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Identification of Novel Bioactive Aldehyde-modified Phosphatidylethanolamines Formed by Lipid Peroxidation

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SUMMARY

Lipid aldehydes generated by lipid peroxidation induce cell damage and inflammation. Recent evidence indicates that gamma-ketoaldehydes (isolevuglandins, IsoLG) form inflammatory mediators by modifying the ethanolamine headgroup of phosphatidyl ethanolamine (PEs). To determine if other species of aldehyde-modified PE (al-PEs) with inflammatory bioactivity were generated by lipid peroxidation, we oxidized liposomes containing arachidonic acid and characterized the resulting products. We detected PE modified by IsoLG, malondialdehyde (MDA), and 4-hydroxynonenal (HNE), as well as novel series of *N*-acyl-PEs and *N*-carboxyacyl-PEs in these oxidized liposomes. These al-PEs were also detected in HDL exposed to myeloperoxidase. When we tested the ability of al-PEs to induce THP-1 monocyte adhesion to cultured endothelial cells, we found that PE modified by MDA, HNE, and 4-oxononenal induced adhesion with potencies similar to PE modified by IsoLG (~2 μ M). A commercially available medium chain *N*-carboxyacyl-PE (C11:0CAPE) also stimulated adhesion, while C4:0CAPE and *N*-acyl PEs did not. PE modified by acrolein or by glucose were only partial agonists for adhesion. These studies indicate that lipid peroxidation generates a large family of al-PEs, many of which have the potential to drive inflammation.

Keywords

Oxidative stress; lipid peroxidation; lipid aldehydes; phosphatidylethanolamines

INTRODUCTION

Oxidative stress has been implicated in atherosclerosis, diabetes, neurodegenerative diseases, and various cancers. Peroxidation of lipids generates highly reactive aldehydes including malondialdehyde (MDA), acrolein, 4-hydroxynonenal (HNE), 4-oxononenal (ONE), and isolevuglandins (IsoLG, also given the trivial name of isoketals) [1–5]. Oxidation of glucose [6] and amino acids [7, 8] form additional species of aldehydes [6–8]. Exposure of vascular cells to these aldehydes results in endothelial dysfunction, secretion of inflammatory cytokines, and recruitment of monocytes [9–15], key steps in the initiation of

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inflammation associated with oxidative stress. The inflammatory effects of lipid aldehydes have often been presumed to arise from their modification of protein or DNA. However, recent studies have shown that many of these aldehydes also modify the ethanolamine headgroup of phosphatidylethanolamine (PE) (Fig. 1) and that PE modification increases in conditions associated with oxidative stress [5, 16–27]. For aldehydes like IsoLG, the reaction rate with PE is significantly greater than that for protein or DNA, so that PE is primary target in cells exposed to IsoLG [24]. While the biological significance of this PE modification is only poorly understood, IsoLG modified PE (IsoLG-PE) activates the proinflammatory responses of endothelial cells including surface expression of adhesion molecules, cytokine expression, and adhesion of monocytes [28]. Whether similar inflammatory responses are invoked when PE is modified by other aldehydes such as MDA and HNE is an important unanswered question.

Another important question is whether there are any additional species of aldehyde-modified PEs that are formed in significant abundance by lipid peroxidation besides those already identified. This seems somewhat likely given that the known aldehyde modified-PEs (al-PEs) were all identified by the candidate approach of reacting well-established aldehydes with PE, structurally characterizing the resulting adduct, and then analyzing biological samples for the presence of the specific al-PE in question. We therefore sought to characterize all of the major species of al-PEs formed by the peroxidation of arachidonic acid and to identify which of these exerted proinflammatory effects similar to IsoLG-PE.

EXPERIMENTAL PROCEDURES

Reagents

Human lipoprotein HDL was purchased from Intracel Co. (Frederick, MD). Arachidonic acid was purchased from Cayman Chemical Company (Ann Arbor, MI). LPS and myeloperoxidase from human leukocytes (MPO) were purchased from Sigma–Aldrich (St. Louis, MO). 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (diPPE) was purchased from Avanti Polar Lipids (Alabaster, AL). Organic solvents, including methanol, chloroform, dichloromethane, and acetonitrile, were high-performance liquid chromatographygrade. All compounds were purchased from Sigma–Aldrich otherwise noted specifically.

Measurement of IsoLG-PE and IsoLG-protein in ox-HDL

Human HDL was oxidized by myeloperoxidase (MPO) as previously described for lipoprotein modification [29]. Briefly, HDL (containing 2 mg protein) was incubated at 37 °C in 50 mM sodium phosphate (pH 7.4, 0.5mL), 200 uM diethylenetriaminepentaacetic acid (DTPA), 1 unit MPO, 100 ug/ml glucose, 20 ng/ml glucose oxidase, and 0.05 mM NaNO₂ for 8 h. Oxidation reaction was terminated by addition of 2 equivalent of Folch solution (methanol/chloroform 1:2) containing 0.1% BHT. IsoLG-protein in the aqueous layer was measured as previously described [30]. IsoLG-PE was extracted to the lower organic layer and analyzed as earlier reported [31] with modifications. IsoLG-PE was purified on silica solid phase extraction cartridge and hydrolyzed by 0.35 M sodium hydroxide in methanol (0.5 mL) at room temperature for 1 h. The hydrolysis was terminated with formic acid by adjusting pH to 7. Product was further purified by a C18 solid phase extraction cartridge to remove ions before MS analysis. Final sample was prepared in methanol and analyzed on a ThermoFinnigan Quantum electrospray ionization triple quadrupole mass spectrometer [31].

Oxidation of arachidonic acid in presence of diPPE

Arachidonic acid (1 μ mol) was oxidized by copper sulfate (1 mM)/hydrogen peroxide (10 mM) in the presence of diPPE (0.2 μ mol) in 1 mL solvent of 1 M triethylammonium acetate/chloroform/ethanol (1:1:3). The system was kept in the shaker bath at 37 °C for 24 h. C17:0NAPE (0.1 nmol) was used as internal standard. The solution was concentrated and purified by a silica solid phase extraction cartridge and al-PEs were eluted with 100% methanol. An unoxidized control without copper/hydrogen peroxide was prepared at the same time and kept closed/dark at 4 °C to prevent any auto-oxidation. An aliquot of solution was extracted by Folch solution (methanol: chloroform 1:2) and re-dissolved in methanol for MS analysis.

Preparation of oxidized liposome models

1-stearoyl-2-arachidonyl-glycerolphosphatidylcholine (SAPC, 1 μ mol) was mixed with diPPE (0.2 μ mol). After drying under nitrogen gas, the mixture was re-suspended in 1 mL PBS pH 7.4 and heated up to 65 °C for 1 h with frequent vortexing. The liposome solution was sonicated for 10 min in a water sonicator. An aliquot of liposome solution was kept closed and saved at 4 °C as control and the rest was incubated with the oxidizing reagent copper sulfate/hydrogen peroxide (1 mM and 10 mM, respectively) or 10 mM tert-butylhydroperoxide (tBHP) at 37 °C for 24 h. An aliquot of liposome solution was dissolved in methanol for MS analysis.

Extraction of al-PEs

Al-PEs were extracted by 2 equivalent of Folch solution twice. The organic layer was saved and concentrated into 1 mL 5% methanol in chloroform. The solution was loaded onto a silica solid phase extraction cartridge and washed with 8 mL chloroform. Al-PEs were eluted with 8 mL methanol and dried under nitrogen.

LC-MS analysis of al-PEs

Aldehyde-modified PEs were analyzed on a ThermoFinnigan Quantum electrospray ionization triple quadrupole mass spectrometer operating in negative ion mode, equipped with Surveyor autosampler. Volume of injection was 5 μ L. Mobile phase A consisted of 1 mM ammonium acetate in water/acetonitrile/methanol (1:1:3), and mobile phase B, 1mM ammonium acetate in ethanol. The lipids were chromatographed on a Zorbax XDB-C8 column (50 mm $\times$ 2.10 mm; Agilent) with a constant flow rate of 400 μ L/min. After 0.5 min hold at 1% mobile phase B, the solvent was gradient ramped to 99% B over 6 minutes, held at 99% B for 1 min and return to 1% B over 1 min and held for 1 min before the next injection. The electrospray needle was maintained at -3300 V. The ion-transfer tube was operated at -35 V and 270 °C. The tube lens voltage was set to -180 V. Precursor ion spectra from 700 to 1500 Da were monitored by collision-induced decomposition of the parent ion of m/z 255.

High definition MS analysis

High definition mass spectrometry was used to identify the chemical formulation of unknown al-PEs. Analysis was performed on a Waters Synapt HDMS (Waters, Inc. Milford, MA) instrument. The same LC solvents, gradients and column were applied as before. Data analysis was performed using MassLynx software (Waters).

Identification of al-PEs

Synthetic al-PEs were prepared for HPLC analysis to validate the putative structures of al-PEs. C2:0NAPE was synthesized from acetic anhydride and diPPE (1:1) in pyridine. The reaction was stopped by the addition of water. The product was extracted by ether.

C6:0NAPE was prepared from hexanoylchloride and diPPE following a similar strategies for the synthesis of C17:0NAPE [31]. In brief, diPPE (3 μ mol) was suspended in 0.5 ml chloroform containing 5 mg triethylamine. To this solution (on ice) acyl chloride (10 μ mol, 1.4 μ L) in 0.1 ml chloroform was added dropwise. After the addition was complete, the reaction mixture was allowed to stir overnight in 37 °C water bath while shaking. The reaction was quenched by saturated NaHCO₃ solution. The organic layer was collected and dried over anhydrous Na₂SO₄. IsoLG-PE was synthesized as reported [24, 31]. C4:0CAPE was purchased from Avanti Polar Lipids (Alabaster, AL). Propenal-PE was prepared from reaction of MDA and PE as a minor product. MDA was freshly prepared from 1,1,3,3-tetramethoxypropane [32] and mixed with equal amount of diPPE in a bi-layer solvent system of chloroform/water (1:1) and incubated overnight at 37 °C. The predominant product of MDA-PE, DHP-PE, was further extracted and purified for later studies, and propenal-PE was used for HPLC analysis. Synthetic al-PEs were loaded on a Zorbax XDB-C8 column and the retention time was monitored by MS method described above. The retention time of synthetic compound was used to validate the putative structures of al-PEs observed in lipid oxidation.

Derivatization of al-PEs

To further examine whether unknown al-PE has a carboxylate group, an aliquot of Cu-oxidized arachidonic acid-diPPE solution (200 μ L) was taken for derivatization. C17NAPE (0.1 nmol) was added as internal standard and al-PEs were extracted with 600 μ L of Folch solution. The organic layer was collected and dried under nitrogen. Derivatization of al-PEs was produced by adding 50 μ L of a 10% solution of pentafluoro benzyl bromide (PFBB) in acetonitrile and 50 μ L of a 20% solution of N, N-diisopropylethylamine in acetonitrile. The solution was vortexed and incubated for an hour at room temperature. The solvent was dried under nitrogen and the lipids were extracted with Folch solution (1 mL) and water (0.5 mL) again. The organic phase was collected and dried under nitrogen. The lipids were re-suspended in methanol prior to MS analysis. MS analysis of derivatized al-PEs was similar to that used for underivatized al-PEs except that new precursor ions with an additional m/z of 180 amu for each appropriate species were used in the MRM transitions.

MS Analysis of al-PEs with base hydrolysis

Base hydrolysis of al-PEs was used to remove the two *O*-acyl chains to facilitate MS analysis of biological samples [33]. Extracted and purified al-PEs were dissolved in 0.35 M sodium hydroxide in methanol (0.5 mL) and vortexed for 1 h at room temperature. The hydrolysis was terminated with formic acid by adjusting pH to 7. Products after hydrolysis were further purified by a C18 solid phase extraction cartridge to remove inorganic ions before MS analysis. Final samples were prepared in methanol and analyzed on a ThermoFinnigan Quantum electrospray ionization triple quadrupole mass spectrometer.

Mass spectrometer was operated in negative ion mode and equipped with Surveyor autosampler. The mobile phase solvent A consisted of 10 mM *t*-butyl ammonium acetate in water (pH 4.5) and mobile phase B of 10 mM *t*-butyl ammonium acetate in acetonitrile (pH 4.5). The lipids were chromatographed on a Kinetex C18 column (50 mm $\times$ 2.10 mm, 2.6 μ m, 100 Å; Phenomenex, Torrance, CA) with a constant flow rate of 250 μ L/min. After 0.5min hold at 1% B, the solvent was gradient ramped to 99% B over 5 minutes, held at 99% B for 1.5 min and return to 1% B over 1 min and held for 2 min before the next injection. The electrospray needle was maintained at -3300 V. The ion-transfer tube was operated at -35 V and 270 °C. The tube lens voltage was set to -180 V. Each hydrolyzed al-PEs was monitored by MRMs with the specific precursor ion and a common product ion of m/z 79 (phosphate). The ion was activated by collision with argon in the second quadrupole (10 eV; argon 1.5 mTorr).

Detection of al-PEs in tBHP-oxidized HDL

Human HDL (containing 2 mg protein) was oxidized by incubation with tBHP (10 mM) in PBS pH 7.4 at 37 °C for 24 h. A control with HDL only was prepared and kept closed at 4 °C to prevent any auto-oxidation. Al-PEs were extracted by Folch solution, immediately followed with base hydrolysis and MS analysis.

Preparation of synthetic al-PEs

C2:0NAPE, C6:0NAPE, IsoLG-PE and DHP-PE were synthesized as in earlier studies. C4:0CAPE (glutaryl-PE) and C11:0CAPE (dodecanoyl-PE) were bought from Avanti Polar Lipids. The remaining al-PEs were synthesized following previously reported methods. Acrolein-PE was prepared from acrolein (purchased from Fluka) and 2 equivalent of diPPE in methanol/dimethyl chloride/PBS (2:1:0.8) as described [16]. The reaction mixture was incubated at 37 °C overnight in a water bath shaker. HNE-PE and ONE-PE were prepared from fresh HNE [34] or ONE and 2 equivalent of diPPE in a mixture of 1 M triethylammonium acetate/chloroform/ethanol (1:1:3). The reaction was incubated at 37 °C overnight in a water bath shaker. To synthesize pHA-PE, pHA was first prepared freshly from MPO-oxidized L-tyrosine [35], and its reaction to diPPE was generated as previously described [36]. Am-PE was prepared by incubating diPPE with glucose (300 equivalent) in methanol at 60 °C for 24 h [37]. After reaction, specific al-PE was extracted using Folch solution and concentrated under nitrogen. The product was purified by HPLC. The mobile phase consisted of solvent A (60:20:20 methanol/acetonitrile/1 mM ammonium acetate and solvent B (1 mM ammonium acetate in ethanol). The lipids were chromatographed on a Prevail C18 5 μ (250 mm $\times$ 4.6 mm) column (Alltech, Deerfield, IL) with a constant flow rate of 1 mL/min. After a 2-min hold at 20% B, the solvent was gradient ramped to 100% B over 15 min, held at 100% B for 5 min, and returned to 20% B over 5 min and then held for 5 min.

The fractions containing IsoLG-PE were identified by negative ion LC/MS analysis (m/z 1006.7, m/z 1022.6 and 1038.7 for pyrrole, lactam and hydroxyl lactam) and combined. The concentration of final product was determined by parent scan of m/z 255 in negative ion LC/MS with C_{17:0}N-acyl PE (C_{17:0}NAPE, m/z 942.7) as the internal standard. The structure and purity of each synthesized al-PE was evaluated by LC/MS operating in negative ion and MRM mode.

Cell culture

HUVEC were obtained from the American Type Culture Collection (Manassa, VA). They were cultured in endothelial basal growth medium-2 (Lonza, Basel, Switzerland) supplemented with 2% FBS, 0.4% human FGF-B, 0.1% VEGF, 0.1% recombinant long R Insulin-like Growth Factor-B, 0.1% human Epidermal Growth Factor, 0.1% gentamicin sulfate, 0.04% hydrocortisone, 0.1% ascorbic acid, and 0.1% heparin. Cells from passage 6 were used in this study. THP-1 cells were propagated in RPMI 1640 medium containing 10% FBS, 25 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, L-glutamine and 50 μ mol/L beta-mercaptoethanol.

Cell Adhesion Assay

Each al-PE was prepared in ethanol (< 0.5%), diluted to the appropriate concentration using HBSS containing 0.1% human serum albumin (HSA). The solution was sonicated in a water bath sonicator for 30 min before assays. Adhesion assay was performed as previously reported [28]. HUVEC (passage 6) were seeded on 0.1% gelatin-coated 96-well culture plates and cultured to 80–90% confluence. Cells were washed with DMEM three times and incubated with vehicle (negative control), LPS (positive control, 10 μ g/mL), or the test

compound in DMEM at 37 °C for 4 h. Cells were washed with DMEM three times and 100 μ L calcein labeled THP-1 cells were added and incubated for 1 h at 37 °C. Calcein labeled THP-1 cells were prepared by incubation with 4 μ g/mL calcein acetoxymethyl ester (Invitrogen, Carlsbad, CA) at 37 °C for 30 min, excess label was removed by washing three times, and the THP-1 were resuspended in DMEM at 5×10^6 cell/ml. Non-adherent THP-1 cells were removed from HUVEC by gently washing with PBS twice and the fluorescence of HUVEC-bound THP-1 cells measured (494ex/520em). Extent of treatment induced adhesion for each well was normalized to increase over basal fluorescence in vehicle treated wells induced by LPS. For each compound, three separate experiments with five replicate wells per experiment were performed.

Statistical Analysis

All statistical analysis was performed using Graph Pad Prism 4.03 (GraphPad Software, La Jolla, CA).

RESULTS

PE modification is a major target of endogenously formed aldehyde

Addition of synthetic IsoLG to cultured cells results in significantly more PE modification than protein modification, which suggests that PE is the major target of this aldehyde in biological systems. However, whether significant PE modification occurs when IsoLG is generated endogenously is unknown. To compare the extent of PE and protein modification during endogenous IsoLG formation, we incubated high density lipoprotein (HDL) with myeloperoxidase (MPO). MPO is an oxidative enzyme released by activated neutrophils that associates with circulating HDL and is an important generator of IsoLG modified plasma protein in vivo [36]. While incubation of MPO with HDL generated significant amounts of IsoLG-protein adducts, we also found that it generated at least 10-fold greater amounts of IsoLG-PE (Fig 2). This result confirms that PE is also a major target of endogenously generated IsoLG.

Lipid peroxidation generates novel al-PE species

To date, identification of al-PEs has primarily relied on a candidate approach of reacting well-established aldehydes with PE and examining the resulting products, rather than a discovery approach of isolating al-PEs from oxidized specimens. We hypothesized that there were other species of al-PE generated in significant abundance by lipid peroxidation besides those identified by these candidate approaches. To identify novel al-PE, we oxidized arachidonic acid (AA) using copper sulfate in the presence of dipalmitoyl PE (diPPE) and then characterized the resulting modified PEs by LC/MS/MS. To detect only modified PEs and not unreacted peroxidized arachidonate products, we utilized precursor scanning with the product ion set at m/z 255.1 which selects for the *O*-palmitoyl fragment of *N*-modified PE species. Novel chromatographic peaks were detected in the reaction of oxidized AA with PE not present in the non-oxidized control (Fig 3). Analyses of the spectrums of these chromatographic peaks identified at least ten PE species present in the oxidized mixture with molecular masses higher than that of the starting unmodified PE. The most abundant of these novel ions represented mass shifts of 28, 42, 54, 72, 98, 114, 154, 172, 180, 348 and 364 amu from the starting diPPE (Fig 4). The PE+348 product was identified as the hydroxylactam species of IsoLG-PE based on its mass and co-elution with authentic IsoLG-PE. Interestingly, at least two of the novel al-PE compounds, PE+28 and PE+42 were formed in equal or greater abundance as IsoLG-PE based on signal intensity, with the assumption that ionization efficiency was approximately equal for each al-PE (Fig 5).

To analyze the formation of these al-PEs in a lipid environment that modeled physiological membranes, we constructed liposomes with SAPC and diPPE and oxidized these liposomes with either tert-butylhydroperoxide (tBHP) or with copper sulfate. We then once again monitored formation of al-PE by precursor scanning of m/z 255 product ions. Oxidation of liposomes with esterified arachidonic acid resulted in formation of similar al-PE products as detected in the reaction with free arachidonic acid, although relative abundance differed (Fig 6). For instance, PE+348 was detected in lower relative abundance, which is consistent with our expectation that IsoLG-PE forms from non-fragmented AA which would therefore remain esterified to the PC. In addition to the ion peak at PE+348, other species which showed relatively lower abundance when esterified arachidonic acid was used included PE +114, PE+154, and PE+180, suggesting these were also esterified products. For the most part, oxidation by tBHP and copper sulfate gave fairly similar results.

Structural characterization of al-PEs

We employed high resolution MS to unambiguously define the elemental composition of compounds initially identified by precursor scanning. The resulting high accuracy masses were matched to potential elemental compositions that included the diPPE moiety ($C_{37}H_{73}NO_8P$) as a minimal component and where the maximum error tolerance was 10 ppm. The most probable chemical formula for each of the observed al-PE species based on these criteria is listed in Table 1.

Using these chemical formulas and previously described reaction pathways for lipid peroxidation, we assigned the most probable identity for PE+28 as *N*-formyl-PE, for PE+42 as *N*-acetyl-PE (C2:0NAPE), for PE+54 as *N*-propenal-PE, and for PE+72 as *N*-oxoacetyl-PE (Fig 7). We interpreted PE+172 ($C_{46}H_{89}NO_{11}P$) to be the oxidized form of the previously described Michael adduct of HNE-PE (oxHNE-PE). PE+364 ($C_{57}H_{101}NO_{14}P$) is consistent with a further oxygenated form of IsoLG-PE hydroxylactam. PE+98 ($C_{43}H_{83}NO_9P$) is consistent with *N*-hexanoyl-PE (C6:0NAPE) which was recently characterized and shown to form during oxidation by Osawa et al [38]. That work showed that C6:0NAPE forms via 15-HPETE. Analogous reactions for 5-HPETE and 8-HPETE, which like 15-HPETE form during arachidonic acid peroxidation, predicts the formation of *N*-glutaryl-PE ($C_{42}H_{79}NO_{11}P$) and *N*-7-carboxyhept-2-enoyl-PE ($C_{45}H_{83}NO_{11}P$) which are consistent with PE+114 and PE+154, respectively (Figure 8). Formation of 11-HPETE, although expected to be a minor and unstable product, would give rise to *N*-9-carboxynona-3,5-dienoyl-PE which is consistent with PE+180 ($C_{47}H_{85}NO_{11}P$).

Synthesis of several of the putative al-PEs formed in oxidized liposomes could be performed using readily available compounds. C2:0NAPE and C6:0NAPE were generated by the reaction of acetic anhydride and hexanoyl chloride with diPPE, respectively. In our LC/MS analysis, these authentic compounds co-eluted with the PE+42 and the PE+98 products from the oxidized liposomes, respectively (Fig 9). While the dihydropyridine-PE (DHP-PE) is the major reaction product formed during the reaction of MDA with PE, propenal-PE is also formed as a minor product of this reaction and this propenal-PE co-eluted with the PE+54 peak of oxidized liposomes. Commercially available glutaryl-PE (*N*-4-carboxybutanoyl-PE, C4:0CAPE) co-eluted with the PE+114 product. Although we were not able to isolate sufficiently pure samples from the oxidized liposome samples to completely validate the identity of these compounds by NMR, the co-elution of the authentic compounds with our identified products support our tentative assignments. We were unable to devise simple synthesis schemes from readily commercially available compounds for the other abundant putative *N*-carboxyacyl-PEs (CAPEs) including *N*-oxoacetyl-PE (C1:0CAPE), *N*-7-carboxyhept-2-enoyl-PE (C7:1CAPE) and *N*-9-carboxynona-3,5-dienoyl-PE (C9:2CAPE). To confirm that these putative CAPEs contained carboxylate groups, we treated the oxidation mixture with pentafluorobenzyl bromide (PFBB), which derivatizes the carboxyl

groups to pentafluorobenzyl esters and leads to a mass shift of 180 amu per carboxyl group. Reaction with PFBB diminished the MRM signals for PE+72, PE+114, PE+154, PE+172, PE+180 and PE+348, while the signals of PE+28, PE+42, PE+54 and PE+98 were unaffected (Fig 10A). Conversely, when we monitored for PFB esters by MRM, reaction with PFBB produced peaks at the appropriate 180 amu mass shift for PE+72, PE+114, PE+154, PE+172, PE+180, and PE+348 (Fig 10B), confirming that these compounds contain a carboxyl group.

Novel al-PEs are present in oxidized HDL

In order to assess the extent of al-PE formation in a biological membrane relevant to disease, we oxidized HDL and then measured the resulting al-PE after deacylation of the *O*-acyl chains by base hydrolysis. This deacylation strategy potentially offers two principal advantages over measuring intact al-PEs in HDL and other biological samples. First, although we can measure intact al-PE species relatively easily in liposomes where the only PE species is diPPE, in HDL and other biological samples there are many different species of PE due to variations in the *O*-acyl chains. Therefore, accurate quantitation of each form of al-PE would necessitate measuring each of these PE variants. In contrast, deacylation would allow us to measure al-PEs with the same headgroup but different *O*-acyl chains as a single species. Furthermore, since some al-PEs like IsoLG-PE and the CAPEs are at least initially esterified to PC, their base hydrolysis should allow us to better quantify these al-PE as well. Prior to analysis of oxidized HDL, we first determined the feasibility of measuring al-PEs after deacylation to their respective glycerophosphate analogs (al-GPs) by base hydrolysis with methanolic sodium hydroxide of tBHP oxidized SAPC/diPPE liposomes. The resulting al-GPs were monitored in MS/MS using their common product ion of *m/z* 79 (phosphate) for individual MRM transitions. MS analysis of the base hydrolysate of tBHP-oxidized SAPC/diPPE liposomes detected all of the expected al-GPs as found for al-PEs (Fig 11). Of note, however, was that the relative abundance of some of the smaller species such as PE+28 and PE+42 were considerably lower for the al-GP measurement than when intact al-PEs were measured, which is likely attributable to their poorer retention during solid phase extraction.

We then analyzed the formation of al-PEs in tBHP-oxidized HDL. We detected IsoLG-PE, propenal-PE, C6:0NAPE, C4:0CAPE, and C7:1CAPE, with IsoLG-PE being the most abundant species (Fig 12A). To determine if these al-PEs were formed under physiologically relevant conditions, we incubated HDL with myeloperoxidase as in our initial studies, and were also able to detect significant formation al-PEs (Fig 12B). These results confirm that lipid peroxidation generates a large number of al-PEs including several that have not been previously identified.

Many al-PEs are biologically active

The biological relevance of this al-PE formation depends on whether such lipids mediated important biological effects attributed to aldehydes. Because our previous studies demonstrated that IsoLG-PE activated the proinflammatory response of endothelial cells, we examined whether other al-PEs shared this same bioactivity. Each al-PE was synthesized by reaction of the appropriate aldehyde or reactive carbonyl with diPPE, the formation of the expected al-PE confirmed by mass spectrometry, and the purified product tested for the ability to stimulate THP-1 monocyte adhesion to endothelial cells. We used commercially available C11:0CAPE as a surrogate for long chain CAPE species such as C9:2CAPE and C7:1CAPE. We found that DHP-PE (Fig 13A), HNE-PE (Fig 13B), ONE-PE (Fig 13C), and C11:0CAPE (Fig 13D) dose-dependently increased THP-1 adhesion to HUVEC with potencies very similar to what we had previously determined for IsoLG-PE (IC₅₀ of 2.5μM). PHA-PE induced full endothelial activation, but required substantially higher concentration

(Fig 13E). Both acrolein-PE (Fig 13F) and am-PE (Fig 13G) were partial agonists of endothelial cell activation. Neither C2:0NAPE (Fig 13H), C6:0NAPE (Fig 13I), or C4:0CAPE (Fig 13J) were able to activate endothelial cells.

DISCUSSION

Our findings demonstrate that there is a much larger family of al-PEs generated by lipid peroxidation than previously recognized and that a wide variety of al-PEs are able to induce endothelial cell activation. This suggests that PE modification may be an important pathway linking oxidative stress to inflammation, so that blocking PE modification or accelerating the degradation of al-PEs may be a useful strategy in reducing inflammation.

Of particular interest is our identification of a large family of amide linked al-PEs in addition to the previously reported C6:0NAPE. C6:0NAPE forms as the major stable product of the reaction of 15-HPETE with PE and is readily detected in the liver of rodents treated with carbon tetrachloride [38]. Three of the additional amide linked al-PEs that we identified, C4:0CAPE, C7:1CAPE, and C9:2CAPE, can be accounted for directly by analogous reactions with 5-HPETE, 8-HPETE, and 10-HPETE, respectively. Because of the instability of its bis-allylic peroxy radical precursor, formation of 10-HPETE during peroxidation of arachidonic acid is expected to be less than for 5-HPETE and 8-HPETE in the absence of hydrogen donors such as α -tocopherol [39, 40]. The other amide-linked al-PE species detected, C2:0NAPE and *N*-oxoacetyl-PE, can be rationalized as secondary products resulting from beta-scission of adjacent double bonds after initial formation of amide-linked al-PEs.

While our studies successfully characterized a number of novel al-PEs, there may be still other al-PEs that transiently form during peroxidation of arachidonic acid but that are insufficiently stable to have been detected by our methods. For instance, we did not detect significant amounts of the reversible HNE-PE Schiff base product previously described during the reaction of HNE with PE [20, 21, 23], but detected instead a more oxidized form of the more stable HNE-PE Michael adduct. This difference may in part be the result of our choice not to use reducing agents like sodium borohydride to stabilize highly reversible reaction products. We did this to avoid artificially increasing the levels of these products and because the physiological significance of any biological activity induced by the reduced products would be unclear. Additionally, our oxidation conditions may have also favored more oxidized form of some adducts. For instance, we predominantly detected the hydroxylactam and oxidized hydroxylactam forms of IsoLG-PE, rather than the pyrrole and lactam forms which tend to be most abundant when IsoLG is reacted with PE in organic solvents [5, 24, 28]. Thus while our studies significantly expand the total repertoire of known alPEs, it would be premature to assume that every bioactive al-PEs formed from peroxidation of arachidonic acid has now been identified or that species not detected in our liposome oxidation experiments do not form under other conditions. Additionally, we would anticipate that oxidation of other PUFA besides arachidonic acid would generate additional amide-linked species.

Our identification of additional al-PEs takes on particular significance because of our finding that a variety of al-PEs share with IsoLG-PE the ability to induce the inflammatory response of endothelial cells. Future studies are needed to determine the mechanism of endothelial cell activation by these other al-PEs. We recently found that the pyrrole form of IsoLG-PE significantly altered membrane curvature and that this effect could be mimicked by modifying PE with 4-oxo-pentanal which forms a pyrrole headgroup that lacks sidechains [28]. This led us to speculate that the aromatic character of this pyrrole headgroup caused it to bury into the membrane/water interface and exert lateral pressure that

resulted in positive membrane curvature. This change in membrane curvature appears to be highly important, as IsoLG-PE is rapidly trafficked to the ER membrane and induces ER stress, while inhibitors of ER stress block the ability of IsoLG-PE to activate the inflammatory responses. Thus perturbation of ER membrane seems likely to be a primary mechanism leading to the induction of inflammatory responses. We postulate that aromatic headgroups like DHP-PE and pHA-PE would similarly create lateral pressure, however, the mechanism by which these and other al-PEs exert their inflammatory effects clearly needs further elucidation.

Additional studies are also needed to simultaneously assess the levels of each al-PEs to determine if their overall levels are sufficient to directly invoke endothelial activation and inflammatory responses. The individual effects of each inflammatory al-PE are likely to be additive, so that measurement of only one or a few al-PEs underestimates their overall effect. Still, previous studies looking at select al-PEs suggest that they are present at concentrations near the 0.2% of total PE that was required to alter curvature in model membranes with IsoLG-PE [28]. For instance, approximately 0.14% of all PE in the retinas of diabetic rats is modified by hydroxyalkenals (e.g. HNE-PE) [23]. 0.45% of PE in LDL isolated from atherosclerotic lesions is pHA-PE [27]. 0.1% of PE in the red blood cells of human type I diabetics is amadori-PE [26]. In plasma from patients with age-related macular degeneration, IsoLG-PE levels are 5.2 ng/ml [25]. Thus, systematic measurement of all al-PEs seems likely to confirm that total al-PEs formed during various conditions associated with oxidative stress are sufficient to initiate inflammatory responses.

It is important to note that al-PEs are likely to participate in a range of biological activities, so that our finding that short- chained CAPEs and NAPEs did not stimulate endothelial activation does not preclude these al-PEs from being bioactive. For instance, HNE-PE has been shown to promote platelet prothrombinase activity [41], C16:0NAPE has been shown to inhibit macrophage phagocytosis through inhibition of Rac1 and Cdc42 [42], and amadori-PE has been shown to induce a structural rearrangement in reconstituted membrane proteins that makes them more sensitive to thermal unfolding [43]. Thus, additional studies will be needed to determine the effects of our novel al-PEs on these and other bioactivities.

In summary, our studies indicate that lipid peroxidation generates a large family of al-PEs, several of which are potent activators of endothelial cells and which therefore have the potential to mediate inflammation associated with oxidative injury and others whose biological contribution remains to be elucidated.

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The abbreviations used are

PE	phosphatidylethanolamine
IsoLG	isolevuglandins
NAPE	N-acyl PE
CAPE	N-carboxyacyl-PEs
al-PE	aldehyde-modified PE

MDA	malondialdehyde
HNE	4-hydroxynonenal
ONE	4-oxynonenal
am-PE	amadori-modified PE
pHA-PE	p-hydroxyphenylacetaldehyde-modified PE
DiPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
SAPC	1-stearoyl-2-arachidonyl-glycerolphosphatidylcholine
N-glutaryl PE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl)
t-BHP	tert-butylhydroperoxide
al-GPs	glycerophosphate analogs of al-PEs after base hydrolysis
ox-HDL	oxidized HDL
PFBB	pentafluorobenzyl bromide
HUVEC	human umbilical cord endothelial cells

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Highlights

- Novel aldehyde-modified phosphatidylethanolamines were identified in oxidized liposomes.
- Formation of aldehyde-modified phosphatidylethanolamines was detected in myeloperoxidase-oxidized HDL.
- A variety of the identified aldehyde-modified phosphatidylethanolamines induced endothelial activation.

Figure 1.

Aldehydes known to form al-PEs. Well-established aldehydes from lipid peroxidation including 4-hydroxynonenal (HNE), 4-oxononenal (ONE), acrolein, malondialdehyde (MDA), isolevuglandin (IsoLG), p-hydroxyphenylacetaldehyde (pHA), and glucose have been shown to modify amino headgroup of PE to form various types of al-PEs.

Figure 2.

PE modification is a major target of endogenously formed IsoLG in oxidized HDL. Human HDL was oxidized by myeloperoxidase (MPO). The modified protein and PE were separated by folch extraction. The organic layer containing modified PE and the protein layer were analyzed by MS. Significantly greater levels of IsoLG-PE than IsoLG-modified protein were detected (two-tailed *t* test, $p = 0.0015$).

Figure 3.

Formation of al-PEs by oxidation of arachidonic acid (AA) in the presence of dipalmitoyl PE (diPPE). AA was oxidized by copper(II)/hydrogen peroxide in the presence of diPPE and the resulting modified PEs were characterized by LC/MS/MS. (A) Schematic of reaction and analysis. LC/MS/MS precursor scanning with the product ion set at m/z 255.1 selects for the *O*-palmitoyl fragment of *N*-modified PE species. (B) Six novel chromatographic peaks were detected in the reaction of oxidized AA with PE that were not present in the non-oxidized control. C17:0NAPE used as internal standard and eluted at 5.35 min.

Figure 4. Mass spectrum of each novel chromatographic peak identified from oxidized arachidonic acid and diPPE. The most abundant al-PE species were selected for analysis (labeled by m/z). The m/z1039 ion was identified as the hydroxylactam species of IsoLG-PE based on its mass and retention time identified in earlier studies.

Figure 5.

Relative abundance of al-PEs in copper oxidized versus unoxidized arachidonic acid and diPPE. The relative abundance was calculated as the ratio of individual al-PE peak height to internal standard peak height. The most abundant al-PEs in MS analysis represented mass shifts of 28, 42, 54, 72, 98, 114, 154, 172, 180, 348 and 364 amu from the starting diPPE.

Figure 6.

Relative abundance of Al-PEs is oxidized SAPC/diPPE liposomes. Liposomes were oxidized by copper(II) (Cu-ox) or t-butylhydroperoxide (tBHP) and formation of al-PEs were monitored by MS precursor scanning of m/z 255 product ions. Oxidation of esterified arachidonic acid resulted in formation of same al-PE products as detected in the reaction with free arachidonic acid, although relative abundance differed. The ion peaks including PE +348, PE+114, PE+154, and PE+180 showed relatively lower abundance when esterified arachidonic acid was used, suggesting that these al-PEs may be esterified products.

Figure 7.

Putative structures of novel al-PEs generated in lipid peroxidation. These structures were based on chemical formulas identified by MS and previously described reaction pathways for lipid peroxidation. For C7:1CAPE and C9:2CAPE, the structures shown represent only one of a number of possible regioisomers in terms of the position and stereochemistry of double bonds that would be consistent with the data.

Figure 8.

Potential unifying mechanism of formation of C6:0NAPE, C4:0CAPE, C7:1 CAPE and C9:2CAPE. Formation of C6:0NAPE via 15-HPETE suggests C4:0CAPE and C7:1 CAPE as products from 5-HPETE and 8-HPETE, respectively. They are consistent with PE+114 and PE+154 detected in oxidized liposomes. Formation of a minor product, 11-HPETE, would give rise to C9:2 CAPE which is consistent with PE+180.

Figure 9.

LC/MS/MS analysis of (A) synthetic al-PEs or (B) al-PEs generated by oxidation of SPC-diPPE liposomes. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode, mass transitions with the product ion of m/z 255.1 for the specific parent ion at collision energy of 50 eV were monitored. The chromatographic results were processed in Xcaliber software (ThermoFinnigan) using 9-point Gaussian smoothing.

Figure 10.

Derivatization of al-PE with pentafluorobenzyl bromide (PFBB) confirms presence of carboxylate moiety for specific al-PEs. (A) Effect of reaction with PFBB on LC/MS/MS signal intensity for individual al-PEs generated by oxidized arachidonic acid in the presence of diPPE. (B) Reaction of this oxidized lipid mixture with PFBB (right column) generates ions consistent with PFB esters that are not present in the absence of PFBB treatment (left column). The mass spectrometer was operated in the MRM mode using transitions from the appropriate parent ion to m/z 255.1 with collision energy of 50 eV. For MRM of PFB esters, appropriate parent ions were chosen by adding 180 amu to the mass of each alPE whose putative structure includes a carboxylate moiety.

Figure 11.

Relative abundance of corresponding aldehyde-modified ethanolamine glycerophosphates (al-GPs) after base hydrolysis of al-PE generated by tBHP oxidation of SAPC/diPPE liposomes. Al-PEs from oxidized liposomes were hydrolyzed by sodium hydroxide in methanol. The resulting al-GPs were purified by C18 solid phase extraction cartridge and monitored by MS operating in the MRM mode using m/z 79 product ion (phosphate) for individual MRM transitions.

Figure 12.

Al-PEs generated in oxidized HDL. Isolated human HDL was oxidized by (A) tBHP and (B) MPO and then analyzed by MS with base hydrolysis. The oxidized HDL generated same al-PE species, which were hydrolyzed by NaOH to remove the two *O*-acyl chains. The resulting al-GPs were monitored by MS operated in the MRM mode, using individual negative precursor ion mass and *m/z*79 (phosphate) product ion.

Figure 13.

Induction of THP-1 monocyte adhesion to HUVEC by individual al-PE species. HUVEC were treated with varying concentrations of each al-PE (0–25 μM), and activation of HUVEC was measured by adhesion of calcein-labeled THP-1 using LPS (10 $\mu\text{g/mL}$) as positive control. (A) DHP-PE (one-way ANOVA, $p < 0.0001$); (B) HNE-PE (one-way ANOVA, ns); (C) ONE-PE (one-way ANOVA, $p = 0.0027$); (D) C11:0CAPE (one-way ANOVA, $p < 0.0001$); (E) pHA-PE (one-way ANOVA, $p < 0.0001$); (F) acrolein-PE (one-way ANOVA, ns); (G) amadori-PE (one-way ANOVA, ns); (H) C2:0NAPE (one-way ANOVA, $p = 0.03$); (I) C6:0NAPE (one-way ANOVA, ns); and (J) C4:0CAPE (one-way ANOVA, ns).

Table 1

High definition MS of al-PEs in oxidized SAPC-diPPE liposomes. Elemental composition analysis of al-PE required fitting two criteria: 1) included the diPPE moiety ($C_{37}H_{73}NO_8P$) as a minimal component, 2) maximum 10 ppm error for measured versus calculated mass. The excess diPPE was used as an internal standard. Only one possible chemical formula (as listed) for each al-PE fit these criteria.

	Parent scan MS	Measured Mass	Calc. Mass	Fit elemental composition
PE+28	718-->255	718.5054	718.5023	$C_{38}H_{73}NO_9P$
PE+42	732-->255	732.5164	732.5179	$C_{39}H_{75}NO_9P$
PE+54	744-->255	744.5251	744.5179	$C_{40}H_{75}NO_9P$
PE+72	762-->255	762.4868	762.4921	$C_{39}H_{73}NO_{11}P$
PE+98	788-->255	788.5875	788.5805	$C_{43}H_{83}NO_9P$
PE+114	804-->255	804.5342	804.5391	$C_{42}H_{79}NO_{11}P$
PE+154	844-->255	844.5692	844.5704	$C_{45}H_{83}NO_{11}P$
PE+172	862-->255	862.6165	862.6173	$C_{46}H_{89}NO_{11}P$
PE+180	870-->255	870.5916	870.5860	$C_{47}H_{85}NO_{11}P$
PE+348	1038-->255	1038.7037	1038.7011	$C_{57}H_{101}NO_{13}P$
PE+364	1054-->255	1054.4956	1054.6960	$C_{57}H_{101}NO_{14}P$
diPPE	690-->255	690.5090	690.5074	$C_{37}H_{73}NO_8P$