chloroform, methanol, ethyl acetate, and water (all Optima grade) were obtained from Fisher Scientific, Pittsburgh, PA. Gelatin (endotoxin-free, tissue culture grade), porcine liver esterase, calcium chloride, methoxylamine hydrochloride, ammonium acetate, sodium borohydride, platinum(IV) oxide, phospholipase A_2 (*naja naja*), and butylated hydroxytoluene (BHT) were obtained from Sigma. Authentic *L*-α-1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL) or Sigma. Deuterated chloroform (99.99% D), deuterated methanol (99+% D), and deuterium oxide (99.9% D) were obtained from Aldrich Chemical Co. Sodium borodeuteride was obtained from Cambridge Isotope Laboratories, Inc.

Endothelial Cell Cultures—Human aortic endothelial cells at passages 4–7 were cultured as described in medium 199 supplemented with 10% fetal bovine serum (9, 12).

Monocyte Adhesion Assay—These studies were performed essentially as described previously (9). Blood monocytes were isolated from a large pool of healthy human blood donors by a modification of the Recalde procedure (19). For monocyte adhesion assays, human aortic endothelial cells were incubated with test medium for 4 h at 37 °C. The test medium was removed, the endothelial cells were washed, and a suspension of human monocytes was added for 12–15 min after which nonadherent monocytes were removed. Bound monocytes were counted and expressed as monocytes/microscopic field.

Lipid Oxidation—PAPC was oxidized by transferring 1 mg in 100 μl of chloroform to a clean 16 × 125-mm glass test tube and evaporating the solvent under a stream of nitrogen. The lipid residue was allowed to autoxidize while exposed to air for 24–72 h at room temperature. The extent of oxidation was monitored by flow injection ESI-MS.

High Performance Liquid Chromatography—Normal phase high performance liquid chromatography (NP-HPLC) was performed by injecting oxidized phospholipid preparations (resuspended in chloroform) onto a silica column (Adsorbosphere, 250 × 22-mm, 5 μm; Alltech Associates, Inc.) and eluting isocratically with a mobile solvent of acetonitrile/methanol/water (77:8:15, v/v/v, pH 5.0 with formic acid) at a flow rate of 18.0 ml/min. Typically, Ox-PAPC produced from 25–35 mg of PAPC was applied for each run. Reverse phase HPLC (RP-HPLC) of oxidized phospholipids was performed with a C_8 column (Betasil, C_8, 250 × 10-mm, 5 μm, Keystone Scientific, Inc.). Phospholipids were eluted with a mobile phase of 80% methanol that was changed linearly over a period of 60 min to 100% methanol at 5 ml/min. Fractions containing oxidized phospholipids of interest were collected by monitoring ultraviolet absorbance and ESI-MS (LC/MS). Oxidized free fatty acids were separated by RP-HPLC using a C_{18} column (Betasil, C_{18}, 250 × 10-mm, 5 μm, Keystone Scientific, Inc.). A mobile phase of 60% methanol containing 1 mM ammonium acetate changed linearly over 60 min to 100% methanol containing 1 mM ammonium acetate was used. When isolating lipids for NMR analysis, solvents without ammonium acetate were used. UV absorbance was detected with a diode array detector (L-3000, Hitachi, Ltd., Tokyo, Japan) scanning from 200 to 350 nm at 2.5 nm resolution.

Phospholipase A_2 Hydrolysis—Phospholipid fractions collected by HPLC were dried under argon to a lipid residue and resuspended in 1 ml of phosphate-buffered saline containing 5 mM $CaCl_2$. To this solution was added 5 units of phospholipase A_2. The solution was mixed and incubated at 37 °C for 45 min. After incubation, the lipids were extracted with 1 ml of ethyl acetate containing 0.01% BHT after acidification with formic acid to pH 3.0.

Methoxylamine Derivatization—Phospholipid fraction containing 828.5 (i2) was isolated by sequential NP-HPLC and RP-HPLC from 5 mg of Ox-PAPC. The fraction was dried under argon, and 1 ml of 0.92 mM methoxylamine hydrochloride in 1× phosphate-buffered saline was added. The solution was mixed thoroughly and incubated for 45 min at 37 °C. After incubation the lipids were extracted with $CHCl_3$/MeOH + BHT and analyzed by positive ion ESI-MS.

Lipid Reduction—Chemical reduction of lipids was achieved by addition of 600 μl of a 70 mM solution of sodium borohydride or sodium borodeuteride in acetonitrile at room temperature for 30 min. Following incubation, 1 ml of ethyl acetate containing 0.01% BHT and 1 ml of water was added. The solution was mixed thoroughly and centrifuged at 2,000 × *g* for 5 min. The ethyl acetate phase was transferred to a clean glass tube, and 30 μl of formic acid was added to displace sodium from phospholipid sodium salts.

Lipid Hydrogenation—Lipids were hydrogenated by exposure to hydrogen gas in the presence of platinum(IV) oxide (20). Oxidized lipids were resuspended in 300 μl of ethyl acetate and transferred to a 25-ml round bottom flask. Platinum(IV) oxide (1 mg) was added and the flask was covered with a rubber septum. Two 18-gauge hypodermic needles were placed through the septum, and the flask was flushed by introduction of hydrogen through one of the needles. After flushing, one of the needles was removed and a balloon containing hydrogen gas was attached to the other. The samples were incubated with constant stirring at room temperature for 45 min. The reaction mixture was transferred to a 13 × 100-mm glass test tube and dried under argon gas. The lipid residue was resuspended in 1 ml of chloroform/methanol (2:1, v/v), 400 μl of water, and 20 μl of concentrated formic acid and then the lipids were recovered from the chloroform phase after mixing and centrifugation.

Carboxylic Acid ^{18}O Labeling—^{18}Oxygen exchange experiments were performed by incubation of the free fatty acids with porcine liver esterase in $H_2{}^{18}O$ (21). To the oxidized fatty acid residue was added 100 μl of $H_2{}^{18}O$ and 23 units of porcine liver esterase. The contents were mixed thoroughly and incubated for 60 min at 37 °C with occasional mixing. Lipids were extracted by addition of 300 μl of chloroform/methanol (2:1, v/v) to the reaction mixture.

Mass Spectrometry—ESI-MS was performed using an API *III* triple-quadrupole biomolecular mass analyzer (Perkin-Elmer Sciex Instruments, Norwalk, CT) fitted with an articulated, pneumatically assisted nebulization probe and an atmospheric pressure ionization source. Details of calibration and tuning have been described previously (18). Phospholipids were introduced into the mass spectrometer by direct flow injection analysis (FIA) in acetonitrile/water/formic acid (50:50:0.1, v/v/v) or via liquid chromatography (LC/MS) and analyzed as the protonated molecule [M + H^+] in positive ion mode. The mass spectrometer was set to scan from *m/z* 450 to 950 with an orifice voltage of +65, a step size of 0.3, a dwell time of 3 msec, and a scan speed of ~4 s. Fatty acids were analyzed as carboxylate anions [M^-] by FIA in methanol/water (50:50, v/v) with 1 mM ammonium acetate or by LC/MS in chromatography solvent. For negative ion ESI-MS/MS, a solvent of 100% methanol with 1 mM ammonium acetate was used, and daughter ion spectra were obtained by colliding the Q1 selected ion of interest with argon in Q2, and scanning Q3 to analyze the fragment ion products. Reconstructed selected ion chromatograms were produced by software supplied by PE Sciex.

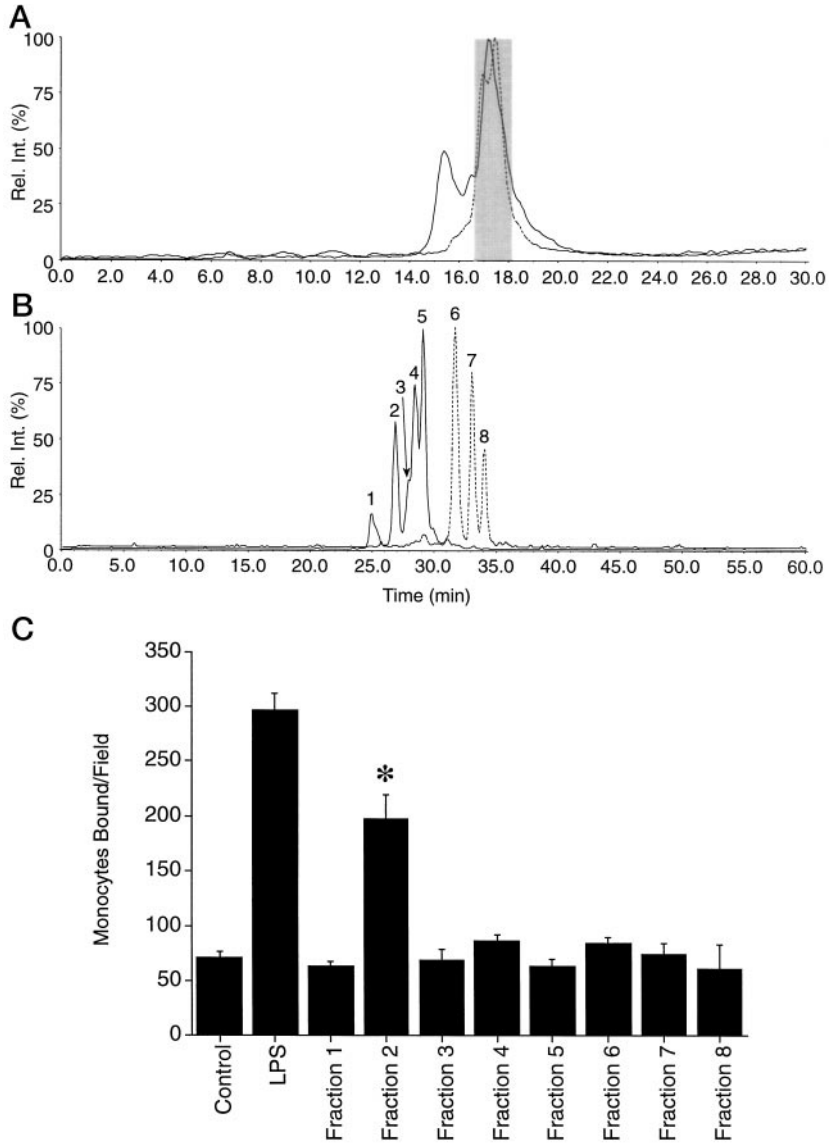


FIG. 1. **Electrospray ionization-liquid chromatography/mass spectrometry (ESI-LC/MS) of oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (Ox-PAPC).** Ox-PAPC produced by autoxidation of 25 mg of PAPC was applied to a preparative NP HPLC column and eluted isocratically with acetonitrile/methanol/water (77:8:15, v/v/v) at 18 ml/min (*A*). A fraction (~1/50th) of the eluant was diverted to an electrospray mass spectrometer, and the balance was recovered in a fraction collector. The fraction shaded in *panel A* was collected, evaporated to dryness, resuspended in methanol, and analyzed by reverse phase ESI-LC/MS (*B*). Lipids were eluted with a linear gradient of 80% methanol to 100% methanol over 60 min. Reconstructed selected ion chromatograms of *m/z* 828.5 (*solid line*) and *m/z* 810.5 (*dashed line*) are shown during NP-LC/MS (*A*) and RP-LC/MS (*B*). Numbered peaks in *panel B* were collected and analyzed for the ability to induce endothelial cells to bind monocytes (*C*). LPS (1 ng/ml) was used as a positive control.

High Resolution-Fast Atom Bombardment/Mass Spectrometry—High resolution-fast atom bombardment/MS spectra were obtained using a VG ZAB-SE fast atom bombardment mass spectrometer (Micromass, Manchester, UK) equipped with a 11/250 data system. HPLC fractions containing oxidized phospholipids of interest were dried under argon and resuspended in an aqueous solution of 0.1% trifluoroacetic acid. To the static fast atom bombardment probe containing 1–2 μl of liquid matrix (*m*-nitrobenzylalcohol/thioglycerol/trifluoroacetic acid, 50:50:0.5, v/v/v) was added 1–2 μl of the oxidized phospholipid solution. Spectra were recorded using a 8 kV accelerating potential, cesium bombardment at 22 kV and 1–2 μA, and a mass resolution of 3,000 (10% valley, M/ΔM). The mass spectrometer was set to scan from *m/z* 200–1,000, and ~10 scans were collected into a multichannel analyzer. The data were smoothed, centroided, and mass measured using cesium iodide ion clusters for calibration.

Nuclear Magnetic Resonance Spectroscopy—All proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker ARX-500 MHz spectrometer using a microprobe (2.5 mm) NMR tube. Proton chemical shifts were reported in parts per million (ppm) on the δ scale with reference to $CHCl_3$ (δ 7.24). ^{1}H NMR spectral data were tabulated in terms of multiplicity of proton absorption (s, singlet; d, doublet; dd, doublet of a doublet; dt, doublet of a triplet; t, triplet; q, quartet; m, multiplet; br, broad), coupling constants (Hz), and number of protons. Purified lipids were dried under argon, resuspended in $CDCl_3$ (90 μl) and transferred to a microprobe NMR tube for analysis. Proton-proton homodecoupling experiments were performed using a power level of 55 dB.

UV-visible Spectrophotometry—Absorbance spectra in the 190–500 nm range for various oxidized phospholipids were measured using a Shimadzu Biospec-1601 UV-visible spectrophotometer (Shimadzu Scientific Instruments, Inc, Columbia, MD). For extinction coefficient determination, the isomers of 828.5 and 810.5 were isolated by sequential normal phase and reverse phase HPLC and quantified by FIA-ESI-MS using dimyristoyl phosphatidylcholine as an internal standard (22). The UV-visible absorbance was scanned for *m/z* 828.5 and 810.5 isomers in methanol (1 ml) at three different concentrations. Using these absorbance values, molar extinction coefficients were calculated (23) for isomers of *m/z* 828.5 and 810.5.

RESULTS

Effect of Isomers of m/z 828.5 and 810.5 on Monocyte-Endothelial Interactions—We have previously separated the phospholipid components of Ox-PAPC by NP-HPLC and found that the second peak enriched in *m/z* 828.5 induced endothelial cells to bind monocytes *in vitro* (18). We repeated these experiments and collected the active fraction between 16.5 and 18.0 min (Fig. 1*A*) that contained mostly *m/z* 828.5 and 810.5 (mass spectrum not shown). The lipids in this fraction were then applied to a reverse phase column, which effectively separated several isomers of *m/z* 828.5 and 810.5 (Fig. 1*B*). Each major peak was collected, dried under argon, resuspended in tissue culture medium, and tested for the ability to induce endothelial cells to bind monocytes. The only peak that showed significant

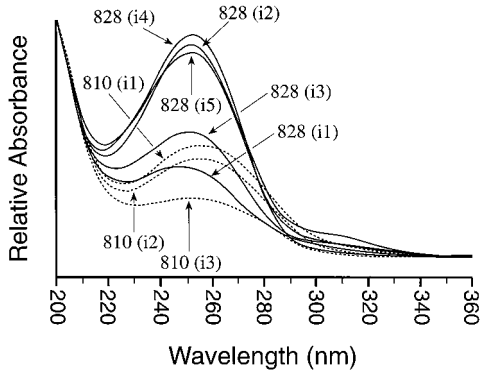


FIG. 2. **Ultraviolet spectroscopy of *m/z* 828.5 and 810.5.** Ultraviolet spectra were obtained by scanning between 200 and 350 nm during RP-LC/MS analysis of the NP-LC/MS fraction containing *m/z* 828.5 and 810.5. These measurements were made in approximately 90% methanol and 10% water.

biological activity above control was the second of the peaks containing *m/z* 828.5 (Fig. 1*C*). This isomer caused a dose-dependent increase in monocyte binding reaching a statistically significant increase over control as low as 380 ng/ml. The fatty acid hydrolyzed from the *sn*-2 position of the biologically active isomer did not induce monocyte-endothelial interactions (data not shown). As a convention throughout this article, the five major isomers of *m/z* 828.5 resolved by RP-LC/MS will henceforth be abbreviated 828.5 (i1–5) and the three major isomers of *m/z* 810.5 will be abbreviated 810.5 (i1–3).

UV-visible Spectrophotometry—All isomers with *m/z* 828.5 possessed nearly identical UV maxima at 252 nm, and all isomers of the *m/z* 810.5 possessed identical UV maxima at 257 nm (Fig. 2). These UV maxima were consistent with a specific conjugated system within all of these molecules. Extinction coefficients (ϵ) at room temperature in methanol for the various isomers were calculated to be: 828.5 (i1) = 24,070; 828.5 (i2) = 18,632; 828.5 (i3) = 17,975; 828.5 (i4) = 22,275; 828.5 (i5) = 15,917; 810.5 (i1) = 20,503; 810.5 (i2) = 19,572; and 810.5 (i3) = 14,950.

Fast Atom Bombardment-High Resolution-Mass Spectrometry—To confirm the molecular formula of *m/z* 828.5 we analyzed the molecule by high resolution-fast atom bombardment/MS. The experimental mass of the ion was determined to be 828.5391, which closely matched the mass of a molecule with the elemental composition of $C_{44}H_{79}NO_{11}P$ (calculated mass = 828.5381). Because unoxidized PAPC has an elemental composition of $C_{44}H_{81}NO_8P$, we concluded that during oxidation this molecule acquired three oxygen atoms and lost two hydrogen atoms. Based on the mass of the products observed after phospholipase A_2 hydrolysis of this molecule, we further concluded that the only oxidized part of the PAPC molecule was the arachidonic acid in the *sn*-2 position of PAPC. Thus, the molecular formula of this oxidized fatty acid in the *sn*-2 position was $C_{20}H_{30}O_5$, compared with the elemental formula of arachidonic acid, $C_{20}H_{32}O_2$.

Dehydration of m/z 828.5 to 810.5—Periodically, positive ion ESI-MS was performed on stored preparations of Ox-PAPC and it was noticed that the relative ratio of 828.5 to 810.5 decreased over time, suggesting that 810.5 may be a decomposition product of 828.5. To test this, we isolated isomers of 828.5 by RP-LC/MS and allowed each of them to undergo spontaneous dehydration. After 48 h at 4 °C in chloroform, we reanalyzed the sample by RP-LC/MS using the same chromatographic conditions that were used for original isolation. Two isomers of 828.5 were collected, the biologically active isomer, 828.5 (i2), and a biologically inactive isomer, 828.5 (i5), (Fig. 3*A*). After 48 h at 4 °C, 828.5 (i2) had partially decomposed to a molecule

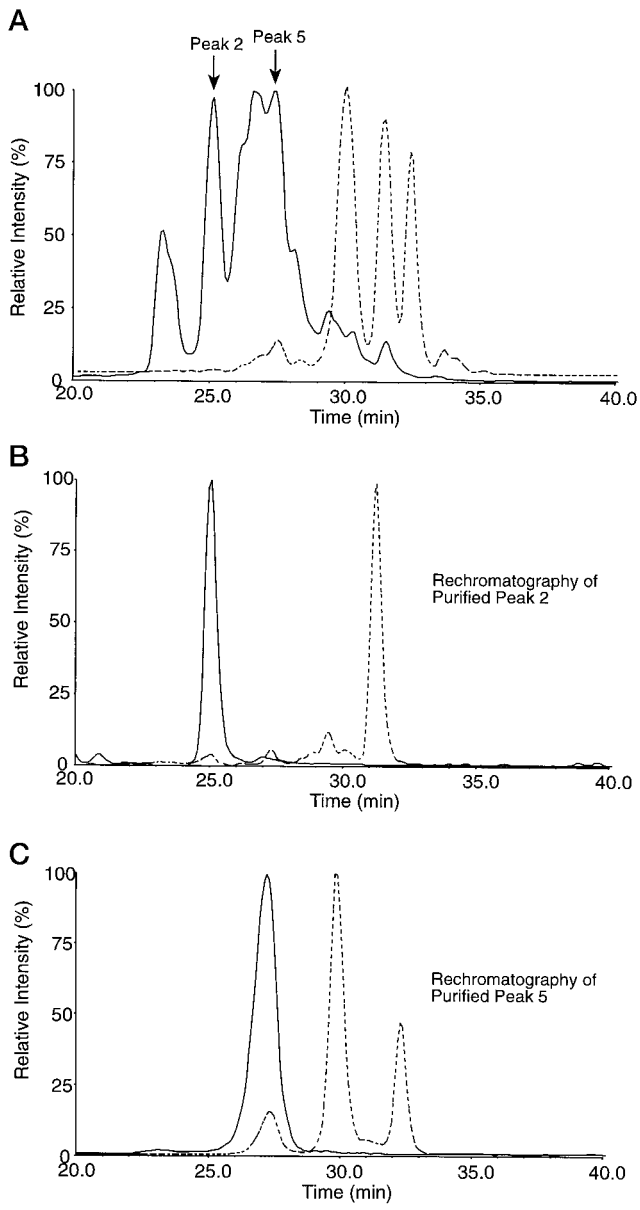


FIG. 3. **Specific dehydration of *m/z* 828.5 to 810.5.** Isomers of *m/z* 828.5 (*solid lines*) and 810.5 (*dotted lines*) were separated by RP-LC/MS (*A*). The peak containing 828.5 (i2) was collected, allowed to undergo spontaneous dehydration, and analyzed by identical RP-LC/MS conditions as described above (*B*). In a similar experiment, the peak containing 828.5 (i5) was collected, allowed to undergo spontaneous dehydration, and analyzed by RP-LC/MS (*C*).

that co-migrated with 810.5 (i2) eluting at 31.5 min (Fig. 3*B*). In contrast, the tube containing 828.5 (i5) contained a mixture of two isomers of 810.5 that co-eluted at 30.0 min and 32.5 min with 810.5 (i1) and 810.5 (i3), respectively (Fig. 3*C*). This experiment showed that 828.5 (i2) underwent dehydration to form 810.5 (i2) and that 828.5 (i5) underwent dehydration to form 810.5 (i1) and 810.5 (i3). In addition, this suggested that the molecular structure of 810.5 (i2) was similar to 828.5 (i2). Derivatization with bis(trimethylsilyl)trifluoroacetamide demonstrated the presence of one hydroxyl group in *m/z* 828.5, which was lost in *m/z* 810.5 (data not shown).

Methoxylamine Hydrochloride Derivatization—As a measure of the number of carbonyl or epoxide groups we examined the derivatization of the molecule with methoxylamine hydrochloride, which adds 47 mass units to carbonyl group and reactive epoxide groups. Treatment of 828.5 (i2) with methoxylamine

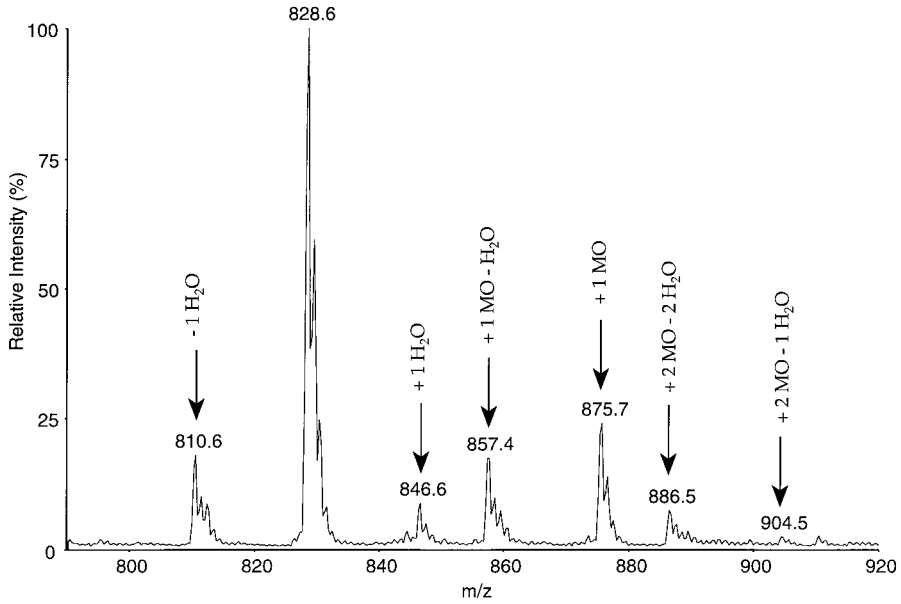


FIG. 4. **Methoxylamine derivatization of 828.5 (i2).** By sequential NP-HPLC and RP-HPLC, 828.5 (i2) was isolated from 5 mg of Ox-PAPC. 1 ml of 0.92 mM methoxylamine hydrochloride in 1× phosphate-buffered saline was added to the dry lipid residue. The solution was mixed thoroughly and incubated for 45 min at 37 °C. After incubation the derivatized lipids were extracted with $CHCl_3$/MeOH + BHT and analyzed by positive ion ESI-MS.

hydrochloride yielded several compounds (Fig. 4). An ion at *m/z* 857.4 was produced by the addition of a methoxylamine group and subsequent loss of water ($[M + H^+] + 47 - 18$). An ion at *m/z* 875.7 was produced by the addition of a methoxylamine without the loss of water ($[M + H^+] + 47$). An ion at *m/z* 886.5 was produced by the addition of two methoxylamine groups with subsequent loss of two waters $\{[M + H^+] + 2(47) - 2(18)\}$. An ion at *m/z* 904.5 was produced by the addition of two methoxylamines with the loss of one water. When the reaction with methoxylamine hydrochloride was allowed to proceed, the most abundant ion was *m/z* 904.5 (data not shown). These data indicate the presence of two groups on *m/z* 828.5, which react with methoxylamine hydrochloride.

Sodium Borohydride and Sodium Borodeuteride Reduction—Reduction of the molecules (*m/z* 810.5 and 828.5) by sodium borohydride and sodium borodeuteride was used to confirm the number of reducible oxygen groups. Sodium borohydride can effectively reduce hydroperoxides, ketones, aldehydes, and some epoxides to hydroxyl groups, thereby altering the molecular weight of the molecule in a predictable manner. Individual isomers of 828.5 and 810.5 were isolated by RP-LC/MS and then treated with sodium borohydride, re-extracted, and analyzed by positive ion FIA-ESI-MS. Each reactive group adds two hydrogens. After reduction with sodium borohydride, the molecular weight of each isomer of 828.5 was increased by 4 Da to *m/z* 832.5. This suggested that all isomers of 828.5 possessed two reducible oxygen-containing functional groups such as aldehydes, ketones, and/or reactive epoxides. Fig. 5 shows the positive ion ESI-MS of the purified biologically active isomer, 828.5 (i2), before (Fig. 5*A*) and after (Fig. 5*B*) sodium borohydride reduction. Some 1-palmitoyl-lysophosphatidylcholine (*m/z* 496.2) and its corresponding sodium salt (*m/z* 518.1) were produced by partial saponification of the phospholipid during the reduction procedure. The reduction was likely incomplete because of the presence of a signal at *m/z* 830.4.

Treatment with sodium borodeuteride, in addition to reducing an aldehyde, ketone, or reactive epoxide, will simultaneously add two deuterium atoms to the group, one associated with oxygen. Because ESI-MS was performed in a solvent containing H_2O, the deuterium bound to the oxygen will undergo rapid exchange with protons in the solvent. Indeed, when 828.5 (i2) was treated with sodium borodeuteride the major product was an ion at *m/z* 834.5 rather than *m/z* 832.5 as seen when the molecule was treated with sodium borohydride (data not shown). These deuterium additions are consistent with the presence of two reducible oxygen functionalities. When 810.5 (i2) was treated with sodium borohydride, the mass was increased by six mass units to 816.6 (data not shown), and with sodium borodeuteride, the mass was increased by nine mass units to 819.6 (Fig. 6*A*). Because this molecule only possessed two reducible oxygen groups, a reactive double bond of the enone was also reduced. It is known that treatment of reactive enones with sodium borohydride results in the reduction of both carbonyl groups as well as double bonds (24). Furthermore, we hypothesize that the reduced double bond of 819.6 contained one deuterium and one hydrogen. We then analyzed *m/z* 819.6 by positive ion FIA-ESI-MS/MS (Fig. 6*B*). The spectrum showed the expected sequential losses of water from the parent (*m/z* 819.6 to 801.5 to 783.4) in addition to two daughter fragments at *m/z* 594.2 and 609.3, which correspond to a 5-carbon aldehyde and a 6-carbon deuterium-labeled aldehyde at the *sn*-2 position, respectively (Fig. 6*B*, *inset*). The production of these two ions can best be explained by the reduction of a molecule with an epoxide at the 5,6 position and the alternate association of the hydroxyl group with the 5 or 6 position.

Hydrogenation—Hydrogenation with platinum(IV) oxide and hydrogen gas was used to determine whether the oxidized fatty acid in the *sn*-2 position was linear or cyclic. Based on high resolution-fast atom bombardment/MS data, the molecular formula of the fatty acid in the *sn*-2 position of 810.5 (i2) was predicted to be $C_{20}H_{28}O_4$ containing four oxygen atoms and six double bond equivalents. In addition, sodium borohydride data had shown that the oxygen-containing groups were reducible. Therefore, if the oxidized fatty acid was linear we would expect hydrogenation of the four double bonds (addition of eight protons) plus reduction of the two oxygen-containing groups (addition of four protons), which would be reflected by an increase of 12 mass units from *m/z* 331.2 to 343.2. If the oxidized fatty acid contained a cycle, we would expect that this cycle would not be hydrogenated, and the fatty acid would maintain one double bond equivalent and, therefore, give a signal at *m/z* 341.2 rather than *m/z* 343.2 expected with a linear molecule.

We isolated the fatty acid from 810.5 (i2) by hydrolysis with phospholipase A_2 and subsequent RP-ESI/MS. The oxidized free fatty acid was then incubated in the presence of hydrogen gas either with or without platinum(IV) oxide and then analyzed by negative ion FIA-ESI-MS. The oxidized fatty acid that

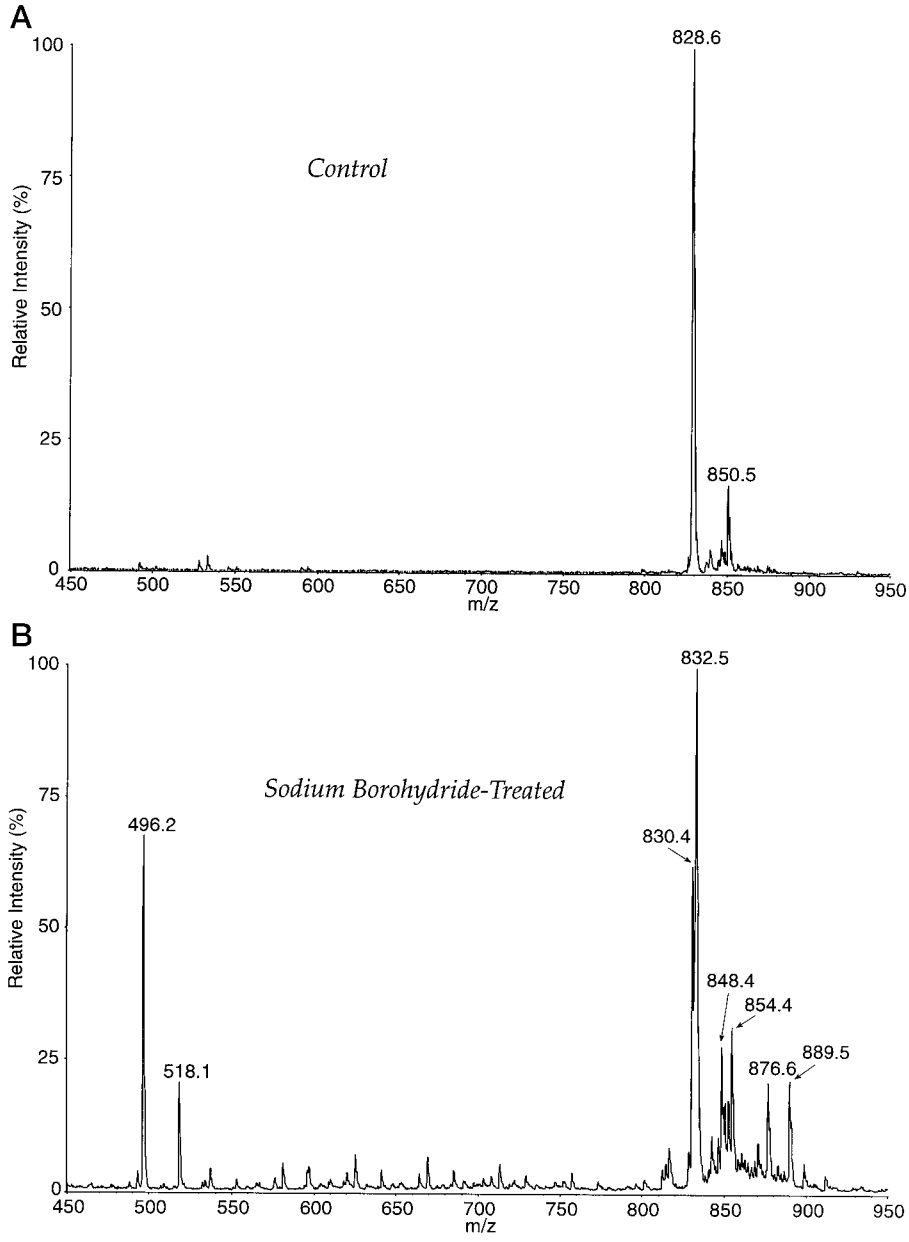


FIG. 5. **Electrospray ionization-mass spectrometry of *m/z* 828.5 (i2) reduced with sodium borohydride.** Purified 828.5 (i2) obtained by sequential NP-LC/MS and RP-LC/MS was distributed evenly between two tubes and treated with no additions (*A*) or sodium borohydride (*B*) in acetonitrile for 30 min at room temperature. Lipids were extracted with ethyl acetate/water with formic acid and analyzed by positive ion ESI-MS.

was incubated in the absence of platinum(IV) oxide contained the unaltered fatty acid with *m/z* 331 (Fig. 7*A*) and the oxidized fatty acid incubated in the presence of platinum contained major ions at *m/z* 339 and 341 (Fig. 7*B*). Obtaining a mixture of major ion at *m/z* 341 implied hydrogenation of five reducible functional groups, *e.g.* double bonds or reducible oxygens. Presence of an ion at *m/z* 339 implied incomplete hydrogenation of the molecule and no peak was found at *m/z* 343, which is expected for a linear molecule. The retention of a double-bond equivalent suggested that the molecule possessed a ring structure and was not a linear molecule.

ESI-MS/MS of m/z 828.5 (i2) and m/z 810.5 (i2) Fatty Acids—Purified isomers of *m/z* 828.5 (i2) and 810.5 (i2) were treated with phospholipase A_2, and the released fatty acids purified by RP-LC/MS. The free fatty acid had identical UV absorbance characteristics compared with the esterified form (data not shown). The isolated oxidized fatty acids were then analyzed by negative ion FIA-ESI-MS/MS (Fig. 8*A*). The fatty acid isolated from 810.5 (i2) was incubated with porcine liver esterase in the presence of $H_2{}^{18}O$, which resulted in replacement of the ^{16}O on the carboxyl end of the fatty acid with ^{18}O. The exchange did not go to completion and the fraction of molecules that contained zero, one, and two ^{18}O atoms was approximately 6, 41, and 53%, respectively (data not shown). The doubly labeled fatty acid (*m/z* 353) was analyzed by negative ion ESI-MS/MS and the fragmentation pattern was compared with the unlabeled molecule (Fig. 8, *A* and *B*). Because of the exchange of two ^{16}O atoms with two ^{18}O atoms, the labeled molecule was 4 Da larger than the unlabeled molecule. Daughter ions that possessed the carboxylic acid portion of the molecule maintained this 4 Da difference (Fig. 8*B*, *arrows*). Conversely, many of the daughter ions of both compounds had identical *m/z* and were presumably formed by loss of the carboxyl end of the molecule. The fragmentation profile obtained was consistent with the structure of an oxidized fatty acid with an epoxide at the 5,6 position (*m/z* 115 for ^{16}O-labeled molecule and *m/z* 119 for ^{18}O labeled molecule), a covalent bond between the 8 and 12 carbon (*m/z* 191 and 220 for ^{16}O-labeled molecule and *m/z* 195 and 224 for ^{18}O-labeled molecule), a ketone at the 9 position, and a double bond at the 10, 11 position (*m/z* 97 for both ^{16}O- and ^{18}O-labeled molecules). This structure was confirmed by ^1H NMR spectroscopy.

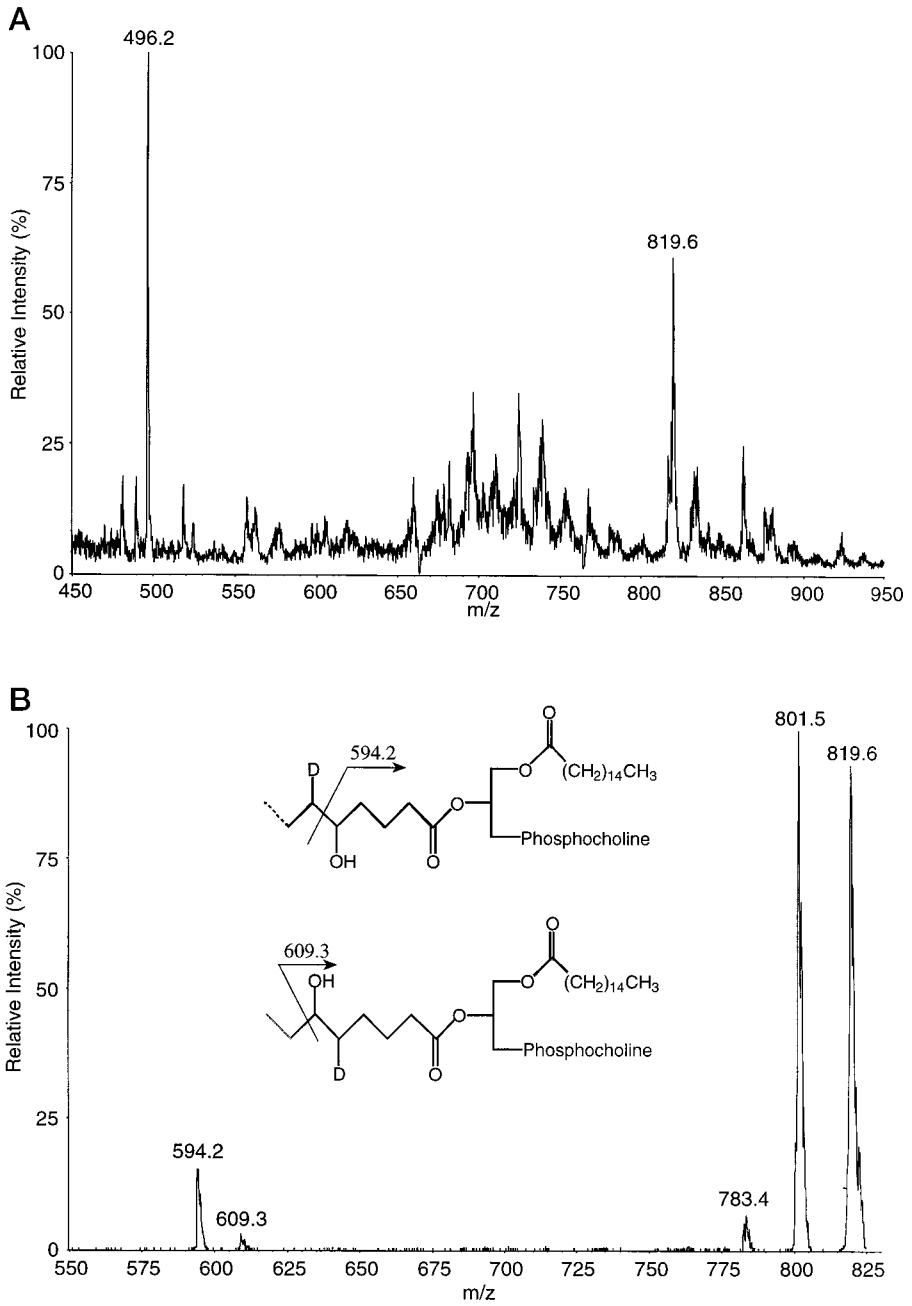


FIG. 6. **ESI-MS and MS/MS of sodium borodeuteride-treated 810.5 (i2).** Purified 810.5 (i2) was treated with sodium borodeuteride as described in Fig. 4 and analyzed by positive ion flow injection ESI-MS (*A*). The *m/z* 819.6 ion was then analyzed by positive ion ESI-MS/MS (*B*).

Proton NMR Spectra of m/z 828.5 (i2), 810.5 (i2), and Free Fatty Acid from 810.5 (i2)—The ^{1}H NMR spectra of *m/z* 828.5 (i2) and 810.5 (i2) provided some structural information on these molecules. One of the major differences between the ^{1}H NMR spectra of *m/z* 828.5 (i2) and 810.5 (i2) was the presence of an α,β-unsaturated carbonyl group in 810.5 (i2) as shown by proton resonances at δ 7.53 and 6.34 (data not shown). The ^{1}H NMR spectrum of the free fatty acid obtained from *m/z* 810.5 (i2) (332 (i2)) suggested the presence of an α,β-unsaturated carbonyl group (δ 7.53 and 6.34), a trisubstituted alkene (δ 6.16), a *cis*-alkene (δ 5.49 and 5.32), and an epoxide (δ 3.39 and 2.99) (Fig. 9). Chemical shift values from ^{1}H NMR spectra of known molecules, prostaglandin A_2, prostaglandin E_2, prostaglandin J_2, Δ^{12}-prostaglandin J_2, and (±)-5,6-EET, which are structurally similar to 332 (i2) were useful in assigning proton resonances. The structure of these molecules are provided for reference (Fig. 10).

The connectivity between various functional groups were assigned based on ^{1}H-^{1}H homodecoupling experiments for 332 (i2). Irradiation of proton resonances at δ 7.53 (dd, C_{11}-H) resulted in the conversion of the dd (J = 1.7, 6 Hz, C_{10}-H) at 6.34 to a d (J = 1.7 Hz) suggested that these two protons (C_{11}-H and C_{10}-H) were coupled to each other. This irradiation also showed some changes on the multiplet at δ 3.65 (C_{12}-H). Irradiation of proton resonances at δ 6.34 (dd, C_{10}-H) resulted in the conversion of the dd (J = 2, 6 Hz) at δ 7.53 (C_{11}-H) to a d (J = 2 Hz) suggesting these two protons were coupled to each other (Fig. 11*A*). Irradiation of proton resonances at δ 6.16 (d, J = 8.4 Hz, C_7-H) resulted in the conversion of dd (J = 1.9, 8.4 Hz) at δ 3.39 (C_6-H) to a d (J = 1.9 Hz), which suggested these two protons are coupled to each other. Irradiation of proton resonances at δ 5.49 (m, C_{15}-H) resulted in changes in δ 5.32 (m, C_{14}-H) and *vice versa*. This suggested that these two proton resonances correspond to *cis*-alkene hydrogens. In addition to the above changes, irradiation of proton resonances at δ 5.49 (m, C_{15}-H) resulted in the conversion of an apparent q (J = 7.4 Hz) at δ 1.94 (C_{16}-H) to a triplet. This suggested the presence of allylic hydrogens (δ 1.94, C_{16}-H) adjacent to a *cis*-alkene hy-

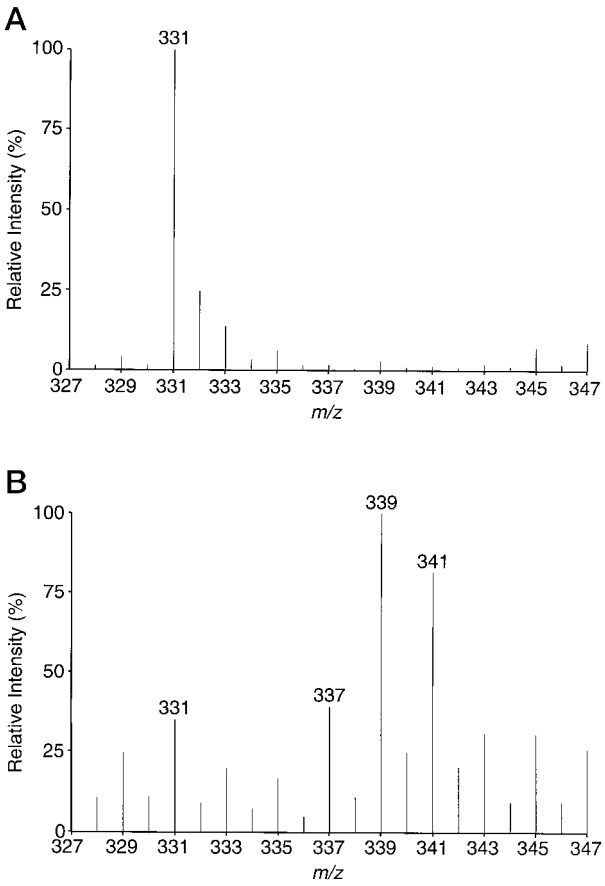


FIG. 7. **Hydrogenation of the fatty acid obtained from the dehydration product of the biologically active *m/z* 828.5 isomer.** The dehydration product of the biologically active isomer of *m/z* 828.5 was isolated by sequential NP and reverse phase chromatography and treated with phospholipase A_2. The free fatty acid was isolated by reverse phase HPLC and treated with platinum(IV) oxide under hydrogen. The *m/z* of the carboxylate anions (M^-) of the untreated (*A*) and PtO_2/H_2-treated (*B*) fatty acid were then measured by negative ion ESI-MS.

drogen (C_{15}-H). Irradiation of proton resonances at δ 5.32 (m, C_{14}-H) resulted in changes in the proton resonances at δ 2.80 and 2.65 (two C_{13}-H). Irradiation of proton resonances at δ 3.65 (m, C_{12}-H) resulted in the conversion of the dd (J = 2.0, 6.0 Hz) at δ 7.53 (C_{10}-H) to a d (J = 6 Hz) and the dd (J = 1.7, 6.0 Hz) at δ 6.34 (C_{11}-H) to a d (J = 6 Hz) suggesting these three protons were coupled to each other. This irradiation also resulted in changes in the proton resonances at δ 2.80 and 2.65 (C_{13}-Hs). The protons at δ 2.80 and 2.65 were assigned as the diastereotopic allylic hydrogens adjacent to both C_{14}-H and C_{12}-H.

Irradiation of proton resonance at δ 3.39 (dd, J = 1.9, 8.4 Hz) resulted in the conversion of the d (J = 8.4 Hz) at δ 6.16 to a singlet (Fig. 11*B*) and some minor changes in the multiplet at δ 2.99 (C_5-H). This result suggested that the dd at δ 3.39 was the hydrogen on C_6 in the 5,6-epoxide group. The coupling constant value for the 5,6-epoxide hydrogen coupling (J = 2.1 Hz) suggested the *trans*-epoxide. Irradiation of the proton resonances at δ 2.80 and 2.65 (C_{13}-Hs) resulted in changes in the proton resonances at δ 5.32 and 3.39. Proton resonance at δ 2.44 (dt, J = 3.2, 7.1 Hz) was assigned as C_4-H. The proton resonance at δ 2.33 were characteristic for C_2 methylene hydrogens and a triplet at δ 0.87 (t, 6.5 Hz) was characteristic of a terminal methyl group. The proton resonances at δ 1.24 were characteristic of C_{17}-C_{19} and C_3 hydrogens. The proton resonances corresponding to C_4-Hs were not clearly seen due to the broad signals from water at δ 1.8–1.4.

DISCUSSION

These studies have demonstrated that the biologically active molecule of *m/z* 828.5 (i2) is 1-palmitoyl-2-(5,6)-epoxyisoprostane E_2-*sn*-glycero-3-phosphocholine (PEIPC). This molecule was 1 of the 5 isomers of *m/z* 828.5 present in Ox-PAPC (Fig. 1). Accurate mass measurements by high resolution fast atom bombardment/MS analysis demonstrated that *m/z* 828.5 was an arachidonic acid derivative with the addition of three oxygens and the loss of two hydrogens. The identification of the active isomer was made by performing structural analysis on *m/z* 828.5 (i2), its dehydration product *m/z* 810.5 (i2) (Fig. 3), and the fatty acids liberated from these phospholipids. It was necessary to use both the native molecule and dehydration product because of the difficulty of recovering the liberated fatty acid from *m/z* 828.5 (i2). Dehydration of a β-hydroxy ketone (*m/z* 828.5 (i2)) to an α,β-unsaturated enone (*m/z* 810.5 (i2)) was demonstrated by ^{1}H NMR analysis of the intact phospholipids (data not shown) and has been described previously for several prostaglandins (*i.e.* conversion of prostaglandin E_2 to prostaglandin A_2) (25). Derivatization with bis(trimethylsilyl)trifluoroacetamide also demonstrated the presence of one hydroxyl group in *m/z* 828.5 (i2), which was lost in *m/z* 810.5 (i2) (data not shown). Studies with sodium borohydride where four hydrogens were added to the molecule suggested the presence of two reducible oxygens (Fig. 5). This was also consistent with addition of two methoxylamine to the molecule (Fig. 4). Previous studies have shown that both epoxide and carbonyl groups add amines (26, 27). ESI-MS/MS analysis of the fatty acid isolated from *m/z* 810.5 (i2) (Fig. 8) and *m/z* 828.5 (i2) (data not shown) with O^{18} labeling demonstrated that there was a reactive oxygen at the 5 position. Further evidence for reactive oxygen at this position was the ability of *m/z* 810.5 (i2) and 828.5 (i2) to form POVPC under MS/MS conditions. MS/MS analysis of *m/z* 810.5 (i2) reduced with sodium borohydride and sodium borodeuteride suggested that this oxygen was an 5,6-epoxide. This was suggested by the association of the reduced oxygen with either the 5 or 6 carbon during fragmentation (Fig. 6). The fact that the fatty acids liberated from *m/z* 810.5 (mass 331) was cyclic, was shown by the addition of only 10 hydrogens during hydrogenation rather than 12 hydrogens, which would be expected if the molecule were linear (Fig. 7).

The detailed structure of the liberated fatty acid was determined by proton NMR analysis (Fig. 9). The ^{1}H NMR spectrum of the 332 (i2) showed the presence of α,β-unsaturated carbonyl group (δ 7.53 and 6.34), a trisubstituted alkene (δ 6.16), a *cis*-alkene (δ 5.49 and 5.32), and an epoxide (δ 3.38 and 2.99). The chemical shift and coupling constant values of 332 (i2) indicated that it was a cyclopentenone containing an exocyclic allylic epoxide. The connectivity between various functional groups in 332 (i2) was obtained based on ^{1}H-^{1}H homodecoupling experiments (Fig. 11). All isomers with *m/z* 828.5 possessed nearly identical UV maxima at 252 nm (mean ε = 19,774) and all isomers of *m/z* 810.5 possessed an identical UV maxima at ~257.5 nm (mean ε = 18,342), which was attributable to the presence of an γ,δ-epoxy, α,β-unsaturated enone, or γ,δ-epoxy, α,β-unsaturated dienone. These values were comparable with the structurally related Δ^{12}-PGJ_2 (λ_{max} = 248 nm, ε = 17,000), which contains a δ-hydroxy, α,β-unsaturated enone (28) (Fig. 10).

A number of groups have identified isoprostanes as a major product of autoxidation of arachidonic acid (Refs. 29 and 30; reviewed in Ref. 31). F_2 isoprostanes are produced during *in vitro* oxidation of LDL by a variety of free radical generating systems including copper (32), peroxynitrite (33), and endothe-

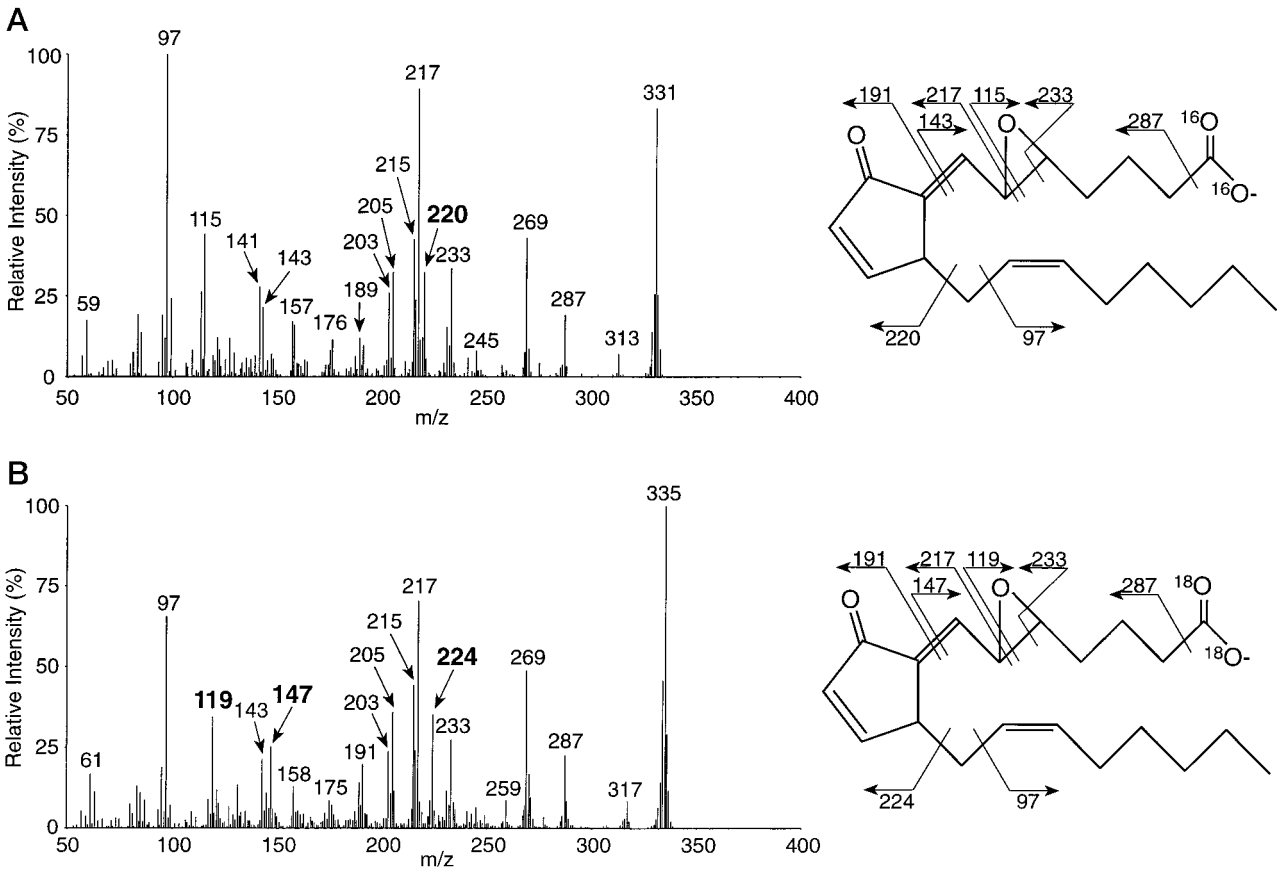
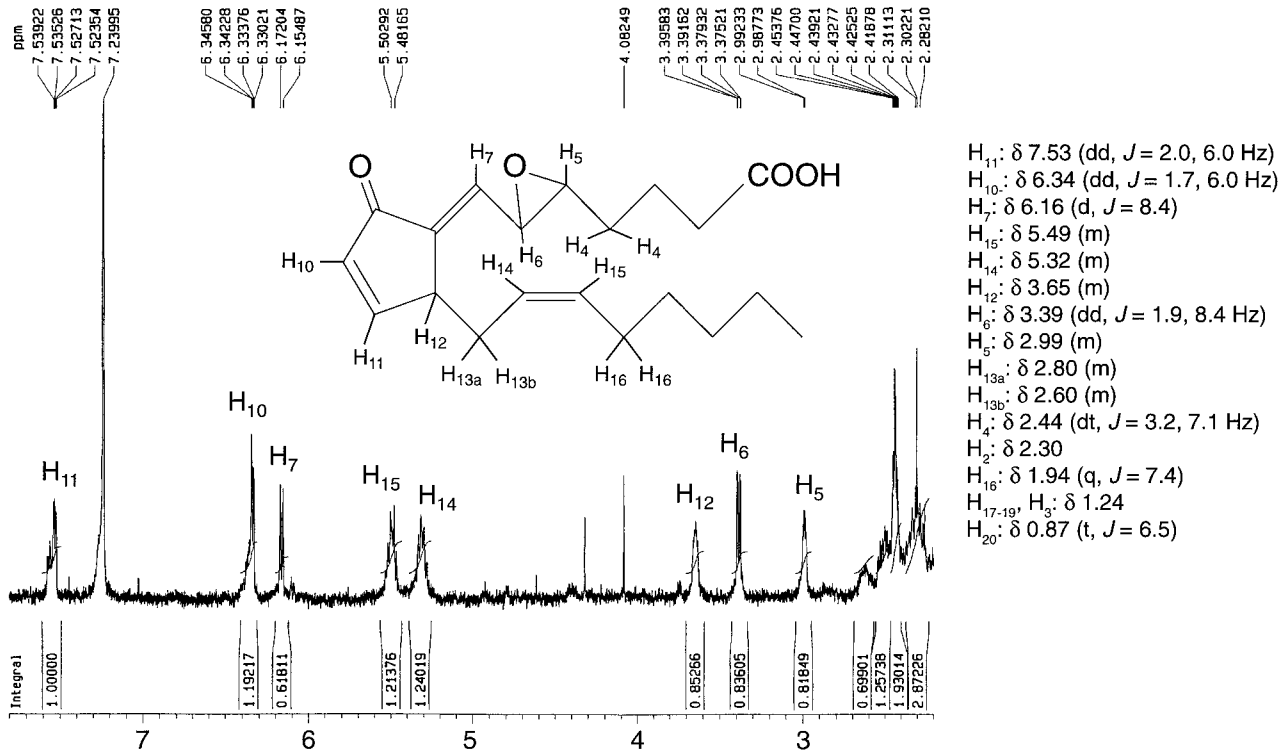


FIG. 8. **Electrospray ionization tandem mass spectrometry of the fatty acid released from 810.5 (i2).** The dehydration product of 828.5 (i2) was isolated by sequential NP-LC/MS and RP-LC/MS and treated with phospholipase A_2. The fatty acid was isolated by a second RP-LC/MS run, collected, and analyzed by ESI-MS/MS in the negative mode (*A*). The free fatty acid obtained by a second RP (C_{18})-LC/MS run was incubated with porcine liver esterase in $H_2{}^{18}O$. The contents were mixed thoroughly and incubated for 60 min at 37 °C with occasional mixing. Lipids were extracted by addition of 300 μl of chloroform/methanol (2:1, v/v), dryed, and analyzed by ESI-MS/MS in the negative ion mode (*B*).



H_{11}: δ 7.53 (dd, J = 2.0, 6.0 Hz)
H_{10}: δ 6.34 (dd, J = 1.7, 6.0 Hz)
H_7: δ 6.16 (d, J = 8.4)
H_{15}: δ 5.49 (m)
H_{14}: δ 5.32 (m)
H_{12}: δ 3.65 (m)
H_6: δ 3.39 (dd, J = 1.9, 8.4 Hz)
H_5: δ 2.99 (m)
H_{13a}: δ 2.80 (m)
H_{13b}: δ 2.60 (m)
H_4: δ 2.44 (dt, J = 3.2, 7.1 Hz)
H_2: δ 2.30
H_{16}: δ 1.94 (q, J = 7.4)
$H_{17\text{-}19}$, H_3: δ 1.24
H_{20}: δ 0.87 (t, J = 6.5)

FIG. 9. **^{1}H NMR of the fatty acid obtained from *m/z* 810.5.** The 500 MHz ^{1}H NMR spectrum of the 332 (i2) in $CDCl_3$ indicates the presence of an α,β-unsaturated carbonyl group (δ 7.53 and 6.34), a tri-substituted alkene (δ 6.16), a *cis*-alkene (δ 5.49 and 5.32), and an epoxide (δ 3.39 and 2.99).

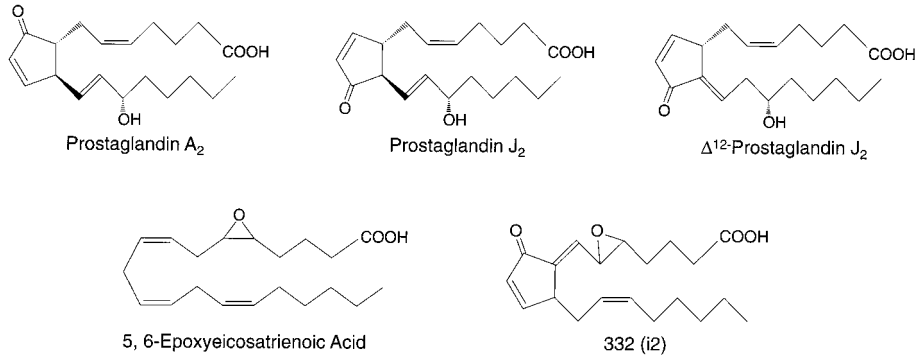


FIG. 10. **Arachidonic acid metabolites with structural similarity to 332 (i2).**

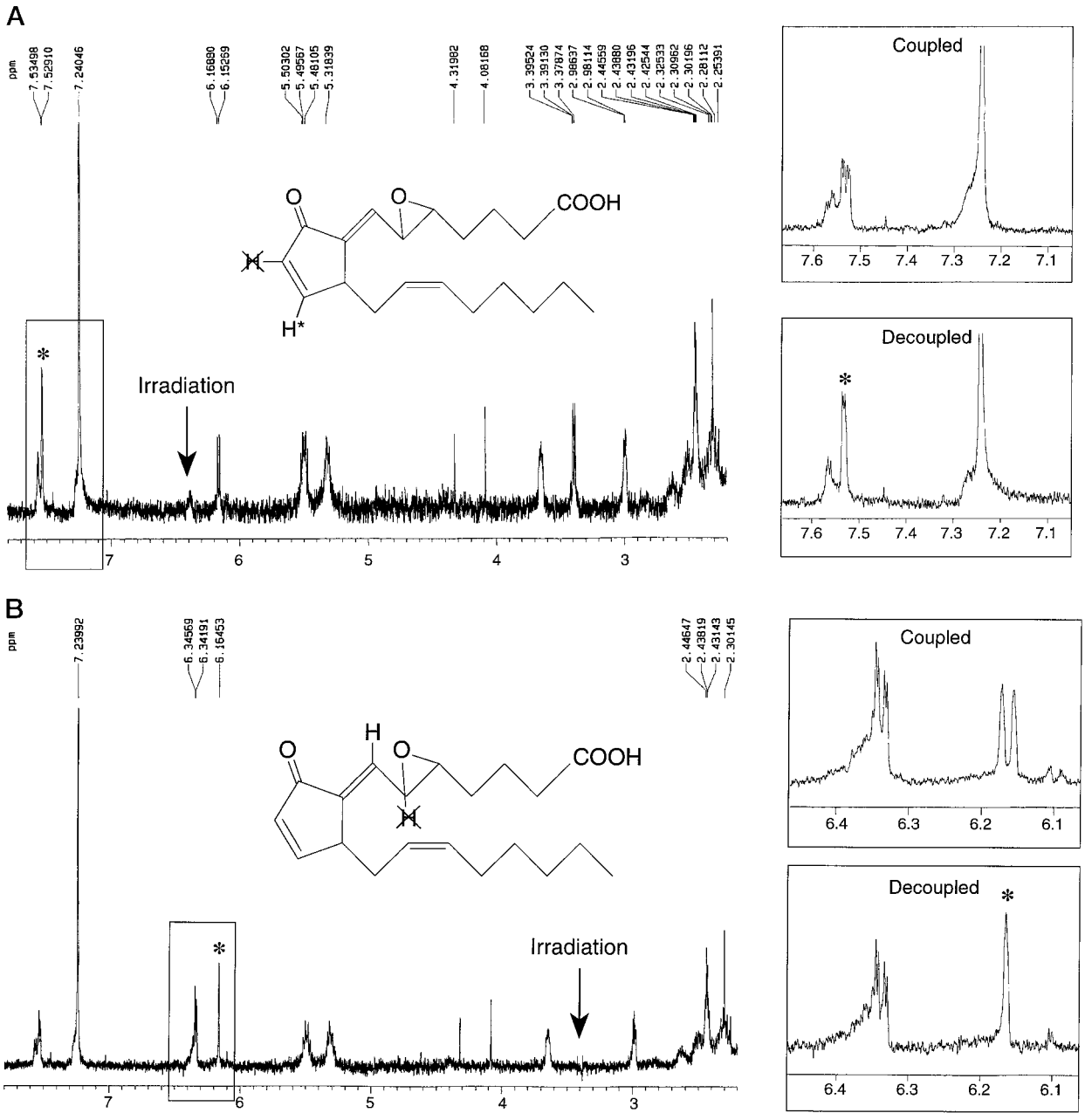


FIG. 11. **Representative ^{1}H-^{1}H Homodecoupling spectra of 332 (i2).** Irradiation of proton resonances at δ 6.34 (dd, C_{10}-H) resulted in the conversion of the dd at δ 7.53 to a d suggests these two protons are coupled to each other (*panel A*). Irradiation of proton resonances at δ 3.39 resulted in the conversion of the doublet at δ 6.16 to a singlet and some minor changes in the multiplet at δ 2.99 (C_5-H) (*panel B*).

lial cell cultures (34). F_2 isoprostanes were shown to be initially formed from arachidonic acid esterified to phospholipids, and free isoprostanes can then be released by hydrolysis (35). Increased levels of isoprostanes have been found in atherosclerotic lesions (36). Recently, association of F_2 isoprostanes with atherosclerosis has been examined. In patients with hypercho-

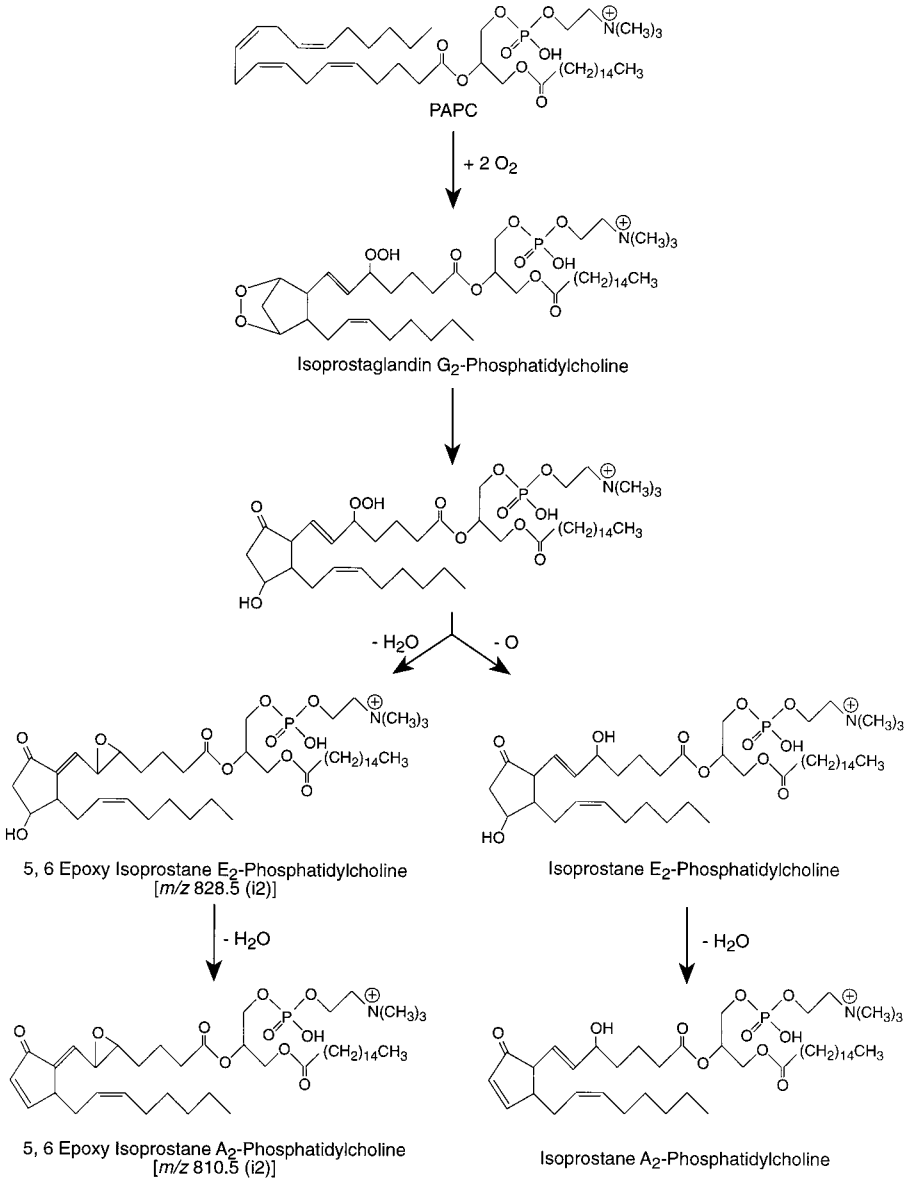


FIG. 12. **Proposed mechanism by which epoxyisoprostane-containing phospholipids are produced from arachidonic acid-containing phospholipids.**

lesterolemia the levels of 8-iso-$PGF_{2\alpha}$ were approximately twice those of age matched controls (37). In another study, a nearly 50-fold increase in 8-iso-$PGF_{2\alpha}$ and $IPF_{2\alpha}$-I levels were found in atherectomy specimens from lesions as compared with nonlesion vascular tissue (38).

More than 64 isoprostanes have been shown to be randomly produced by arachidonic acid oxidation (31). The amount of D_2/E_2 and F_2 isoprostanes differ considerably depending upon the type of oxidative stress (39). Although large numbers of isoprostanes have been described, no epoxyisoprostanes have previously been identified. PEIPC was one of the most prominent products during the autoxidation of PAPC (18). The lack of previous identification of epoxyisoprostanes may be attributed to the harsh conditions typically used for the release of fatty acids from phospholipids. Base hydrolysis and acidification during extraction can alter or destroy the reactive allylic epoxide functionality present in these molecules. It is known that molecules containing allylic epoxide functionality can add nucleophiles such as methanol and amines (26, 40) and undergo rearrangements (41). We observed addition of CD_3OD to PEIPC when lipids were extracted with $CHCl_3/(CD_3)_3OD$ under certain conditions (data not shown). Autoxidation of unesterified arachidonic acid also generated ions with *m/z* 349 and *m/z* 331. However, UV and HPLC characteristics of the *m/z* 349 and *m/z* 331 derived from arachidonic acid autoxidation were very different than that of the free fatty acids released from *m/z* 828.5 or *m/z* 810.5. This suggested that the mechanism by which arachidonic acid and arachidonoyl phospholipids autoxidize were not identical.

The proposed mechanism for the generation of epoxyisoprostane during autoxidation of PAPC is shown in Fig. 13. Oxidation of arachidonoyl phospholipids were shown to generate four regioisomers of F_2 or E_2/D_2 isoprostanes via the proposed prostaglandin endoperoxide phospholipid intermediates (G_2-IsoP-PC and H_2-IsoP-PC) (42). It is known that allylic hydroperoxides can undergo dehydration to generate allylic epoxides (43, 44). Decomposition of endoperoxide nucleus and rearrangement of allylic hydroperoxide of 5-G_2-IsoP-PC is expected to form an allylic epoxide containing 5,6-epoxyisoprostane E_2 phospholipid (*m/z* 828.5). Dehydration of 5,6-epoxyisoprostane E_2-PC generates an epoxycyclopentenone isoprostane (*m/z* 810.5). Alternatively, reduction of allylic hydroperoxide and decomposition of endoperoxide nucleus in 5-G_2-IsoP-PC has been shown to generate 5-E_2-IsoP-PC. Dehydration of 5-E_2-IsoP-PC is expected to generate the corresponding cyclopentenone isoprostane 5-A_2-IsoP-PC. It is likely that the proposed

hydroperoxide rearrangements of other regioisomers of G_2-Iso-PC could generate different regioisomers of epoxyisoprostane phospholipids.

These studies have demonstrated that only one of five isomers of the phospholipid of mass 828.5 induce monocyte-endothelial interactions. This isomer was more potent in the activation of monocyte binding than the two previously identified biologically active phospholipids found in MM-LDL (POVPC and PGPC). Significant bioactivity of PEIPC was detected at <0.5 μM as compared with the 5 μM levels necessary for bioactivity of POVPC and PGPC. The dehydration products of all the *m/z* 828.5 isomers were inactive as were the liberated fatty acids from *m/z* 810.5 and *m/z* 828.5 (data not shown). Thus, this epoxyisoprostane is active on monocyte binding as the phospholipid rather than its fatty acid oxidation product. POVPC and PGPC are present in MM-LDL at approximately 8-fold higher concentration than PEIPC (18), though this varied between preparations. The amount of these three lipids recovered from MM-LDL can account for essentially all of the monocyte binding activity in the PC fraction of MM-LDL. Other isoprostanes have been shown to be active as free fatty acids, though others have not, to our knowledge, tested the isoprostane phospholipids for activity.

There is evidence to suggest that the *m/z* 828.5 molecules may have an important regulatory role(s) in inflammatory processes *in vivo*. Molecules of *m/z* 828.5 were prominent components of atherosclerotic lesions of rabbits (18). Antibodies to this molecule were present in the plasma of ApoE null mice. In addition to mediating monocyte binding, molecules of *m/z* 828.5 have been shown to induce hemoxygenase-1 in co-cultures of endothelial-smooth muscle cells and to induce MCP-1 synthesis (45). Importantly, initial studies suggest that the *m/z* 828.5 is less easily hydrolyzed by two lipoprotein associated enzymes platelet-activating factor, acetylhydrolase and paraoxonase (46). Thus, the half-life of *m/z* 828.5 may be extended relative to other phospholipid oxidation products. All of these studies were performed with the mixed isomers of *m/z* 828.5; effects of specific isomers will be tested in future studies.

In summary, we have identified a novel epoxyisoprostane-containing phospholipid with defined structural requirements for biological activity. Because this molecule is formed by free radical oxidation, it may be important in the regulation of pro-inflammatory processes involving oxidative stress.

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