Low-Concentration Ozone Reacts with Plasmalogen Glycerophosphoethanolamine Lipids in Lung Surfactant

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Ozone is a common environmental toxicant to which individuals are exposed to on a daily basis. While biochemical end points such as increased mortality, decrements in pulmonary function, and initiation of inflammatory processes are known, little is actually understood regarding the chemical mechanisms underlying changes in pulmonary health, especially for low concentrations of ozone. This study was undertaken to investigate ozone-induced oxidation of endogenous lipids that are potentially exposed to environmental ozone within lung, specifically focusing on plasmalogen glycerophospholipids present in pulmonary surfactant. Sensitive liquid chromatography−mass spectrometry methods were developed to follow oxidation of diacyl and plasmalogen phosphatidylethanolamine (PE) phospholipids and to identify and quantitate products generated by ozonolysis. Using a unilamellar vesicle system containing a 1:1 molar mixture of 1-*O*-octadec-1′-enyl-2-octadecenoyl-PE and 1,2-dihexadecanoyl-PC, these studies revealed that the vinyl ether bond of plasmalogens was oxidized preferentially at low concentrations of ozone (100 ppb), when compared to olefinic bond oxidation on ω-9 of the fatty acyl chain in the same phospholipids. Major phospholipid products generated were identified as 1-formyl-2-octadecenoyl-PE and 1-hydroxy-2-octadecenoyl-PE. Heptadecanal and heptadecanoic acid production was also quantitated using gas chromatography−mass spectrometry, and production was consistent with oxidation of the vinyl ether, at low concentrations of ozone. Analysis of murine lung surfactant from C57Bl/6 mice revealed several plasmalogen PE lipid species, encompassing ∼38% of total PE species. Upon exposure of ozone (0 and 100 ppb) to murine surfactant, plasmalogen PE molecular species preferentially reacted, as compared to diacyl PE molecular species. Lysophospholipids, pentadecanal, and nonanal were found to be the primary products of surfactant ozone oxidation.

Introduction

Ozone is a chemically reactive gas present in air pollution and, as such, is one of the most common environmental toxicants (*1*). Ozone is generated through various photochemical-driven reactions between oxygen and nitrogen oxides or volatile organic compounds (*2*) and, therefore, more prominent during summer months in most areas. Concentrations of ozone found in many suburban areas often reach 100 ppb or higher, commonly exceeding the current EPA limit of 75 ppb (*1*). Epidemiological studies have linked increases in environmental ozone with increased mortality, especially in susceptible populations such as asthmatics or individuals with chronic obstructive pulmonary disease (*3*). Other effects on pulmonary function, such as neutrophilia, acute inflammatory responses, decrements in peak expiratory flow rates, forced expiratory volumes and forced vital capacities, eye and airway irritation, decreased phagocytosis by macrophages, and increased incidence of asthma attacks in children (*4*−*10*) have also been associated with concentrations of ozone as low as 100 ppb. Despite the wealth of knowledge to date regarding the biochemical and physiological end points of ozone, little is currently known regarding the underlying mechanisms that link the chemistry of ozone with subsequent biological effects.

Because of its high reactivity, ozone is not thought to directly act with pulmonary epithelium but instead react with components of the surfactant layer covering the respiratory tract or cells that are not always covered by the surfactant, such as alveolar macrophages. Therefore, the damaging effects are not directly due to ozone *per se* but, rather, are mediated by a cascade of secondary ozonation products generated by reactions with these targets (*11*). Recently, particular interest has been focused on the oxidation of lipids within the surfactant, which has been shown to generate secondary signaling cascades within the lung (*12, 13*). For instance, ozone-induced oxidation of cholesterol generates products such as the 5β,6β-epoxy cholesterol, both in vitro and in vivo, which can cause necrosis in bronchial epithelial cell lines (*14*). Another product of surfactant oxidation, 1-hexadecanoyl-2-nonanal-PC, has been shown to initiate apoptosis of peripheral monocytes at low nanomolar concentrations (*15*).

Another potential surfactant target of ozone oxidation could be plasmalogen glycerophospholipids. Plasmalogens are a chemically unique type of ether lipid that possess a vinyl ether linkage at the *sn*-1 position of the glycerol backbone (Figure 1A, 18:0p/18:1-PE). The vinyl ether bond has been shown to have a higher reactivity to most reactive oxygen species, including superoxide, peroxyl radicals, and HOBr/HOCl, generated by myeloperoxidase (*16, 17*). The *sn*-2 position in plasmalogen molecular species generally contains a highly polyunsaturated fatty acid such as docosahexanoic or arachidonic acid, and the *sn*-3 position is usually an ethanolamine or choline headgroup. Plasmalogens are found in variable concentrations in the various tissues; in human lung homogenates, plasmalogens

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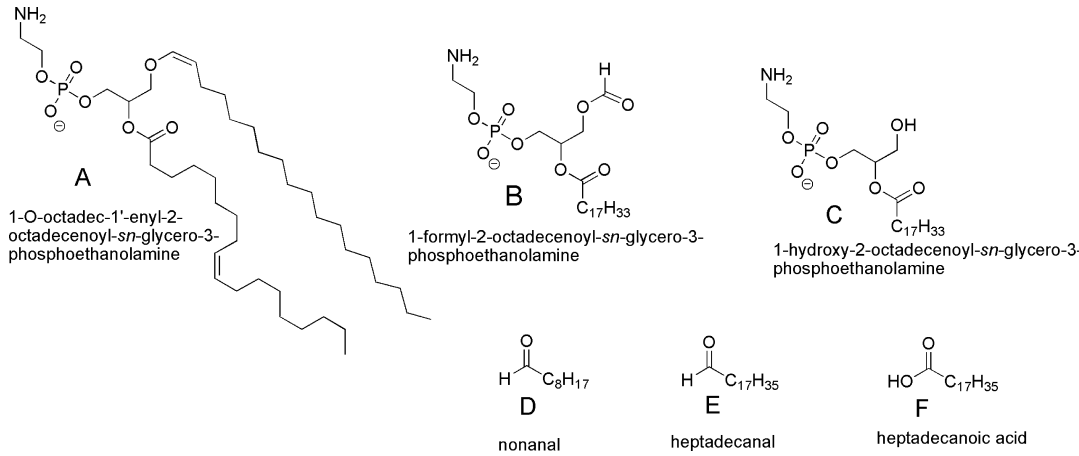

Figure 1. Structure of the ozonolysis products derived from 18:0p/18:1-PE (A), in a vesicular model system exposed to 100 ppb ozone (1 h), yielded five primary products: 1-formyl-2-octadecenoyl-PE (B), 1-hydroxy-2-octadecenoyl-PE (C), nonanal (D), heptadecanal (E), and heptadecanoic acid (F).

are reported to total ~12% of all phospholipids, but the exact location or cell type containing these unique lipids was not investigated (*18*). While the exact function of plasmalogens has not been completely elucidated, they have been suggested to serve a role as endogenous antioxidants within cell membranes and lipoproteins (*19, 20*).

Plasmalogens have also been shown to be present within lung surfactant; however, the exact content of various species has not been previously examined. Choline and ethanolamine plasmalogens have been reported to comprise ~1% of total surfactant phospholipids in rat (*21*). Plasmalogens were also reported to be significantly lower in patients with respiratory distress syndrome; however, the analysis used was unable to distinguish specific lipid classes or fatty acyl chains (*22*). The work described here investigates one of the potential contributions of plasmalogens within lung surfactant and studies their susceptibility to oxidation by levels of ozone that typically occur in environmental settings.

Materials and Methods

1-*O*-octadec-1′-enyl-2-octadecanoyl-*sn*-glycero-3-phosphatidylethanolamine (18:0p/18:1-PE), 1,2-dihexadecanoyl-PC (16:0a/16:0-PC), 1-tetradecanoyl-2-hydroxy-PE (14:0a/OH-PE), 1,2-tetradecanoyl-PE (14:0a/14:0-PE), 1-octadecanoyl-2-hydroxy-PE (18:0a/OH-PE), 1,2-di-(3,7,11,15-tetramethylhexadecanoyl)-PE, 1-octadecanoyl-2-octadecenoyl-PE (18:0a/18:1-PE), and (13,14,15,16-d_9)-hexadecanal (48.9 atom %, deuterated hexadecanal) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). HPLC solvents, formic acid, methanolic HCl, dodecanal, pentafluorobenzyl bromide, and triethylammonium buffer (1 M) were purchased from Sigma Aldrich (Milwaukee, WI). *O*-(2,3,4,5,6-Pentafluororbenzyl) hydroxylamine hydrochloride (99+%) was purchased from Alfa Aesar (Ward Hill, MA). The synthesis of *N*-hydroxy-succinimide-dimethylaminobenzoic acid (DMABA) reagents (deuterium labeled) has been previously described (*23*). Heptadecanoic acid was purchased from Supelco (Bellefonte, PA) and (18-d_3)-octadecanoic acid (99.5 atom %, deuterated stearic acid) was purchased from MSD Isotopes (Montreal, Canada).

Ozonolysis. Ozone, at precise concentrations, was generated using a model 306 Ozone Calibration source (2B Technologies, Boulder, CO), which delivered gas flow into a modified vacuum desiccator, which served as the exposure chamber. Briefly, the source was linked to a Teflon-coated tube, which transferred gas from the generator to the ozonolysis chamber, at a flow rate of 2 L/min. A lab rotator was placed underneath the chamber to allow shaking of samples during ozonolysis experiments (2 revolutions per second). The generator and chamber were given at least 15 min to equilibrate ozone concentrations before any experiments were conducted. The concentration of ozone in delivered gas was confirmed in initial studies using a model 202 Ozone Monitor (2B Technologies) to monitor ozone concentration exiting the chamber.

Vesicle Model System. Small unilamellar vesicles, comprised of 18:0p/18:1-PE and 16:0a/16:0-PC (1:1 mol ratio), were prepared using an extruder (Avanti Polar Lipids), per the manufacturer's instructions. The samples were then sonicated for 30 min. Vesicles were made to a final volume of 1 mL/sample in phosphate-buffered saline and contained no added oxidizing or reducing equivalents. Prior to ozonolysis, vesicles were transferred into a 10 mL beaker, which was placed inside the ozonolysis chamber for all ozone exposures.

Isolation and Ozonolysis of Bronchoalveolar Lavage (BAL). C57Bl/6 mice (3−8 months) were sacrificed by CO_2 asphyxiation, followed by cervical dislocation. BAL was obtained by tracheal instillation of 1 mL of PBS by means of a safelet catheter (18 gauge needle) and was repeated four times. Lavages were pooled and spun at 2000*g* to remove cells. The supernatant was collected and spun at 10000*g* (*24*) to isolate endogenous surfactant as a pellet. The surfactant pellets collected were pooled and resuspended in PBS, and a 1,2-di-(3,7,11,15-tetramethylhexadecanoyl)-PE internal standard was added to ensure equivalent splitting. The entire pool of surfactant was then split to yield three mice per sample. All procedures were approved by the Animal Care and Use Committee of the University of Colorado Denver.

To ascertain the presence of PE and PC plasmalogens within lung surfactant, the surfactant from two C57Bl/6 mice was isolated, extracted, and separated on aminopropyl solid-phase extraction column (Supelco), according to the method of Kim et al. (*25*). The phospholipids were then separated using normal phase high-pressure liquid chromatography, with mass spectrometry detection (NP-HPLC/MS). Negative ion, full mass spectral scans were run, and 1 min fractions were collected. PE and PC lipid classes were pooled separately and infused for positive and negative ion collision-induced dissociation (CID) experiments. Identification of individual PE phospholipids present in lavage was confirmed according the method of Zemski Berry (*26*). Additional verification of plasmalogen phospholipids was done by treating a portion of the sample with methanolic HCl, which hydrolyzes the vinyl ether bond of all plasmalogens within each sample, leaving only diacyl phospholipids to be detected (*27*). Upon identification of all plasmalogen lipids present within surfactant, multiple reaction monitoring (MRM) methods were developed for quantitation of each molecular species.

Ozonolysis (0 and 100 ppb) of surfactant was conducted on pooled surfactant extracts, equivalent to 3 mice per sample. Samples (1 mL) were ozonized or exposed to air (scrubbed of ozone) in a 10 mL beaker that was placed inside of the ozone chamber for 1 h. Samples were then extracted (see Lipid Extractions for the method) and derivatized with either pentafluorbenzyl (PFB) hydroxylamine

(aldehydic compounds) or with dimethylaminobenzoic acid reagents (aminophospholipids).

Lipid Extractions. Following ozonolysis, internal standards (14:0a/14:0-PE, 14:0a/OH-PE, and deuterated hexadecanal) were added to each sample. Aldehydes were extracted by the addition of acetonitrile and hexane (1:0.5:3 buffer/acetonitrile/hexane). The sample was vortexed for 30 s, and the upper hexane layer was removed. The extraction procedure was repeated three times. The remaining aqueous layer, which contained the phospholipids and polar products of ozonolysis, was extracted using a modified Bligh and Dyer extraction, substituting methylene chloride for chloroform (*28*). Extraction was conducted three times per sample, and the organic layer was pooled.

Aldehyde Detection (GC/MS). The aldehydes removed in the hexane extraction were taken to dryness using a stream of nitrogen and then were derivatized to a PFB-oxime, using pentafluorbenzyl hydroxylamine HCl, according to the method of Luo et al. (*29*). The samples were analyzed by capillary column gas chromatography-negative ion electron capture ionization mass spectrometry (GC-NI-ECMS). GC-MS experiments were carried out using a 30 m (30 m × 0.2 mm i.d. × 0.25 mm film thickness) ZB-1 polydimethysiloxane capillary gas chromatograph column (Phenomenex, Torrance, CA) attached to a ThermoFinnigan (San Jose, CA) Trace DSQ mass spectrometer. Helium was used as the carrier gas, at a flow rate of 1.5 mL/min, and methane was used as the collision gas; samples were injected by splitless injection, with an injection port at 230 °C. The column temperature was initially set at 70 °C for 2 min, then increased by 10 °C/min until 310 °C, and then held at 310 °C for 6 min. The source parameters were as follows: temperature, 200 °C; and electron energy, 70 eV. A specific ion for each derivative was selected, corresponding to the loss of $[M - HF]^-$ or $[M - HFNO]^-$ for selected ion monitoring (SIM), with a dwell time of 100 ms per mass. Standard curves were made using dodecanal as a reference standard and an internal standard of deuterium-labeled hexadecanal. The linear regression generated was directly applied to the signal for heptadecanal and nonanal relative to the added internal standard.

Fatty Acid Extraction. Separate small unilamellar vesicles were used for fatty acid analysis. Following vesicular air or ozone exposure, deuterated stearic acid was added as an internal standard. Fatty acids were extracted from buffer by the addition of ethyl acetate (2:1 ethyl acetate/buffer). The sample was vortexed for 10 s, and the upper ethyl acetate was removed. The extraction procedure was repeated three times.

Fatty Acid Analysis. The fatty acids extracted were derivatized to a PFB-ester using pentafluorbenzyl bromide (PFB-Br), according to the method of Hadeley et al. (*30*). A specific ion for each derivative was selected, corresponding to the loss of $[M - PFB]^-$ for SIM, with a dwell time of 100 ms per mass. Standard curves were made using heptadecanoic acid as a reference standards and internal standard deuterated stearic acid. The linear regression generated was directly applied to the signal for heptadecanoic acid relative to the added internal standard.

Ozonolysis Phospholipid Product Analysis. Phospholipid samples extracted in methylene chloride layer were taken to dryness under a stream of nitrogen and were separated using NP-LC/MS (electrospray ionization), according to the method of Zemski Berry (*26*), on a 150 mm × 2.0 mm Ultremex 3 Silica column (Phenomenex). Products of ozonolysis were identified by their molecular ions, CID of both the positive and negative molecular ion, and HPLC retention time.

The synthesis of 1-formyl-2-octadecenoyl-PE was carried out according to previously published methods (*31*) with modifications for the formylation of free hydroxyl, rather than acetylation. Briefly, 1-hydroxy-2-octadecenoyl-PE (100 μg) was suspended in formic acid (250 μL). The solution was stirred overnight, at room temperature, and then extracted as detailed above (modified Bligh and Dyer). The product was characterized by positive and negative ion CID mass spectrometry (yielding CID spectra identical to Figure 3C,D) experiments, with an approximate yield of 40%.

Quantitation of all phospholipids was carried out using RP-LC/MS/MS, on a 150 mm × 2.0 mm C_{18} (5 μm) column (Phenomenex) and API QTRAP tandem quadruple mass spectrometer (PE Sciex, Toronto, Canada). The HPLC was operated at a flow rate of 0.2 mL/min with a mobile phase of methanol/acetonitrile/ water 1 mM aqueous ammonium acetate (6:2:2) (solvent A) and methanol made to 1 mM ammonium acetate (solvent B). The gradient was run from 10 to 99% (solvent B) over 15 min, held at 99% for 15 min, and then returned to 10% for re-equilibration. MRM was carried out in negative ion mode with an electrospray voltage of −4000 V, a declustering potential of −80 V, a collision energy of −40 V, a collision gas of nitrogen, and a dwell time of 100 ms per mass. Masses monitored included *m/z* 728.7→281.1 (18:0p/18:1-PE), 478.3→281.1 (1-hydroxy-2-octadecenoyl-PE), 506.4→281.1 (1-formyl-2-octadecenoyl-PE), 634.3→227.2 (14:0a/14:0-PE), 424.3→227.2 (14:0a/OH-PE), 618.4→171.1 [1-*O*′-octadec-1′-enyl-2-nonanal-PE (18:0p/9al-PE)], 368.2→171.1 (1-hydroxy-2-nonanal-PE), and 396.2→171.1 (1-formyl-2-nonanal-PE).

The 18:0p/18:1-PE and 16:0a/16:0-PC, using each as their own reference standard and using 14:0a/14:0-PE as the internal standard, were used to generate standard curves. Lysophospholipids were quantitated using the standard curve generated from 18:0a/OH-PE as a reference standard and 14:0a/OH-PE as an internal standard. For quantitation of the formyl products, synthetic 1-formyl-2-octadecanoyl-PE was used as a reference standard as described above for 14:0a/OH-PE as an internal standard.

DMABA Derivatization. Deuterium-labeled DMABA reagents were synthesized as previously described, and derivatization methods were followed according to the method of Zemski Berry (*23*). A solution of d_4-DMABA reagent for control and d_{10}-DMABA for ozone samples was added to each sample for aminophospholipid derivatization. After derivatization, re-extraction was conducted using the modified Bligh and Dyer described above to remove excess reagent. A detailed work flow diagram has been included in the Supporting Information to schematically indicate steps used to differentially label experimental samples. Quantitation of DMABA-labeled phospholipids was achieved by creating standard curves of d_{10}-DMABA 18:0p/18:1-PE, d_{10}-DMABA 18:0a/18:1-PE, and d_{10}-DMABA 18:0a/OH-PE as reference standards and d_{10}-DMABA 14:0a/14:0a-PE and d_{10}-DMABA 14:0a/OH-PE as internal standards.

Statistics. Data are expressed as means ± SEMs. Individual comparisons between groups (ozone-treated versus control) were confirmed by the two-tailed Student's *t* test. Reported differences were calculated using GraphPad Prism 4 (La Jolla, CA), and a p value of less than 0.05 was considered to be statistically significant.

Results

Ozone Oxidation of Plasmalogen PE. A unilamellar vesicle system of defined phospholipid content was first examined to evaluate plasmalogen oxidation using low concentrations of ozone. The plasmalogen PE molecular species studied (18:0p/18:1-PE, Figure 1A) was not the one found in murine surfactant (see below) but did contain two different unsaturated, a vinyl ether moiety connecting the *sn*-1 radyl chain to the glycerophospholipid backbone as well as an ω-9 olefinic bond of the octadecenoyl chain. The progress of ozonolysis was followed by product formation and loss of substrate. We hypothesized that the vinyl ether bond of plasmalogens, in an ordered structure such as a vesicle, would be preferentially oxidized at low concentrations of ozone, when compared to fatty acyl chain olefinic double bonds. Therefore, small unilamellar vesicles comprised of 18:0p/18:1-PE and 16:0a/16:0-PC, in a mole ratio of ∼1:1, were made in PBS and exposed for 1 h to ozone at 0, 50, 100, 300, or 1000 ppb.

Concentration-dependent oxidation was observed by monitoring a decrease in 18:0p/18:1-PE content (Figure 2A). A loss of approximately 19% 18:0p/18:1-PE was observed at 50 ppb, 31% at 100 ppb, 36% at 300 ppb, and 52% at 1000 ppb ozone. The

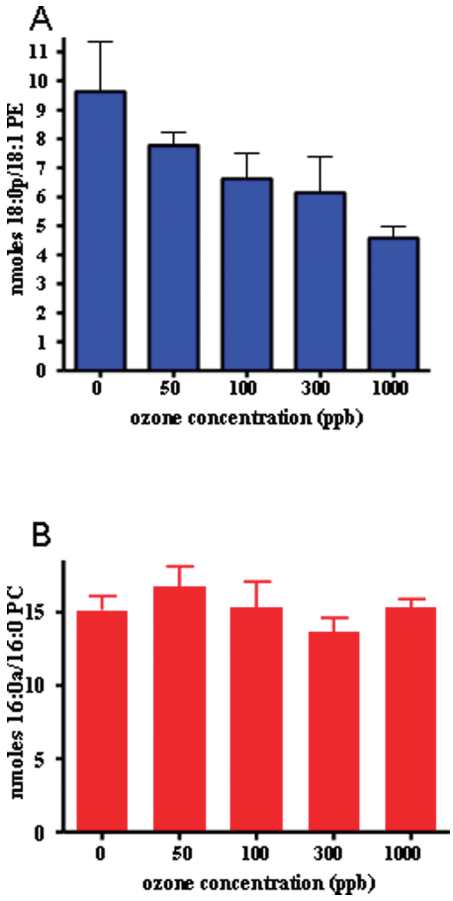

Figure 2. Ozone concentration effect: Small unilamellar vesicles comprised of 18:0p/18:1-PE and 16:0a/16:0-PC were ozonized for 1 h, at 0 (air scrubbed of ozone), 50, 100, 300, and 1000 ppb. (A) Loss of 18:0p/18:1-PE as measured by quantitative LC/MRM analysis. (B) Quantity of 16:0a/16:0-PC in the same vesicles. Results are reported as means ± SEMs ($n = \geq 3$).

quantity of 16:0a/16:0-PC present in these vesicles did not change significantly (Figure 2B). To begin identification of ozonolysis products, control and ozonized total ion chromatograms (of NP-LC/MS) were overlaid, allowing changes in ion abundances to be compared (data not shown). Areas of increased ion abundances were noted for identification of products during infusion of fractions collected from NP-LC/MS. The products generated were identified using positive and negative ion CID experiments, and the alkyl aldehydic products and fatty acids generated were derivatized and quantitated by GC/MS. The main products formed included 1-formyl-2-octadecenoyl-PE (Figure 1B), 1-hydroxy-2-octadecenoyl-PE (Figure 1C), nonanal (Figure 1D), heptadecanal (Figure 1E), and heptadecanoic acid (Figure 1F). The formation of the 1-formyl-2-octadecenoyl-PE (Figure 1B) and aldehydes (Figures 1C and 1D) follows typical Criegee ozonolysis mechanisms. The production of the 1-hydroxy-2-octadecenoyl-PE (Figure 1C) is proposed to occur by loss of performic acid from the hydroxyhydroperoxy intermediate, on carbon-1 of the vinyl ether group.

Structural characterization of phospholipid products was based upon product ions generated by CID experiments of observed molecular ions, as well as HPLC retention time. Characterization of the products in positive ion CID demonstrated that both the 1-formyl-2-octadecenoyl-PE and the 1-hydroxy-2-octadecenoyl-PE fragmented to lose the phosphoethanolamine headgroup (Figure 3B,D, [M + H − 141]) (*30*). Negative ion CID yielded *m/z* 281 as the primary fragment, indicative of an intact oleic acid in the *sn*-2 position of the backbone (Figure 3A,C). Further confirmation of identity was achieved by comparing retention times of the commercially available standard, 1-hydroxy-2-octadecenoyl-PE, as well as synthetic 1-formyl-2-octadecenoyl-PE to the ozonolysis products (data not shown).

Two major phospholipid products were found to be produced in the vesicular model system at 100 ppb, 1-formyl-2-octadecenoyl-PE (Figure 4A) and 1-hydroxy-2-octadecenoyl-PE (Figure 4B). This was consistent with the hypothesis of the vinyl ether bond having a unique susceptibility to low concentrations of ozone. At higher concentrations of ozone, these products were still produced; however, further oxidation of the acyl olefinic bond within these products was found, yielding the 1-hydroxy-2-nonanal-PE and 1-formyl-2-nonanal-PE (Supporting Information, Figure 1A,B). The 1-formyl-2-nonanal-PE did not display significant production above control until 300 and 1000 ppb. The 1-hydroxy-2-nonanal-PE increased at 100 and 300 ppb, with the highest levels reached at 1000 ppb.

Fatty aldehydes measured by GC/MS (Figure 5A) included the nonanal, which was likely produced by oxidation of the (ω-9) olefinic double bond in the oleoyl fatty acyl chain, and heptadecanal, produced by oxidation of the vinyl ether moiety of the plasmalogen. Aldehyde formation followed the same trend as the phospholipid products recovered. The heptadecanal was a prominent product at low concentrations of ozone when compared to the nonanal, again consistent with preferential oxidation of the vinyl ether moiety of the plasmalogen to low concentration of ozone. Higher concentrations of ozone displayed oxidation of both olefinic double bond in the oleoyl fatty acyl chain as well as the vinyl ether moiety.

The heptadecanoic acid was also measured by GC/MS as the PFB-ester (Figure 5B). Heptadecanoic acid, produced by oxidation of the heptadecanal, displayed a concentration-dependent production at each concentration measured. Nonanoic acid, a potential product of nonanal hydroxyhydroperoxide oxidation, was observed at all four concentrations of ozone exposure employed; however, it was also abundant in control and procedural blanks at very high levels; therefore, absolute quantitation was not carried out.

Presence of Plasmalogens in Murine Lung Surfactant. To identify the plasmalogen lipid species present within lung surfactant, BALs were taken, and surfactant was isolated according to the method of Oulton et al. (*24*) as described in the Materials and Methods. Because plasmalogen species are most commonly found as PE or PC phospholipids, identification of plasmalogens within lung surfactant focused on these two lipid classes. The PE and PC lipids were separated from neutral and anionic lipids using the aminopropyl solid-phase extraction column (*25*). The collected eluant containing PE and PC was then separated on NP-LC/MS/MS, and fractions were collected for direct infusion and collisional activation of both positive and negative molecular ions.

Infusion of the PE fraction (Figure 6A) showed numerous molecular species ($[M - H]^-$). The most abundant lipid species (*m/z* 722) was identified as 1-*O*-hexadec-1′-enyl-2-eicosatetraenoyl-PE (16:0p/20:4-PE), as confirmed by CID experiments (Figure 6B,C). As shown, in positive ion CID (Figure 6B), abundant fragmentation to *m/z* 361 and 364, corresponds to the *sn*-2 and *sn*-1 fragments. Fragmentation of PE plasmalogens to these specific fragments has been studied in great detail by Zemski Berry; the production of the *sn*-1 and *sn*-2 fragments was proposed to occur by a complex series of molecular rearrangements upon CID (*26*). In negative ion CID, the arachidonate carboxylate anion (*m/z* 303) was the most prominent fragment ion (Figure 6C), followed by loss of the

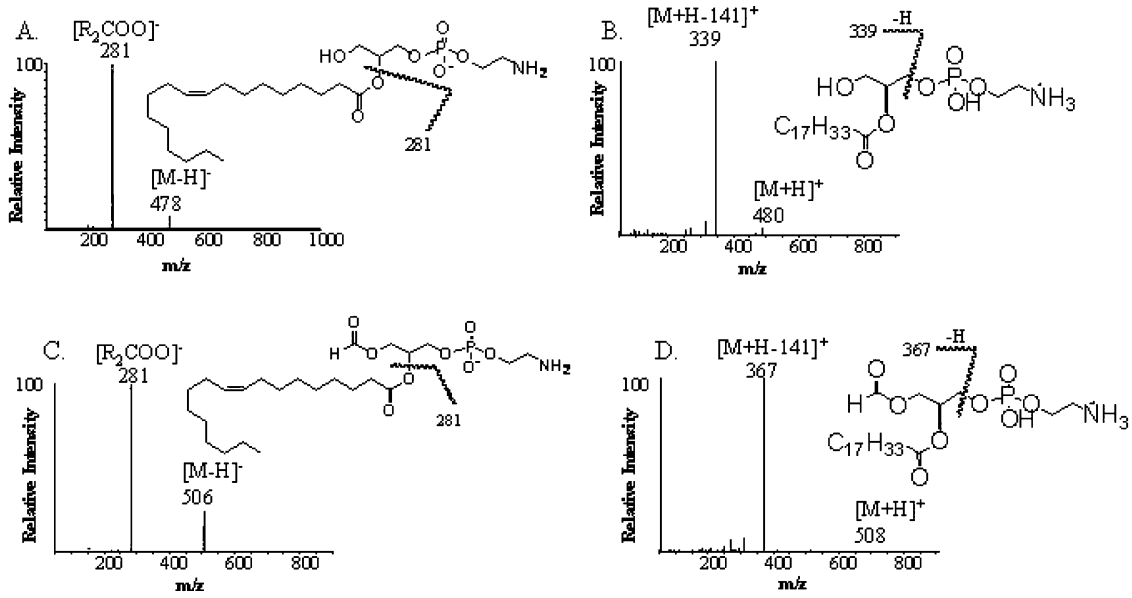

Figure 3. Characterization of phospholipid products produced by exposure of small unilamellar vesicles to 100 ppb ozone: Products of ozonolysis were extracted and separated using NP-LC/MS. Fractions were infused for positive and negative ion CID mass spectrometric experiments. Major products found are as follows: (A) 1-hydroxy-2-octadecenoyl-PE (negative ion mode), (B) 1-hydroxy-2-octadecenoyl-PE (positive ion mode), (C) 1-formyl-2-octadecenoyl-PE (negative ion mode), and (D) 1-formyl-2-octadecenoyl-PE (positive ion mode).

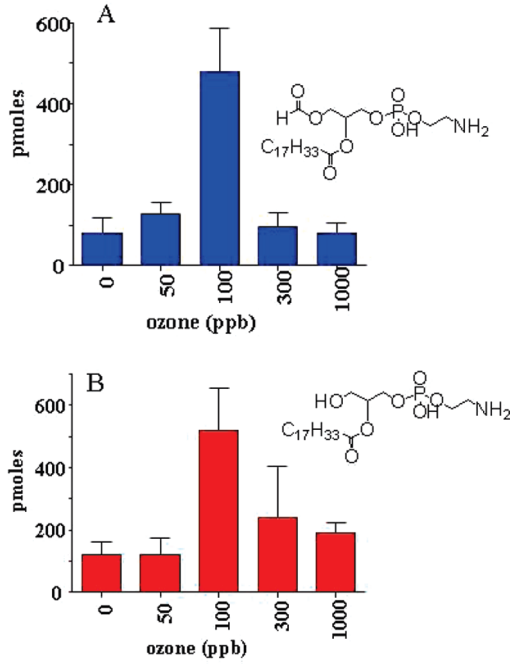

Figure 4. Phospholipid products generated by ozone exposure (0, 50, 100, 300, and 1000 ppb) of 18:0p/18:1-PE. Products were measured using LC/MRM analysis. (A) 1-Formyl-2-octadecenoyl-PE (blue, *m/z* 506→281) and (B) 1-hydroxy-2-octadecenoyl-PE (red, *m/z* 478→281). Quantitation was based on standard curves. Results are expressed as means ± SEMs ($n = \geq 3$).

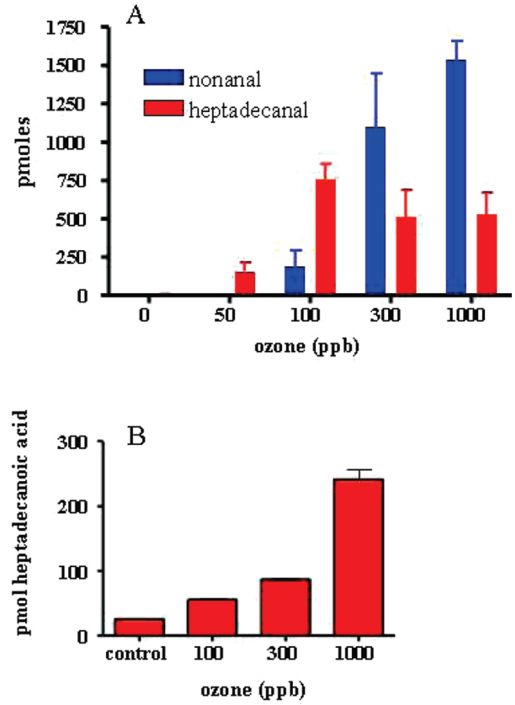

Figure 5. Concentration response of aldehydes produced by exposure of small unilamellar vesicles to 0 (air control), 50, 100, 300, and 1000 ppb ozone for 1 h. (A) Aldehydes were extracted and derivatized to the PFB-oxime; GC-MS was used for quantitation of aldehydes, monitoring the $[M - HFNO]^-$ ion. Heptadecanal (red) was monitored at *m/z* 399, and nonanal (blue) was monitored at *m/z* 287. (B) Heptadecanoic acid produced by exposure of small unilamellar vesicles to 0 (air control), 100, 300, and 1000 ppb ozone for 1 h. Fatty acids were extracted and derivatized to the PFB-ester; GC-MS was used for quantitation of the heptadecanoic acid, monitoring the carboxylate anion $[RCOO]^-$ at *m/z* 269. All results are expressed as means ± SEMs ($n = 3$).

arachidonate as a neutral ketene (*m/z* 436). Other plasmalogens species found included 1-*O*-hexadec-1′-enyl-2-docosahexaenoyl-PE (16:0p/22:6-PE), 1-*O*-hexadec-1′-enyl-2-octadecenoyl-PE (16:0p/18:1-PE), 1-*O*-hexadec-1′-enyl-2-hexadecenoyl-PE (16:0p/16:1-PE), 1-*O*-hexadec-1′-enyl-2-eicosapentaenoyl-PE (16:0p/22:5-PE), 1-*O*-hexadec-1′-enyl-2-eicosatetraenoyl-PE (16:0p/22:4-PE), and 1-*O*-octadec-1′-enyl-2-eicosatetraenoyl-PE (18:0p/20:4-PE). Diacyl species were also identified and confirmed using CID (Table 1), with the most abundant species confirmed as 1-hexadecanoyl-2-octadecadienoyl-PE (16:0a/18:2-PE) and 1-hexadecanoyl-2-octadecenoyl-PE (16:0a/18:1-PE). The 15 most abundant molecular species (accounting for >95% of all PE phospholipids identified) is presented in Table 1. The 18:0p/18:1-PE molecular species used in the vesicular model was not found in the mouse lung surfactant. In total, plasmalogens encompassed over ~38% of the total PE phospholipids present;

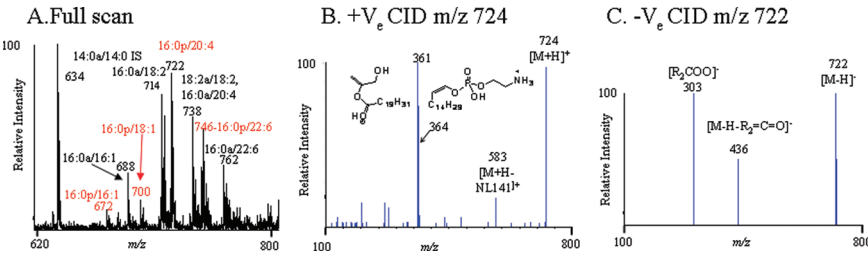

Figure 6. Identification of PE phospholipid molecular species by mass spectrometry. Isolated surfactant from two mice was pooled and separated on an aminopropyl solid-phase extraction column (see the Materials and Methods for conditions). The collected eluant was additionally separated using NP-LC/MS, fractions were collected, and all PE phospholipids were pooled for individual lipid species identification. (A) Pooled PE phospholipids were infused. Shown is a negative ion full mass spectrum from *m/z* 620 to 800. Plasmalogen species are indicated in red text. (B) Positive ion CID of *m/z* 724 (16:0p/20:4-PE) yielded abundant fragment ions at *m/z* 361 and *m/z* 364, corresponding to the *sn*-1 and *sn*-2 fragments indicative of plasmalogens. A fragment corresponding to the NL 141 at *m/z* 583 was also observed. (C) Negative ion CID of *m/z* 722 (16:0p/20:4-PE) confirmed *sn*-2 arachidonate carboxylate anion (*m/z* 303) as the most prominent fragment, as well as a neutral loss as a ketene (*m/z* 436).

Table 1. Listed Are the 15 Most Abundant Lipid Species, Which Were Monitored by LC/MRM Analysis, in Negative Ion Mode[a]

PE lipid species	MRM transistion used (*m/z*)	mol % total PE phospholipids
16:0p/16:1	672→253	1.9
16:0a/16:1	688→253	5.9
16:0p/18:1	700→281	2.5
16:0a/18:2	714→279	20.4
16:0a/18:1	716→281	12.4
16:0p/20:4	722→303	23.8
16:0a/20:4	740→303	1.8
18:0a/18:1	744→281	1.4
16:0p/22:6	746→327	1.7
16:0p/22:5	748→329	2.3
16:0e/22:6	748→327	1.3
16:0p/22:4	750→331	2.0
18:0p/20:4	750→303	4.7
16:0a/22:6	762→327	10.5
18:0a/20:4	766→303	2.6

[a] MRM transitions used for identification are listed in column two, and percent contribution of each species to total PE phospholipids is listed in column three. All identifications were confirmed using both positive and negative ion CID experiments. Results are expressed as means ± SEMs ($n = 3$).

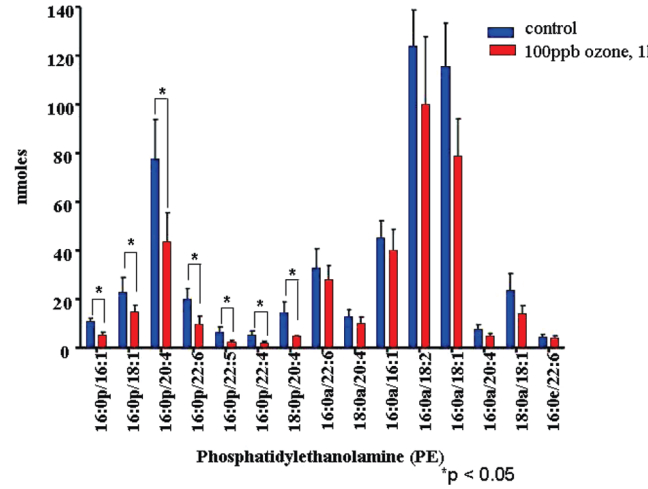

Figure 7. PE molecular species in murine lung surfactant and changes caused by exposure to 100 ppb ozone for 1 h. Control samples were derivatized with d_4-dimethylaminobenzoic acid (DMABA), and ozonized samples were derivatized with d_{10}-DMABA. Samples were then re-extracted, pooled together, separated from PC using NP-LC/MS, collected, and quantitated using RP-LC/MRM. Shown in blue are control data, and shown in red are ozone data. Plasmalogens are shown on the left side, and diacyl phospholipids are shown on the right. Data are expressed as means ± SEMs ($n = 3$). *Significant oxidation as compared to control values ($p < 0.05$).

on the basis of the published composition of each lipid species contribution (*24*) to total surfactant composition, plasmalogens comprise approximately 1−2% of all of the lipid species. Analysis of the PC fractions revealed no plasmalogen species to be present (data not shown). The surfactant from male and female mice was also compared and revealed no significant differences in PE species content (data not shown).

Ozonolysis of Surfactant. Finding that plasmalogens comprised ∼38% of all murine surfactant PE lipid species, it was of interest to monitor the effect of low concentrations of ozone on lipid species present in this more complex system. The surfactant was extracted and pooled according to the methods described in the Materials and Methods. Samples were individually exposed to air (scrubbed of ozone) or ozone (100 ppb) for 1 h, and a differential display method was used to label control versus experimentally treated samples (see the Supporting Information, Scheme 1). DMABA labeled with four deuterium atoms was used for control samples, while a label with 10 deuterium atoms was used to tag ozonolysis samples. This allowed for a combination of samples to decrease inter-run variability and increase the mass of each phospholipid monitored by 151 and 157 mass units, respectively. The conversion of the ion abundance ratios of labeled PE species to the internal standard was used with a standard curve for quantitation.

When the d_4/d_{10} ratios were measured for 100 ppb ozone, the only PE components that revealed significant oxidation were plasmalogen species (Figure 7). Plasmalogens were found to decrease on average ∼53%, whereas diacyl molecular species decreased, however, not significantly as compared to control values. The oxidation of the 16:0p/20:4-PE, the most abundant plasmalogen, was reduced ∼56% following exposure to 100 ppb ozone. In comparison, 16:0a/18:2-PE, the most abundant diacyl species, did not significantly decrease even though it contained two double bonds.

Lyso and formyl products were also measured in response to 100 ppb ozone (Figure 8A). Interestingly, significant increases in the 1-hydroxy-2-eicosatetraenoyl-PE (20:4a/OH-PE) and 1-hexadecanoyl-2-hydroxy-PE (16:0a/OH-PE) were observed. The 16:0a/OH-PE was not an expected product because no plasmalogens were identified that contained a saturated *sn*-2 with palmitate; therefore, further experimentation was conducted to confirm the regiochemistry (*sn*-1 versus *sn*-2) of the lysophospholipids formed. Chromatographically, lysophospholipids can be separated by regiochemistry, using the reverse phase system described in the Materials and Methods (*32*). The DMABA-labeled lysophospholipids, however, will not separate in this manner. Therefore, additional samples were exposed to control air or 100 ppb of ozone, not labeled using DMABA, and run by RP-LC/MRM to monitor regiochemistry of the increased

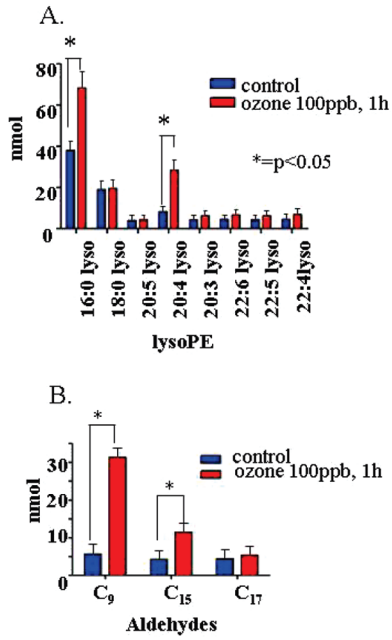

Figure 8. Lysophospholipid molecular species and aldehydes generated from murine surfactant exposure to 100 ppb ozone for 1 h. (A) Control samples were derivatized with d_4-DMABA, and ozonized samples were derivatized with d_{10}-DMABA. Samples were then re-extracted, pooled together, separated from PC using NP-LC/MS, collected, and quantitated using RP-LC/MRM. The appearance of lysophospholipids was measured in control vs ozone samples. (B) Appearance of aldehydes generated during ozonolysis. Three aldehydes were found as measured by GC-MS as the PFB-oxime. Significant increases were found in two of the three aldehydes measured. Data are expressed as means ± SEMs ($n = 3$). *Significant oxidation ($p < 0.05$).

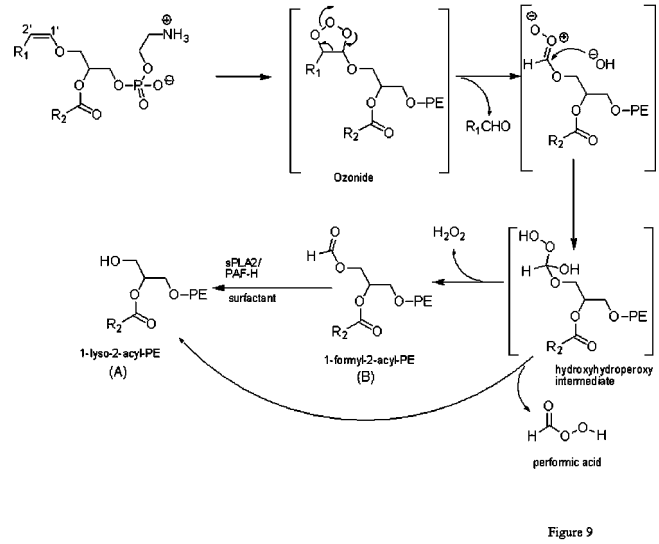

Figure 9. Proposed mechanism of formation of plasmalogen PE ozonolysis products.

lysophospholipid products formed by ozone oxidation. As shown in the Supporting Information, Figure 2, the increase in 20:4a/OH-PE was predominantly *sn*-1 lyso, whereas the increase in 16:0a/OH-PE was *sn*-2 lyso (*32*). This supported increased arachidonyl lysophospholipid, resulting from oxidation of the vinyl ether moiety, whereas the 16:0a/OH-PE was likely a result from the removal of a *sn*-2 fatty acid by secretory phospholipases A_2 ($sPLA_2$) or a PAF-acetylhydrolase, known enzymatic components of surfactant (*33*−*35*). Other lysophospholipid compounds measured showed only negligible production. The formation of formyl products was also measured; however, no increase in the formation of product was observed for ozonized surfactant, as compared to controls, and standard deviations of data were high (data not shown).

Aldehydic products were also measured in this experiment of control (air) and ozone-treated (100 ppb) surfactant (Figure 8B). Aldehydes measured included nonanal (C_9), a product of oleoyl fatty acyl chain oxidation, pentadecanal (C_{15}) and heptadecanal (C_{17}), products of oxidizing a 1-*O*′-hexadecyl-1′-enyl or 1-*O*′-octadecyl-1′-enyl bond within plasmalogens. The nonanal and pentadecanal both showed significant increases (25 and 7 nmol, respectively), in ozonized samples, as compared to their control. The heptadecanal did not increase significantly, an expected result considering there was only one 1-*O*-octadecy-1′-enyl containing plasmalogen found within the surfactant (18:0p/20:4-PE).

Discussion

In this study, a small unilamellar vesicle system (lipid bilayer system) of known composition was used to evaluate the reactivity of ozone. This vesicle system contained a plasmalogen PE that had a vinyl ether bond, as well as an olefinic bond in the *sn*-2 fatty acyl group distant from the glycerol backbone. Products of ozonolysis were characterized using liquid and gas chromatography with sensitive mass spectrometric techniques. When low concentrations of ozone were exposed to these vesicles, the major products observed were 1-hydroxy-2-octadecenoyl-PE, 1-formyl-2-octadecenoyl-PE, and heptadecanal, consistent with preferential attack of the vinyl ether moiety by ozone. Attack at the fatty acyl double bond yielded nonanal as a product, which increased in abundance as the concentration of ozone increased above 100 ppb.

Previous studies have shown that ozonolysis of short chain vinyl ethers, such as ethyl 1-propenyl ether, has higher reaction rate constants for reaction with ozone, as compared to olefinic double bonds in compounds such as methyl methacrylate (*36, 37*). Other published studies showed that ozonolysis of alkenes, in which the double bond was connected to an electron-donating group, reacted many times faster than typical alkenes and those connected to electron-withdrawing groups (*38*). Phospholipid products of ozonolysis identified within this vesicular plasmalogen model exhibited the same unique susceptibility of the vinyl ether bond; additionally, this effect was specific to low concentrations of ozone (e.g., 100 ppb), suggesting that when plasmalogens were present in lipid bilayers, they were very likely initial targets of reactions with ozone at the lipid interface.

The phospholipid products (1-formyl-2-octadecenoyl-PE and 1-hydroxy-2-octadecenoyl-PE) could both arise from an initial ozonide formed at the vinyl ether moiety 18:0p/18:1-PE and decomposition by the Criegee mechanism (*39*), giving rise to an aldehydic product and an unstable hydroxyhydroperoxy intermediate (Figure 9). When the aldehydic product is formed at the 2′-carbon atom of the vinyl ether, the product would be heptadecanal, while the hydroxyhydroperoxy product (shown as an intermediate in brackets in Figure 9) would be a rather unique ortho perracid intermediate, which would be expected to be correspondingly unstable. The lyso PE product (A) would directly result from decomposition of this intermediate through the loss of performic acid. The loss of hydrogen peroxide from this intermediate would lead to the formation of the 1-formyl-PE product (B). This same product (B) would be formed if the initial aldehydic product formed was at the 1′ carbon atom of the vinyl ether in the initial ozonide. The nonanal would arise from an ozonide formed at the ω-9 double bond of the fatty

acyl group at *sn*-2, from starting material or subsequent phospholipid products above.

The analysis of surfactant was then carried out because of it being a likely target for ozonolysis reactions in the lung. Detailed analysis of murine surfactant phospholipid molecular species revealed a complex mixture of molecular species. The most abundant phospholipids were the expected PC molecular species 1-hexadecanoyl-2-hexadecenoyl-PC, 1-hexadecanoyl-2-octadecenoyl-PC, and 1-hexadecanoyl-2-octadecadienoyl-PC as previously reported (*40*). Major PE molecular species in mouse lung surfactant contained unsaturated and polyunsaturated fatty acyl groups esterified to the *sn*-2 glycerol backbone, as well as plasmalogen PE species. Interestingly, no saturated PE molecular species were detected in contrast to the major PC species observed in lung surfactant. It was found that plasmalogen PE accounted for 38% of all PE phospholipid species in the surfactant isolated from C57/BL6 mice, with the major plasmalogen molecular species containing a polyunsaturated fatty acid at *sn*-2. Only two plasmalogen PE molecular species were found that contained an acyl group with a single double bond.

Because surfactant was a considerably more complex mixture of lipid molecules than the vesicle system, the strategy of using a differential display of PE molecular species was used to monitor the formation of new products, as well as the decrease in the PE molecular species when murine surfactant was exposed to ozone. Lysophospholipid molecular species were found to be a primary ozonolysis product; however, the corresponding formylated products observed in the small unilamellar vesicles were not found as major components. An important difference between the model vesicle system and the ozone exposed surfactant system was likely the fact that the isolated lung surfactant still could contain many active proteins and enzymes. Any 1-formyl products could be a substrate for enzymes such as PAF-acetylhydrolase or $sPLA_2$ (Figure 9), enzymes known to be present in lung surfactant and have broad specificity for the hydrolysis of short chain acyl groups on both PE and PC (*41*−*43*).

Overall, the mass balance between oxidation of the most abundant plasmalogen (16:0p/20:4-PE, 38 nmol oxidized) and the production of PE lysophospholipids, predominately 1-lyso-2-arachidonoyl-PE (20 nmol), revealed that some products had not been accounted for in this analysis. It was hypothesized that the amount of 1-lyso-2-arachidonoyl-PE initially formed was actually higher than what was eventually measured and that this lysophospholipid was simply being further oxidized by ozone at one or more of the double bonds of the polyunsaturated fatty acyl group at *sn*-2. A shortened time course of ozone exposure of 30 min was carried out and found that indeed higher levels of this 1-lyso-2-arachidonoyl-PE were present (data not shown).

While loss of either diacyl or plasmalogen phospholipids may cause changes in the efficiency of the surfactant barrier in the lung in terms of stability and protection of the underlying epithelial layer from reactive oxygen species, the observed susceptibility of plasmalogen PE to ozone corresponded to only 1−2% of total surfactant phospholipids, making such an effect of loss of phospholipids minimal. Rudiger et al. (*44*) show that as little as 2 mol % of added plasmalogens effectively reduced the surface tension of surfactant like phospholipid mixtures. Previous studies have suggested that PE contributes only 3% to total lung surfactant phospholipids at most (*24*). It has been found that even minor increases in the percentage of plasmalogen phospholipids and polyunsaturated fatty acyl groups in pulmonary surfactant systems were protective against the development of bronchopulmonary dysplasia in preterm infants, an effect attributed to protection against oxygen toxicity (*45*). Therefore, it is possible that even small changes in the low concentrations of plasmalogen PE by ozonolysis may have implications on normal respiration.

Perhaps of greater importance is the potential pulmonary toxicity of lysophospholipid products generated during ozone exposure to surfactant. These molecules are known to have biological activities. For example, lysophosphoethanolamine has been shown to play a role in neutrophil calcium flux through G2A receptor (*46*). It has also been reported to have growth factor-regulating effects of growth plate chondrocytes through activation of TGF-β 1 (*47*) and recently has been shown to have antiapoptotic effects on PC-12 cells (*48*). Lysophospholipids in general are also substrates for metabolism to other biologically active lipids such as lysophosphatidic acid. In the lung, lysophosphatidic acid has been shown to be involved in numerous biological events such as pulmonary fibrosis, enhancement of epithelial cell barrier integrity, and stimulation of cytokine secretion (*49*−*51*).

The production of aldehydes may also have biochemical implications both for lipids and proteins within the surfactant as well as underlying cells, since these lipophilic molecules are readily diffusible across lipid membranes. Adduction to proteins can cause decreased activities and, in some cases, complete alteration of protein properties. Nonanal, one of the aldehydes detected in both surfactant and our small unilamellar vesicle system, has been shown to adduct human plasma apolipoprotein A1 (*52*) as well as adduct the heme group of cytochrome P4502B4 (*53*). The reactivity of aldehydes with aminophospholipids, such as phosphatidylethanolamine, has also been shown to lead to inhibition of secreted phospholipase A_2 and phospholipase D (*54*).

It is of interest to consider the low concentration of plasmalogen PE (approximately 1% total lipid) that is present in surfactant and the much higher concentration of other phospholipids containing one or more double bonds in their fatty acyl groups. How can, therefore, plasmalogen PE phospholipids be targets of ozone since this reactive oxygen allotrope would be consumed by olefinic bonds in the fatty acyl chains. However, such considerations do not take into account the fact that *in vivo*, the epithelial lining fluid (surfactant) or even cell membranes do not present molecules in a homogeneous solution but rather in ordered systems into which ozone can diffuse or even react with lipids at the air−liquid interface. These experiments with surfactant preparations exposed to low concentrations of ozone suggest that even though plasmalogen PE accounts for only a small percentage of total unsaturated lipids in surfactant, they can be unique targets even when other fatty acyl groups containing one or more double bonds may be more abundant.

Conclusion

The mechanisms by which oxidants such as ozone initiate and propagate cellular and tissue toxicity are a poorly understood phenomenon. An alternate function of surfactant, other than lowering surface tension, can be seen as a defensive shield for the underlying epithelial cell layer. Reactions of ozone within the surfactant layer are likely to be the source of many of the biochemical products that mediate cellular responses to the inhalation of ozone. The study was conducted to investigate the reaction of ozone with plasmalogen glycerophospholipids, lipids found to be a component of surfactant, and to discover oxidation products that may be formed when low concentrations

of ozone typically encountered in the environment are present. Plasmalogens were found to encompass ~38% of total PE lipids within surfactant, and the vinyl ether bond was shown to react with ozone. Analysis of PE phospholipid products generated in this experiment revealed a significant increase in lysophospholipids, which are known to induce an array of biological activities (*46*−*48*). The oxidation of plasmalogens may therefore be a potential source of secondary bioactive lipids, produced by low concentrations of ozone found in the environment.

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Supporting Information Available: Figure of ozone concentrations of 1-hydroxy-2-nonanal-PE and 1-formyl-2-nonanal-PE, figure of chromatographic separation of increasing lysophospholipids found after ozonolysis of lung surfactant, and work flow diagram to schematically indicate steps used to differentially label experimental samples. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Ciencewicki, J., Trivedi, S., and Kleeberger, S. R. (2008) Oxidants and the pathogenesis of lung diseases. *J. Allergy Clin. Immunol. 122*, 456–468.
(2) Finlayson-Pitts, B. J., and Pitts, J. N. (2000) *Chemistry of the Upper and Lower Atmosphere*, Academic Press, San Diego, CA.
(3) Tong, D. Q., Yu, S., and Kan, H. (2009) Ozone exposure and mortality. *N. Engl. J. Med. 360*, 2788–2789.
(4) Michalec, L., Choudhury, B. K., Postlethwait, E., Wild, J. S., Alam, R., Lett-Brown, M., and Sur, S. (2002) CCL7 and CXCL10 orchestrate oxidative stress-induced neutrophilic lung inflammation. *J. Immunol. 168*, 846–852.
(5) Bernard, A., Carbonnelle, S., Nickmilder, M., and de Burbure, C. (2005) Non-invasive biomarkers of pulmonary damage and inflammation: Application to children exposed to ozone and trichloramine. *Toxicol. Appl. Pharmacol. 206*, 185–190.
(6) Brown, J. S., Bateson, T. F., and McDonnell, W. F. (2008) Effects of exposure to 0.06 ppm ozone on FEV1 in humans: A secondary analysis of existing data. *Environ. Health Perspect. 116*, 1023–1026.
(7) Schelegle, E. S., Morales, C. A., Walby, W. F., Marion, S., and Allen, R. P. (2009) 6.6-h inhalation of ozone concentrations from 60 to 87 parts per billion in healthy humans. *Am. J. Respir. Crit. Care Med. 180*, 265–272.
(8) Hoppe, P., Peters, A., Rabe, G., Praml, G., Lindner, J., Jakobi, G., Fruhmann, G., and Nowak, D. (2003) Environmental ozone effects in different population subgroups. *Int. J. Hyg. Environ. Health 206*, 505–516.
(9) Devlin, R. B., McDonnell, W. F., Mann, R., Becker, S., House, D. E., Schreinemachers, D., and Koren, H. S. (1991) Exposure of humans to ambient levels of ozone for 6.6 h causes cellular and biochemical changes in the lung. *Am. J. Respir. Cell Mol. Biol. 4*, 72–81.
(10) Thurston, G. D., Lippmann, M., Scott, M. B., and Fine, J. M. (1997) Summertime haze air pollution and children with asthma. *Am. J. Respir. Crit. Care Med. 155*, 654–660.
(11) Pryor, W. A. (1992) How far does ozone penetrate into the pulmonary air/tissue boundary before it reacts? *Free Radical Biol. Med. 12*, 83–88.
(12) Dahl, M., Bauer, A. K., Arredouani, M., Soininen, R., Tryggvason, K., Kleeberger, S. R., and Kobzik, L. (2007) Protection against inhaled oxidants through scavenging of oxidized lipids by macrophage receptors MARCO and SR-AI/II. *J. Clin. Invest. 117*, 757–764.
(13) Postlethwait, E. M. (2007) Scavenger receptors clear the air. *J. Clin. Invest. 117*, 601–604.
(14) Pulfer, M. K., and Murphy, R. C. (2004) Formation of biologically active oxysterols during ozonolysis of cholesterol present in lung surfactant. *J. Biol. Chem. 279*, 26331–26338.
(15) Uhlson, C., Harrison, K., Allen, C. B., Ahmad, S., White, C. W., and Murphy, R. C. (2002) Oxidized phospholipids derived from ozone-treated lung surfactant extract reduce macrophage and epithelial cell viability. *Chem. Res. Toxicol. 15*, 896–906.
(16) Stadelmann-Ingrand, S., Pontcharraud, R., and Fauconneau, B. (2004) Evidence for the reactivity of fatty aldehydes released from oxidized plasmalogens with phosphatidylethanolamine to form Schiff base adducts in rat brain homogenates. *Chem. Phys. Lipids 131*, 93–105.
(17) Murphy, R. C. (2001) Free-radical-induced oxidation of arachidonoyl plasmalogen phospholipids: Antioxidant mechanism and precursor pathway for bioactive eicosanoids. *Chem. Res. Toxicol. 14*, 463–472.
(18) Diagne, A., Fauvel, J., Record, M., Chap, H., and Douste-Blazy, L. (1984) Studies on ether phospholipids. II. Comparative composition of various tissues from human, rat and guinea pig. *Biochim. Biophys. Acta 793*, 221–231.
(19) Zoeller, R. A., Lake, A. C., Nagan, N., Gaposchkin, D. P., Legner, M. A., and Lieberthal, W. (1999) Plasmalogens as endogenous antioxidants: somatic cell mutants reveal the importance of the vinyl ether. *Biochem. J. 338* (Part 3), 769–776.
(20) Engelmann, B. (2004) Plasmalogens: targets for oxidants and major lipophilic antioxidants. *Biochem. Soc. Trans. 32*, 147–150.
(21) Rustow, B., Kolleck, I., Guthmann, F., Haupt, R., Kunze, D., and Stevens, P. (1994) Sythesis and secretion of plasmalogens by type II pneumocytes. *Biochem. J. 302*, 665–668.
(22) Schmidt, R., Luboeinski, T., Markart, P., Ruppert, C., Daum, C., Grimminger, F., Seeger, W., and Gunther, A. (2004) Alveolar antioxidant status in patients with acute respiratory distress syndrome. *Eur. Respir. J. 24*, 994–999.
(23) Zemski Berry, K. A., Turner, W. W., VanNieuwenhze, M. S., and Murphy, R. C. (2009) Stable isotope labeled 4-(dimethylamino)benzoic acid derivatives of glycerophosphoethanolamine lipids. *Anal. Chem. 81*, 6633–6640.
(24) Notter, R. (2000) Lung biology in health and disease. In *Lung Surfactants: Basic Science and Clinical Applications* (Lenfant, C., Ed.) Marcel Dekker, New York.
(25) Kim, H. Y., and Salem, N., Jr. (1990) Separation of lipid classes by solid phase extraction. *J. Lipid Res. 31*, 2285–2289.
(26) Zemski Berry, K. A., and Murphy, R. C. (2004) Electrospray ionization tandem mass spectrometry of glycerophosphoethanolamine plasmalogen phospholipids. *J. Am. Soc. Mass Spectrom. 15*, 1499–1508.
(27) Snyder, F., Blank, M. L., and Wykle, R. L. (1971) The enzymic synthesis of ethanolamine plasmalogens. *J. Biol. Chem. 246*, 3639–3645.
(28) Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol. 37*, 911–917.
(29) Luo, X. P., Yazdanpanah, M., Bhooi, N., and Lehotay, D. C. (1995) Determination of aldehydes and other lipid peroxidation products in biological samples by gas chromatography-mass spectrometry. *Anal. Biochem. 228*, 294–298.
(30) Hadley, J. S., Fradin, A., and Murphy, R. C. (1988) Electron capture negative ion chemical ionization analysis of arachidonic acid. *Biomed. Environ. Mass Spectrom. 15*, 175–178.
(31) Clay, K. (1990) Arachidonate related lipid mediators. In *Methods in Enzymology* (Murphy, R. C., Fitzpatrick, F. A., Ed.) Academic Press, San Diego.
(32) Creer, M. H., and Gross, R. W. (1985) Separation of isomeric lysophospholipids by reverse phase HPLC. *Lipids 20*, 922–928.
(33) Hurly, B. P., and McCormick, B. A. (2008) Multiple roles of phospholipase A_2 during lung infection and inflammation. *Infect. Immun. 76* (6), 2259–2272.
(34) De Luca, D., Silvia, B., Vento, G., Piastra, M, Pietrini, D., Romitelli, F., Capoluongo, E., Romagnoli, C., Conti, G., and Zecca, E. (2008) Secretory phospholipases A2 and neonatal respiratory distress: Pilot study on broncho-alveolar lavage. *Intensive Care Med. 34*, 1858–1864.
(35) Arbibe, L., Koumanov, K., Vial, D., Rougeot, C., Faure, G., Havet, N., Longacre, S., Vargaftig, B. B., Bereziat, G., Voelker, D. R., Wolf, C., and Touqui, L. (1998) Generation of lysophospholipids from surfactant in acute lung injury is mediated by type-II phospholipase A2 and inhibited by a direct surfactant protein A-phospholipase protein interaction. *J. Clin. Invest. 102*, 1152–1160.
(36) Grosjean, E, and Grosjean, D. (1999) The reaction of unsaturated aliphatic oxygenates with ozone. *J. Atmos. Chem. 32* (1), 205–232.
(37) Grosjean, E, and Grosjean, D. (1998) Rate constants for the gas phase reaction of ozone with unsaturated oxygenates. *Int. J. Chem. Kinet. 30* (1), 21–29.
(38) Pryor, W. A., Giamalva, D., and Church, D. F. (1985) Kinetics of ozonation. 3. Substituent effects on the rates of reaction of alkenes. *J. Am. Chem. Soc. 107*, 2793–2797.
(39) Criegee, R. (1975) Mechanism of ozonolysis. *Angew Chem. Int. Ed. 14* (11), 745–752.
(40) Bernhard, W., Wand, J., Tschernig, T., Tummler, B., Hedrich, H. J., and von der Hardt, H. (1997) Lung surfactant in a cystic fibrosis animal model: Increased alveolar phospholipid pool size without altered composition and surface tension function in *cftr*$m^{2HGU/m2HGU}$ mice. *Thorax 52*, 723–730.
(41) Nakos, G., Kitsiouli, E., Hatzidaki, E., Koulouras, V., Touqui, L., and Lekka, M. (2005) Phospholipases A_2 and platelet-activating-factor

acetylhydrolase in patients with acute respiratory distress syndrome. *Crit. Care Med. 33*, 772–779.
(42) Kudo, I., and Murakami, M. (2002) Phospholipase A_2 enzymes. *Prostaglandins Other Lipid Mediators 68−69*, 3–58.
(43) Min, J., Wilder, C., Aoki, J., Arai, H., Inoue, K., Paul, L., and Gelb, M. (2001) Platelet activating factor acetylhydrolases: Broad substrate specificity and lipoprotein binding does not modulate the catalytic properties of the plasma enzyme. *Biochemistry 40*, 4539–4549.
(44) Rudiger, M., Kolleck, I., Putz, G., Wauer, R. R., Stevens, P., and Rustow, B. (1998) Plasmalogens effectively reduce the surface tension of surfactant-like phospholipid mixtures. *Am. J. Physiol. 274*, L143–L148.
(45) Rudiger, M., von Baehr, A., Haupt, R., Wauer, R. R., and Rustow, B. (2000) Preterm infants with high polyunsaturated fatty acid and plasmalogen content in tracheal aspirates develop bronchopulmonay dysplasia less ofter. *Crit. Care Med. 28*, 1572–1577.
(46) Frasch, S. C., Zemski-Berry, K., Murphy, R. C., Borregaard, N., Henson, P. M., and Bratton, D. L. (2007) Lysophospholipids of different classes mobilize neutrophil secretory vesicles and induce redundant signaling through G2A. *J. Immunol. 178*, 6540–6548.
(47) Gay, I., Schwartz, Z., Sylvia, V. L., and Boyan, B. D. (2004) Lysophospholipid regulates release and activation of latent TGF-beta1 from chondrocyte extracellular matrix. *Biochim. Biophys. Acta 1684*, 18–28.
(48) Nishina, A., Kimura, H., Sekiguchi, A., Fukumoto, R. H., Nakajima, S., and Furukawa, S. (2006) Lysophosphatidylethanolamine in *Grifola frondosa* as a neurotrophic activator via activation of MAPK. *J. Lipid Res. 47*, 1434–1443.
(49) Tager, A. M., LaCamera, P., Shea, B. S., Campanella, G. S., Selman, M., Zhao, Z., Polosukhin, V., Wain, J., Karimi-Shah, B. A., Kim, N. D., Hart, W. K., Pardo, A., Blackwell, T. S., Xu, Y., Chun, J., and Luster, A. D. (2008) The lysophosphatidic acid receptor LPA1 links pulmonary fibrosis to lung injury by mediating fibroblast recruitment and vascular leak. *Nat. Med. 14*, 45–54.
(50) He, D., Su, Y., Usatyuk, P. V., Spannhake, E. W., Kogut, P., Solway, J., Natarajan, V., and Zhao, Y. (2009) Lysophosphatidic acid enhances pulmonary epithelial barrier integrity and protects endotoxin-induced epithelial barrier disruption and lung injury. *J. Biol. Chem. 284*, 24123–24132.
(51) Medoff, B. D., Landry, A. L., Wittbold, K. A., Sandall, B. P., Derby, M. C., Cao, Z., Adams, J. C., and Xavier, R. J. (2009) CARMA3 mediates lysophosphatidic acid-stimulated cytokine secretion by bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol. 40*, 286–294.
(52) Szapacs, M. E., Kim, H. Y., Porter, N. A., and Liebler, D. C. (2008) Identification of proteins adducted by lipid peroxidation products in plasma and modifications of apolipoprotein A1 with a novel biotinylated phospholipid probe. *J. Proteome Res. 7*, 4237–4246.
(53) Kuo, C. L., Raner, G. M., Vaz, A. D., and Coon, M. J. (1999) Discrete species of activated oxygen yield different cytochrome P450 heme adducts from aldehydes. *Biochemistry 38*, 10511–10518.
(54) Guichardant, M., Bernoud-Hubac, N., Chantegrel, B., Deshayes, C., and Lagarde, M. (2002) Aldehydes from n-6 fatty acid peroxidation. Effects on aminophospholipids. *Prostaglandins, Leukotrienes Essent. Fatty Acids 67*, 147–149.

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