Hypochlorous Acid-Derived Modification of Phospholipids: Characterization of Aminophospholipids as Regulatory Molecules for Lipid Peroxidation†

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ABSTRACT: Hypochlorous acid (HOCl), an inflammatory oxidant derived from neutrophil myeloperoxidase, can chlorinate cytosolic proteins and nuclear DNA bases of target cells by passing through the cell membrane. However, little is known about the consequences of HOCl-derived modification of cell membrane components, including phospholipids. In this study, we characterize the reaction of HOCl with phospholipid molecules and found that aminophospholipids are the key molecules that chemically regulate lipid peroxidation. Upon incubation with HOCl, the peroxidation of egg yolk phosphatidylcholine was significantly enhanced in the presence of phosphatidylethanolamine (PE). In contrast, the peroxidation was significantly inhibited in the presence of phosphatidylserine (PS). On the basis of mass spectrometric and electron paramagnetic resonance characterization, the initiator of the peroxidation was identified as the nitrogen-centered radical originating from PE-derived chloramines, especially N,N-dichlorinated PE, a major product in the HOCl-modified PE. Although PS was also chlorinated upon reaction with HOCl, the formed chloramine rapidly decomposed to phosphatidylglycolaldehyde, a novel class of lipid aldehyde. Formation of phosphatidylglycolaldehyde was also confirmed in the porcine brain PS and erythrocyte cell membrane ghost exposed to HOCl. These results provide a novel mechanism for the HOCl-induced oxidative damage and its endogenous protection in the cell membrane at the site of inflammation.

Oxidants generated by activated phagocyte cells play a central role in host antimicrobial defenses but may also damage host tissues (*1*, *2*). Activation of phagocyte results in the generation of superoxide, which is subsequently dismutated to H_2O_2, and the release of the heme enzyme myeloperoxidase (MPO)[1] (*1*). This enzyme catalyzes the reaction of H_2O_2 with physiological concentrations of Cl^- ions to give the potent oxidant hypochlorous acid (HOCl). HOCl undergoes numerous reactions with biomolecules, including aromatic chlorination (*3*, *4*), double bond addition (*5*, *6*), chloramine formation (*7*, *8*), aldehyde formation (*9*), and oxidation of thiols (*10*). Chlorinated products, such as 3-chlorotyrosine (*4*) and 5-chlorouracil (*11*), have been detected in inflammatory human tissues. In addition, similar halogenation reactions have been observed in cytosolic proteins and nuclear DNA bases in the liver and lungs of lipopolysaccharide-induced inflammation model mice (*12*, *13*). The pK_a of HOCl is 7.59 (*14*); thus, at physiological pH, a mixture of both HOCl and OCl^- is present. Therefore, HOCl, at least in part, can pass through the cell membranes of the target tissues and then chlorinate intracellular biomolecules. Therefore, cell membrane components, especially membrane phospholipids, may be the primary target of HOCl.

A growing body of evidence suggests that many of the effects of cellular dysfunction under oxidative stress are mediated by oxidation products of polyunsaturated fatty acids. Lipid peroxidation proceeds by a free radical chain reaction mechanism and yields lipid hydroperoxides as major initial reaction products. Subsequent decomposition of lipid hydroperoxides generates a number of breakdown products that display a wide variety of damaging actions. Several lines of evidence have suggested that HOCl or the MPO/H_2O_2/Cl^- system induces lipid peroxidation in model phospholipid systems (*15*, *16*) and in lipoproteins, especially low-density lipoproteins (*17*, *18*). In addition to phosphatidylcholine that is generally used in experimental lipid peroxidation, cell membranes in tissues also contain aminophospholipids, such

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[1] Abbreviations: MPO, myeloperoxidase; EYPC, egg yolk phosphatidylcholine; DPPE, 1,2-dipalmitoylphosphoethanolamine; PFBHA, pentafluorobenzylhydroxylamine hydrochloride; DPPC, 1,2-dipalmitoylphosphatidylcholine; DPPS, 1,2-dipalmitoylphosphatidylserine; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; POE, phosphoethanolamine; POS, phosphoserine; EYPC-OOH, egg yolk phosphatidylcholine hydroperoxide; HPLC, high-performance liquid chromatography; DNPH, 2,4-dinitrophenylhydrazine; GC−MS, gas chromatography−mass spectrometry; PGA, phosphatidylglycoaldehyde; TMS, trimethylsilyl; PBN, α-phenyl-*N*-*tert*-butylnitrone; EPR, electron paramagnetic resonance.

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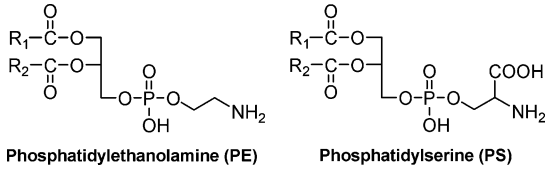


FIGURE 1: Chemical structures of aminophospholipids. R_1 and R_2 are the aliphatic chains of fatty acid esters in the *sn*-1 and *sn*-2 positions, respectively.

as phosphatidylethanolamine (PE) and phosphatidylserine (PS) (Figure 1). Neuronal tissues contain particularly high levels of PS, in comparison to other tissues (*19*). Although it has been suggested that HOCl may react with amino groups in the phospholipid polar head groups much more rapidly than lipid double bonds (*20*), little attention has been given to the reaction mechanism and the subsequent consequences of HOCl-derived modifications of aminophospholipids. In this study, we focused on the reaction of HOCl with phospholipids in vitro and found that aminophospholipids, PE and PS, are the plausible key molecules that chemically regulate the lipid peroxidation reaction in cell membranes.

EXPERIMENTAL PROCEDURES

Materials. Egg yolk phosphatidylcholine (EYPC), 1,2-dipalmitoylphosphoethanolamine (DPPE), Chelex-100 resin, pentafluorobenzylhydroxylamine hydrochloride (PFBHA), and phospholipase D (from *Streptomyces* sp.) were purchased from Sigma-Aldrich Co. (St. Louis, MO). 1,2-Dipalmitoylphosphatidylcholine (DPPC), 1,2-dipalmitoylphosphatidylserine (DPPS), 1-palmitoyl-2-oleoylphosphatidylserine, and porcine brain PS were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). MPO from human sputum was obtained from Elastin Products Co., Inc. (Owensville, MO). Sodium hypochlorite, L-serine, and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phosphoserine was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Hypobromous acid (HOBr) was prepared as previously reported (*21*).

In Vitro Modification of Phospholipids. Multilamellar liposomes comprising EYPC and 1,2-dipalmitoyl phospholipids were prepared as previously described (*22*). Briefly, lipids dissolved in chloroform were dried under a N_2 stream and then emulsified in Chelex-100-treated 0.1 M phosphate buffer (pH 7.4) using an Astrason XL2020 ultrasonic processor (Heat Systems-Ultrasonics, Farmingdale, NY) for 30 s at level 6. The final concentration of EYPC (4 mg/mL) was calculated to be approximately 5.2 mM on the basis of the molecular weight (760.08) of 1-palmitoyl-2-oleoyl-PC, a major molecular species in EYPC. For identification of the modification products of aminophospholipids, liposomes were prepared by replacing EYPC with DPPC. The emulsion was then incubated with HOCl or HOBr at 37 °C. The concentrations of HOCl [$\epsilon_{292} = 350$ M^{-1} cm^{-1} (*14*)] and HOBr [$\epsilon_{329} = 332$ M^{-1} cm^{-1} (*21*)] were determined spectrophotometrically. Reactions with the MPO/H_2O_2/Cl^- system were typically performed in Chelex-100-treated sodium phosphate buffer (pH 4.5) containing 10 nM MPO, 1 mM H_2O_2, and 100 mM NaCl at 37 °C for 1 h. Reactions were initiated by addition of H_2O_2. The concentrations of MPO [$\epsilon_{430} = 178$ mM^{-1} cm^{-1} (*23*)] and H_2O_2 [$\epsilon_{240} = 43.6$ M^{-1} cm^{-1} (*24*)] were determined spectrophotometrically. Reactions were generally terminated with an excess of methionine (4 mM). The modification of phosphoethanolamine (POE) or phosphoserine (POS) was performed by incubating POE or POS (10 mM) and HOCl (0−10 mM) at 37 °C for 1 h in Chelex-100-treated 0.1 M phosphate buffer (pH 7.4). Porcine brain PS (5 mg/mL) was emulsified in Chelex-100-treated 0.1 M phosphate buffer (pH 7.4) and then incubated with HOCl at 37 °C. Spectrophotometric analysis was performed with a Beckman DU640 spectrophotometer.

Lipid Hydroperoxide Assay. The lipid peroxidation reaction was terminated by the addition of 1 mM ethylenediaminetetraacetic acid and 10 μM 2,6-di-*tert*-butyl-*p*-cresol. The EYPC hydroperoxide (EYPC-OOH) was analyzed by reverse-phase high-performance liquid chromatography (HPLC) using TSK-gel ODS 80Ts (4.6 mm × 150 mm, Tosoh, Tokyo, Japan) equilibrated with 95% aqueous methanol at a flow rate of 1 mL/min with UV detection at 234 nm. The retention times for the two peaks of EYPC-OOH were 4.5 and 5.5 min, indicating 1-palmitoyl and 1-stearyl molecules in the *sn*-1 fatty acid esters, respectively (*25*). Authentic EYPC-OOH was prepared by a photosensitized oxidation of EYPC as previously reported (*26*). Briefly, EYPC was dissolved in chloroform and methanol (2/1, by volume) containing methylene blue and illuminated with a tungsten−halogen lamp at 5 °C in air. The formed EYPC-OOH was purified by column chromatography using LiChroprep RP-8 (Merck; octane-binding silica gel powder, 40−63 μm size).

Thin-Layer Chromatography Analysis. For thin-layer chromatography (TLC) analysis, the lipids in the reaction mixtures were extracted twice with an equal amount of chloroform followed by evaporation under a N_2 stream. Thin-layer chromatography (TLC) was performed utilizing a silica gel 60 TLC plate (Merck) and a mobile phase comprising chloroform, methanol, and acetic acid (65/25/4, v/v). After development, lipids were visualized by spraying a ninhydrin reagent (Wako) for detection of primary amino groups, Dittmer-Lester reagent for detection of phospholipids, or 0.2% (w/v) 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl and ethanol (1/1, v/v) for detection of carbonyl/aldehyde groups.

Gas Chromatography−Mass Spectrometry (GC−MS). Gas chromatography and mass spectrometry (GC−MS) were performed with an AQP-5050 mass spectrometer (Shimadzu Co., Kyoto, Japan) equipped with an SPB-1 fused silica capillary column (30 m × 0.25 mm, 10 μm film thickness; Supelco, Bellefonte, PA) in the electron impact ionization mode at 70 eV. The carrier gas, helium, was applied at a flow rate of 1.0 mL/min. The column oven was held at 50 °C for 2 min before being heated to 290 °C at a rate of 10 °C/min and kept constant for 5 min. The molecular separator temperature and ion source temperature were set at 250 and 260 °C, respectively.

For GC−MS analysis of chlorinated POE, 10 μL of POE/HOCl reaction mixture was dried in vacuo and then derivatized with 50 μL of BSTFA at room temperature for 1 h. One microliter of the sample was directly subjected to GC−MS. For GC−MS analysis of phosphatidylglycoaldehyde (PGA), an aliquot of the DPPS/HOCl reaction mixture was treated with phospholipase D (final concentration of 28 units/mL) dissolved in 200 μL of 0.1 M sodium acetate buffer

(pH 5.6) containing 0.1 M $CaCl_2$ and 500 μL of diethyl ether at room temperature for 2 h. After evaporation of diethyl ether with a N_2 stream, 400 μL of a Chelex-100-treated phosphate buffer (pH 7.4)/50 mM PFBHA methanolic solution (1/1, v/v) was added to the reaction mixture and the solution incubated at room temperature for 1 h. The formed PFB derivative of glycolaldehyde was extracted three times with *n*-hexane, evaporated under a N_2 stream, and further derivatized to the trimethylsilyl (TMS) ether with 50 μL of BSTFA at room temperature for 1 h. Authentic glycolaldehyde was prepared by incubating serine (10 mM) and HOCl (10 mM) in Chelex-100-treated phosphate buffer (pH 7.4) at 37 °C for 1 h, followed by derivatization with PFBHA and BSTFA. For analysis of phosphoglycolaldehyde, 200 μL of the phosphoserine/HOCl reaction mixture was mixed with 100 μL of a 50 mM PFBHA methanolic solution and incubated at room temperature for 1 h. Ten microliters of the mixture was evaporated to dryness and incubated with 50 μL of BSTFA. One microliter of each derivatized sample was subjected to GC−MS. The retention times of the PFB/TMS derivatives of glycolaldehyde and phosphoglycolaldehyde were 10.5 and 16.6 min, respectively.

Electron Paramagnetic Resonance. The formation of reactive oxygen species was studied by the α-phenyl-*N*-*tert*-butylnitrone (PBN) spin trapping technique. An electron paramagnetic resonance (EPR) spectrometer (JES-TE300, JEOL Co., Tokyo, Japan) with an ES-UCX2 cavity and a quartz flat cell was employed to collect the EPR signals of PBN spin adducts. POE or POS (100 mM) was mixed with HOCl (15 mM) in Chelex-100-treated phosphate buffer (pH 7.4). After 5 min at room temperature, 20 mg of PBN was added to the reaction mixture (200 μL), and the EPR spectrum was then measured. Spectra were stored on an IBM personal computer for analysis. Hyperfine coupling constants were obtained with Winsim (*27*).

HPLC Analysis of DNPH-Derivatized Aldehydes. For analysis of PGA and plasmalogen-derived aldehydes, an aliquot of the reaction mixture (100 μL) was derivatized with 200 μL of 0.2% DNPH in 2 N HCl at room temperature for 30 min. After incubation, the DNPH adducts were extracted twice with 1 mL of chloroform. The chloroform layer was evaporated under a N_2 stream and then dissolved in 200 μL of methanol. Twenty microliters of the aliquot was injected into a TSK-gel ODS 80Ts column (4.6 mm × 150 mm, Tosoh) equipped with methanol and 2-propanol (9/1) containing 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. The elution profiles were monitored by absorbance at 365 nm. The amount of aldehydes was indirectly estimated using a calibration curve obtained from the analysis of authentic tridecanal. The results were expressed as the means of duplicate determinations. Structural characterization of the PGA−DNPH intermediate via mass spectrometry was performed by syringe pump infusion analysis using a quadrupole tandem mass spectrometer, API-3000 (Applied Biosystems, Inc.), with a turbospray (electrospray) interface in the negative mode.

Human Neutrophils. Neutrophils were prepared from fresh anticoagulated human blood by density gradient centrifugation using Mono-poly resolving medium (Dainippon Pharmaceutical Co., Osaka, Japan) and suspended in Ca^{2+}-free Krebs-Ringer-phosphate solution [0.1 M sodium phosphate buffer (pH 7.4), 0.15 M NaCl, 0.154 M KCl, and 0.154 M $MgCl_2$ (21/100/4/1, v/v)]. The cells (5×10^6 cells/mL) mixed with an equal volume of phospholipid emulsion were stimulated with 200 nM phorbol myristate acetate and maintained in suspension with intermittent inversion at 37 °C for 1 h. After incubation, cells were removed by centrifugation, and the obtained supernatant was derivatized with DNPH for HPLC analysis.

Erythrocyte Cell Membrane Ghost. Blood from rabbits that had been preserved (50 mL) (Nippon Biotest Laboratories Inc., Tokyo, Japan) was mixed with 50 mL of 10 mM phosphate buffer (pH 7.4) containing 152 mM NaCl and centrifuged at 500*g* for 10 min at 4 °C. The precipitate was washed twice with 50 mL of 10 mM phosphate buffer (pH 7.4) containing 152 mM NaCl. The erythrocytes that were obtained were mixed with 75 mL of 10 mM phosphate buffer (pH 7.4). After 15 min at 4 °C, the mixture was centrifuged at 22000*g* for 10 min at 4 °C. The precipitate was washed twice with 75 mL of 10 mM phosphate buffer (pH 7.4). The protein content in the obtained ghost was determined with a BCA protein assay reagent (Pierce). Modification of the ghost was performed upon incubation of the erythrocyte ghost (2.7 mg of protein/mL) and HOCl (0−5 mM) at 37 °C for 1 h. After being incubated, the reaction mixtures were derivatized with an equal volume of 0.2% DNPH in 2 N HCl at room temperature for 30 min. The DNPH adducts were extracted twice with 1 mL of chloroform. The chloroform layer was evaporated under a N_2 stream, dissolved in 200 μL of methanol, and then subjected to HPLC as described above. For GC−MS analysis of the PFB/TMS-derivatized PGA, the lipids in the reaction mixtures were extracted three times with chloroform and methanol (2/1) and evaporated under a N_2 stream. The lipids were treated with phospholipase D (final concentration of 2.8 units/mL) dissolved in 200 μL of 0.1 M sodium acetate buffer (pH 5.6) containing 0.1 M $CaCl_2$ and 100 μL of diethyl ether at 37 °C for 1 h. After evaporation of diethyl ether by a N_2 stream, 200 μL of a Chelex-100-treated phosphate buffer (pH 7.4)/50 mM PFBHA methanolic solution (1/1, v/v) was added to the reaction mixture and the solution incubated at room temperature for 1 h. The formed PFB derivative of glycolaldehyde was extracted three times with 200 μL of *n*-hexane, evaporated under a N_2 stream, and further derivatized with 50 μL of BSTFA at room temperature for 1 h. One microliter of the mixture was subjected to GC−MS.

RESULTS

Effects of Aminophospholipids on the HOCl-Induced Lipid Peroxidation. It has been known that amino groups of biomolecules including proteins and nucleobases are the potential target of HOCl. Thus, we first examined the effects of aminophospholipids on the HOCl-induced lipid peroxidation. Egg yolk phosphatidylcholine (EYPC) was used as the substrate for HOCl-induced peroxidation, and synthetic 1,2-dipalmitoyl phospholipids [phosphatidylcholine (DPPC), phosphatidylethanolamine (DPPE), or phosphatidylserine (DPPS)] were used as additives to clarify the effects of the polar head groups of these phospholipids. The liposome comprising EYPC and an additive were incubated with HOCl at 37 °C in phosphate buffer (pH 7.4), and the formed peroxidized EYPC (EYPC-OOH) was analyzed by HPLC. As shown in Figure 2A, the formation of EYPC-OOH [retention times of 4.5 min for the 1-palmitoyl derivative

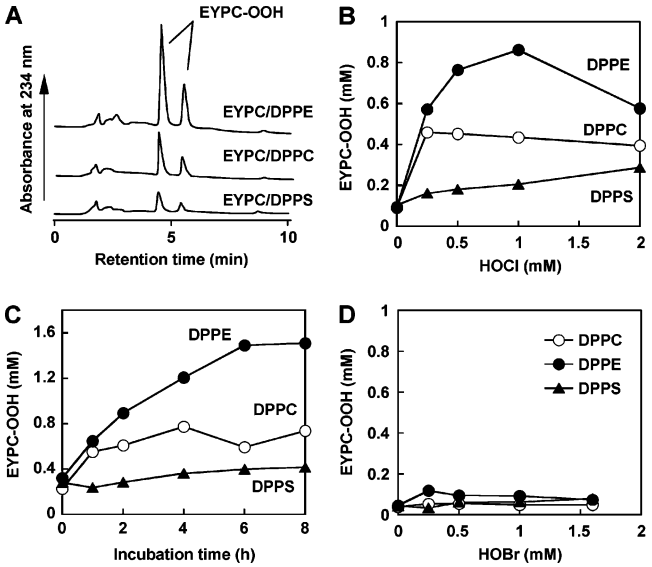


FIGURE 2: Effects of 1,2-dipalmitoyl phospholipids on the HOCl-induced peroxidation of EYPC. (A) HPLC profiles of EYPC hydroperoxide (EYPC-OOH) formed in the reaction of HOCl with a liposome comprising EYPC (4 mg/mL) and a 1,2-dipalmitoyl phospholipid (DPPC, DPPE, or DPPS at 0.5 mM) in phosphate buffer (pH 7.4) at 37 °C for 5 h. (B) Formation of EYPC-OOH in the liposome upon incubation with HOCl (0−2 mM) at 37 °C for 5 h. (C) Time-dependent formation of EYPC-OOH in the liposome upon incubation with HOCl (1 mM) at 37 °C. (D) Formation of EYPC-OOH in the liposome upon incubation with HOBr (0−1.6 mM) at 37 °C for 5 h.

and 5.5 min for the 1-stearyl derivative (*25*)] was significantly enhanced in the presence of DPPE and, in contrast, inhibited in the presence of DPPS. The peroxidation level of EYPC in the presence of DPPC was similar to that in the absence of additives (data not shown), showing that addition of DPPC does not affect the HOCl-induced lipid peroxidation. Similar results were also obtained in a HOCl dose-dependent experiment (Figure 2B) and a time course experiment (Figure 2C). These results showed that HOCl-induced phospholipid peroxidation was crucially affected by aminophospholipids. On the other hand, these two aminophospholipids did not alter the peroxidation levels of EYPC exposed to HOBr, another hypohalous acid derived from a peroxidase of eosinophils (Figure 2D).

Enhancement of HOCl-induced EYPC peroxidation by DPPE was also examined in a DPPE dose-dependent experiment. As shown in Figure 3A, the formation of EYPC-OOH induced by 1 mM HOCl reached a plateau at 0.5 mM DPPE. To examine the inhibitory effect of DPPS on the HOCl/PE-induced lipid peroxidation, DPPS (0−1 mM) was added to EYPC/DPPE liposomes and HOCl-induced peroxidation was then examined. As shown in Figure 3B, we could observe the dose-dependent inhibition of EYPC peroxidation by DPPS. These results suggest that the presence of PS in a biomembrane may play an important role in the inhibition of HOCl/PE-induced lipid peroxidation.

Enhancement of HOCl-Induced Lipid Peroxidation by PE. The enhancement of HOCl-induced peroxidation of EYPC was reproduced when DPPE was initially preincubated with HOCl, extracted with chloroform, and then incubated with EYPC (Figure 4A). In this experiment, we can observe the effects of HOCl-modified phospholipids, but not of HOCl itself, on the peroxidation of EYPC because residual HOCl

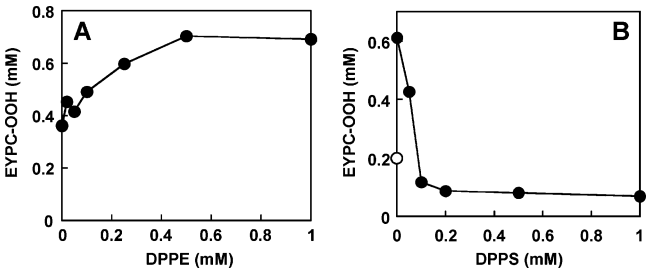


FIGURE 3: Effects of aminophospholipids on the HOCl-induced peroxidation of EYPC. (A) Dose-dependent enhancement of HOCl-induced peroxidation of EYPC by DPPE. Liposome comprising EYPC (4 mg/mL) and DPPE (0−1 mM) was incubated with HOCl (1 mM) at 37 °C for 5 h. (B) Inhibition of HOCl-induced peroxidation of EYPC in the presence of DPPE by DPPS. Liposome comprising EYPC (4 mg/mL), DPPE (0.5 mM), and DPPS (0−1 mM) was incubated with HOCl (1 mM) at 37 °C for 5 h. The white circle denotes formation of EYPC-OOH upon incubation of EYPC alone with HOCl.

was removed during extraction with chloroform. The result suggests that the HOCl-modified product(s) of DPPE initiates the EYPC peroxidation reaction. The HOCl-treated DPPC and DPPS did not influence the peroxidation of EYPC. Complete removal of HOCl during extraction with chloroform was spectrophotometrically confirmed (data not shown). To identify the initiator(s), we analyzed the modification product(s) in the HOCl-modified DPPE using electrospray ionization mass spectrometry and detected the molecular ions of both mono- and dichlorinated DPPE upon reaction with an equimolar HOCl/DPPE mixture (Figure 4B). The intensities of ions at *m*/*z* 725.5 and 727.0 exhibited approximately an expected isotopic ratio (3/1) for the monochlorinated product containing ^{35}Cl and ^{37}Cl, respectively. The intensities of ions at *m*/*z* 758.8, 760.8, and 763.0 exhibited an approximately expected isotopic ratio (9/6/1) for the dichlorinated product. Interestingly, we could detect dichlorinated PE upon reaction with lower concentrations of HOCl (data not shown). Although it would be expected that monochloramine was the major product at a lower concentration of HOCl, dichlorinated PE was detected as the major product. The reactivity of PE with HOCl was also spectrophotometrically examined using phosphoethanolamine (POE), a polar head group derivative of PE. The HOCl-treated POE exhibited characteristic UV spectra with a λ_{max} of 300 nm (Figure 4C), indicating dichloramine formation (*28*, *29*). Interestingly, the similar spectra could also be observed upon incubation with a lower ratio of HOCl. The addition of excess methionine reduced the spectrum with a λ_{max} of 300 nm, showing that the products were chloramine-like species. The formation of dichlorinated POE was confirmed using GC−MS by detecting fragmented ions at *m*/*z* 236 ($[M - TMS - 3CH_3]^+$) and *m*/*z* 266 ($[M - TMS - CH_3]^+$) that indicate the TMS derivative of *N*,*N*-dichloro-POE (data not shown). Under our analytical condition, we could not observe the mass spectrum of monochloramine via GC−MS (data not shown). These results showed that *N*,*N*-dichloramine is a major modification product formed in the reaction of HOCl with PE; therefore, it might be a plausible initiator of lipid peroxidation. Thus, EPR spectroscopy with spin trapping was employed to determine whether radical intermediates are indeed formed in the HOCl-modified PE. Treatment of POE with HOCl followed by the addition of spin trap PBN resulted in the detection of intense EPR signals (Figure 5A).

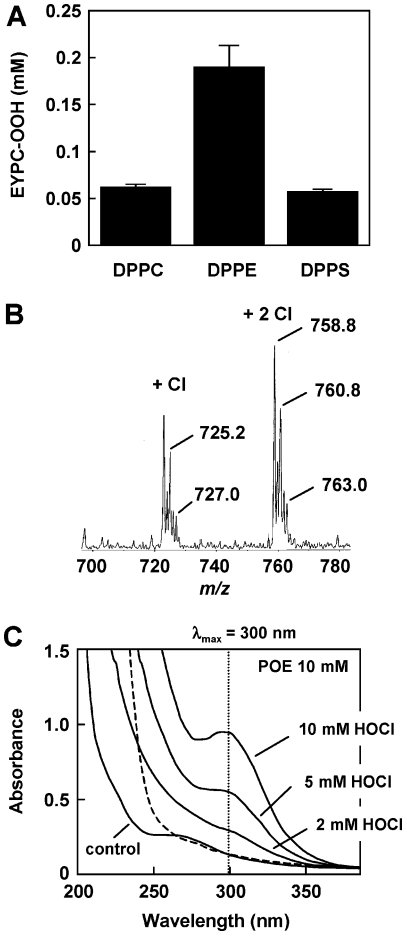


FIGURE 4: Structural characterization of HOCl-modified DPPE that initiates lipid peroxidation. (A) Formation of EYPC-OOH upon incubation with HOCl-treated 1,2-dipalmitoyl phospholipids (DPPC, DPPE, or DPPS). DPPC (1 mM) and a 1,2-dipalmitoyl phospholipid (DPPC, DPPE, or DPPS at 1 mM) were emulsified in Chelex-100-treated phosphate buffer (pH 7.4) and treated with HOCl (1 mM) at 37 °C for 30 min. After incubation, the lipids were extracted twice with chloroform, evaporated, and then emulsified with EYPC (4 mg/mL). After incubation for 5 h, the amount of formed EYPC-OOH was measured by HPLC. The means ± standard deviation ($n = 3$) are presented. (B) Mass spectrometric characterization of HOCl-modified DPPE. DPPE (1 mM) emulsified with DPPC (1 mM) was treated with HOCl (1 mM) in Chelex-100-treated phosphate buffer (pH 7.4) for 10 min and then infused into an electrospray ionization mass spectrometer with the negative mode. The ions at *m*/*z* 725.2 and 727.0 indicate monochlorinated DPPE, and the ions at *m*/*z* 758.8, 760.8, and 763.0 indicate dichlorinated DPPE. (C) Spectrophotometric analysis of HOCl-treated POE. The UV spectra were measured after incubation of HOCl (0−10 mM) and POE (10 mM) in Chelex-100-treated phosphate buffer (pH 7.4) for 30 s. The dashed line indicates the UV spectrum after addition of methionine (25 mM) to HOCl (10 mM)-treated POE.

These signals are assigned to the formation of a mixture of a nitrogen-centered radical ($a^N = 15.34$ G, $a^H = 3.2$ G, $a^{N'} = 0.77$ G), oxidized PBN ($a^N = 14.8$ G, $a^H = 1.4$ G), and a weak carbon-centered radical ($a^N = 14.5$ G, $a^H = 4.7$ G) (*30*, *31*). A computer simulation is shown in Figure 5D. The intense signals of oxidized PBN were also observed in both HOCl-treated POS and HOCl alone (Figure 4B,C), suggesting that the signals may be formed during the nonspecific oxidation of PBN by residual HOCl and/or chloramine species. A nitrogen-centered radical was not observed in HOCl-treated POS (Figure 5B). These results reflect the observation that HOCl-induced peroxidation of EYPC was

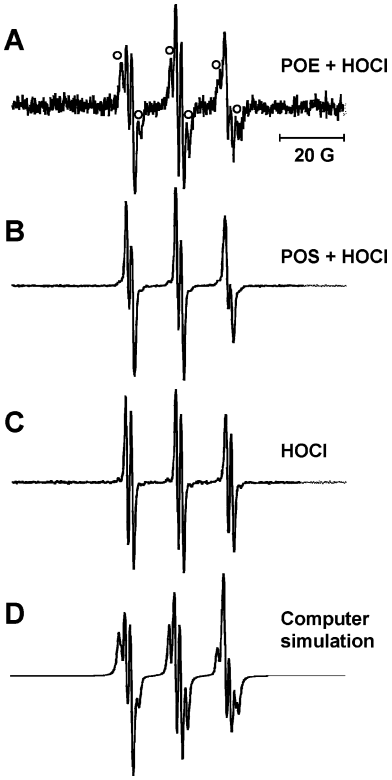


FIGURE 5: EPR spectra of HOCl-treated phospholipid head groups. POE (100 mM) (A), POS (100 mM) (B), or none (C) was treated with HOCl (15 mM) for 5 min at room temperature before addition of 20 mg of PBN. Features in panel A marked with a white circle (○) are assigned to major EPR signals of the PBN−N adduct. Panel D shows the computer simulation for the experimental data in panel A.

enhanced by DPPE but not by DPPS (Figure 2A,B). *N*,*N*-Dichloramine-induced lipid peroxidation was also confirmed by the observation that the level of formation of linoleic acid hydroperoxides was increased in the incubation of linoleic acid with HOCl-modified POE (data not shown).

Inhibition of HOCl-Induced Lipid Peroxidation by PS. To examine the mechanism for the inhibitory effect of PS on HOCl-induced lipid peroxidation, we first analyzed modification products formed in the reaction of DPPS (emulsified in the presence of DPPC) and HOCl by TLC. Upon reaction with different concentrations of HOCl, DPPS (spot b) dose-dependently disappeared with the appearance of an unknown product (spot c) (Figure 6A, left). The product (spot c) could not be formed in HOCl-treated DPPC (data not shown). The disappearance of DPPS was also confirmed by spraying ninhydrin reagent that specifically reacts with primary amino groups (Figure 6A, right). The reaction product (spot c) was not stained with ninhydrin, suggesting that the product was formed through the modification of the amino group of DPPS by HOCl. The reaction product (spot c) was also stained with DNPH, a reagent for detecting carbonyl/aldehydes (Figure S1 of the Supporting Information), suggesting the formation of carbonyl/aldehydic compounds during the reaction of HOCl and PS. DPPC (spot a) was not modified in the reaction with HOCl (left panel and Figure S1 of the Supporting Information). It has been reported that the reaction of HOCl and α-amino groups of amino acids generates a corresponding family of aldehydes in high yield via *N*-monochloramines as the intermediates (*9*). Upon reaction with serine, glycolaldehyde ($HO-CH_2-CH=O$) has

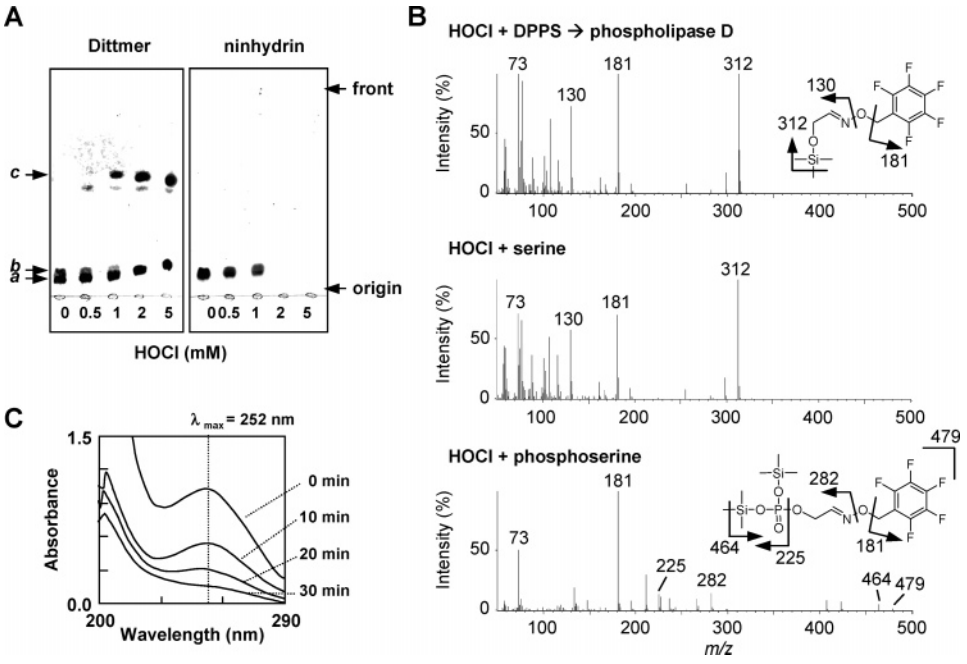


FIGURE 6: Characterization of HOCl-modified DPPS. (A) TLC analysis of HOCl-treated DPPS. Liposome comprising DPPC and DPPS (both at 1 mM) was treated with HOCl (0−2 mM) at 37 °C for 1 h in phosphate buffer (pH 7.4). Lipids were extracted with chloroform and then applied to silica TLC plates: (left) Dittmer reagent detection (for phosphate groups) and (right) ninhydrin detection (for amino groups). (B) Mass spectra of glycolaldehyde derivatives formed in the reaction of HOCl and PS: (top) phospholipase D hydrolysate of HOCl-treated DPPS, (middle) glycolaldehyde formed in the reaction of HOCl and L-serine, and (bottom) HOCl-treated phosphoserine. Samples were derivatized with PFBHA and BSTFA and subjected to GC−MS. (C) Spectrophotometric analysis of HOCl-treated POS.

been identified as a major product (*32*). On the basis of this information, we presumed the formation of a glycolaldehyde-like derivative in the head group of PS upon reaction with HOCl. Thus, the aldehydic compound formed in the reaction mixture of HOCl and DPPS was characterized by GC−MS. The mass spectrum indicating a PFB/TMS derivative of glycolaldehyde was detected in the GC−MS analysis of HOCl-modified DPPS treated with phospholipase D, which releases an alcoholic head group and phosphatidic acid (Figure 6B, top). The characteristic masses at *m*/*z* 312, 181, and 130 completely coincided with the mass spectrum of glycolaldehyde formed in the reaction of serine and HOCl (Figure 6B, middle). Formation of a glycolaldehyde moiety in the head group of PS was also confirmed by GC−MS analysis of the reaction mixture of HOCl and POS, a polar head group derivative of PS. The obtained masses at *m*/*z* 479, 464, 282, 225, and 181 indicated the PFB/TMS derivative of phosphoglycolaldehyde (Figure 6B, bottom). These mass spectrometric characterizations showed that PS was converted to phosphatidylglycolaldehyde (PGA), a novel phospholipid-derived aldehyde, during the reaction with HOCl.

It has been shown that chloramines of the α-amino groups of amino acids are the initially formed intermediates in the formation of amino acid-derived aldehydes (*9*). To confirm the reaction mechanism for the formation of PGA, chloramine formation in the reaction of HOCl and POS was examined by monitoring the UV spectrum. As shown in Figure 6C, a peak with a λ_{max} of 252 nm, which is characteristic of monochloramine formation, was observed immediately after the addition of HOCl and underwent a time-dependent decrease in magnitude during incubation at 37 °C, suggesting that the monochloramine is the intermediate of PGA.

We then established the procedure for HPLC analysis of PGA as the DNPH derivative. As shown in Figure 7A, we could clearly detect a major peak indicating the PGA−DNPH derivative in the HOCl-treated DPPS derivatized with DNPH. This peak could not be observed in HOCl-treated DPPC and HOCl-treated DPPE. Structural identification of the expected PGA−DNPH derivative was performed using electrospray ionization mass spectrometry. The molecular ion peak of the PGA−DNPH derivative was observed at *m*/*z* 869.8 ($[M - H]^-$) (Figure 7B, top). In addition, the product ion spectrum generated from collision-induced dissociation of the molecular ion (*m*/*z* 869.8) was also measured, and the four specific fragmented ions were detected: *m*/*z* 255.4, 391.5, 409.5, and 648.0 (Figure 7B, middle). These ions were identified as illustrated in Figure 7B (bottom). The ion at *m*/*z* 409.5 was presumed to be *m*/*z* 391.5 plus water. The amount of PGA was almost stoichiometrically correlated with the amount of added HOCl up to the concentration of DPPS, 1 mM (Figure 7C). A time course experiment showed that both the formation of PGA (with HPLC analysis of the DNPH derivative) and the decomposition of chloramine, the intermediate of PGA (with TNB assay), in the reaction mixture of DPPS and HOCl reached a plateau within 30 min (data not shown). These results showed that PGA is a major and specific end product of HOCl-modified PS.

To confirm that MPO can generate PGA, DPPS was incubated with MPO, H_2O_2, and Cl^- (complete system) and analyzed by HPLC with DNPH derivatization. As shown in Figure 8A, we could detect the PGA−DNPH derivative in the reaction mixture. The formation of PGA was inhibited in the presence of the heme poison NaN_3. In addition, the formation of PGA required MPO, H_2O_2, and Cl^-. Furthermore, to determine whether HOCl generated by human neutrophils might generate PGA, we activated human neu-

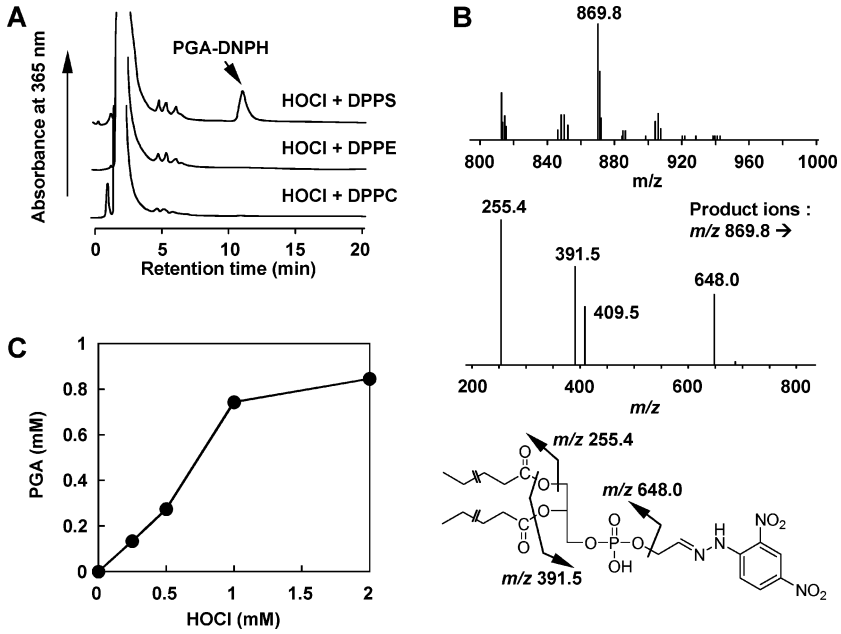


FIGURE 7: HPLC analysis of the PGA−DNPH derivative. (A) HPLC profiles of HOCl-treated 1,2-dipalmitoyl phospholipids after derivatization with DNPH. (B) Mass spectrometric characterization of the PGA−DNPH derivative. HOCl-treated DPPS was derivatized with DNPH and analyzed with an electrospray ionization mass spectrometer in the negative mode: (top) total ion scan, (middle) product ion scan of the ion at *m/z* 869.8, and (bottom) proposed fragmentation pattern of the PGA−DNPH derivative. (C) Formation of PGA upon reaction of DPPS (1 mM) and HOCl (0−2 mM). DPPS was emulsified in the presence of DPPC (1 mM) in phosphate buffer (pH 7.4) and treated with HOCl at 37 °C for 1 h. After incubation, reaction mixtures were derivatized with DNPH and analyzed by HPLC.

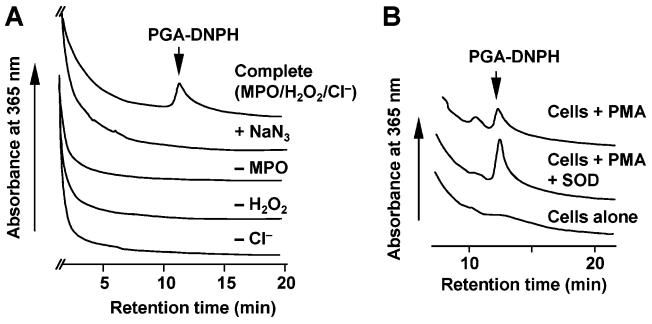


FIGURE 8: Formation of PGA by the MPO/H_2O_2/Cl^- system and activated neutrophils. (A) Formation of PGA in the reaction of DPPS with the MPO/H_2O_2/Cl^- system. DPPS (1 mM) was emulsified in the presence of DPPC (1 mM) in phosphate buffer (pH 7.4) and incubated in the presence of MPO (10 nM), H_2O_2 (1 mM), and Cl^- (100 mM) at 37 °C for 1 h (complete system). Reactions with the complete system without MPO, H_2O_2, or Cl^- and with complete system with NaN_3 (1 mM) were also performed. (B) Formation of PGA in the reaction of DPPS with activated human neutrophils. DPPS (1 mM) was emulsified in the presence of DPPC (1 mM) in phosphate buffer (pH 7.4) and mixed with human neutrophils (final concentration of 2.5×10^6 cells/mL). The reaction was initiated by addition of 200 nM phorbol myristate acetate (PMA) and the mixture incubated at 37 °C for 1 h (Cells + PMA). Reactions in the presence of superoxide dismutase (5.6 units/mL) (Cells + PMA + SOD) and in the absence of PMA (Cells alone) were also performed. All reaction mixtures were derivatized with DNPH and analyzed by HPLC.

trophils with phorbol myristate acetate in the presence of DPPS. As shown in Figure 8B, we detected PGA in the reaction mixture of activated neutrophils and DPPS. The formation of PGA was also inhibited in the presence of the heme poison NaN_3, suggesting the contribution of MPO (data not shown). An increase in the yield of the reaction by superoxide dismutase, by increasing H_2O_2 availability or preventing inactivation of MPO by superoxide, was also confirmed.

Formation of PGA in HOCl-Modified Endogenous PS. In addition to synthetic DPPS, the formation of PGA in the endogenous PS molecule was also examined. As shown in Figure 9A, the formation of PGA was confirmed by HPLC analysis of the reaction mixture of HOCl and porcine brain PS. At a higher concentration of HOCl (5 mM), additional peaks (retention time of 12−13 min) were also detected. On the basis of the information that the brain PS contains oleic acid as the major unsaturated fatty acid esters in the *sn*-2 position, these peaks were suggested to be chlorohydrin derivatives of PGA formed upon addition of HOCl to the double bonds of oleic acid and other unsaturated fatty acid esters. As shown in Figure 9B, exposure of brain PS to HOCl resulted in the dose-dependent formation of PGA and the chlorohydrin−PGA derivative. A similar reaction was also reproduced upon reaction of HOCl and 1-palmitoyl-2-oleoyl-PS (Figure S2 of the Supporting Information).

We also examined the formation of PGA in the erythrocyte membrane. The rabbit erythrocyte membrane ghost was prepared and incubated with HOCl. As shown in Figure 10A, a dose-dependent formation of PGA was observed in the HPLC analysis with DNPH derivatizaton. The formation of PGA was also confirmed by GC−MS analysis after hydrolysis with phospholipase D. As shown in Figure 10B, a mass spectrum indicating the PFB/TMS derivative of glycolaldehyde (see Figure 6B) was observed in HOCl-treated ghost samples. Selected ion monitoring at *m/z* 181 and 312 also showed the formation of PGA in the HOCl-treated erythrocyte ghost (Figure 10C).

DISCUSSION

In this study, we characterized the reaction between HOCl and phospholipid molecules and found that aminophospholipids, PE and PS, are the key molecules that chemically regulate the phospholipid peroxidation reactions. It has been

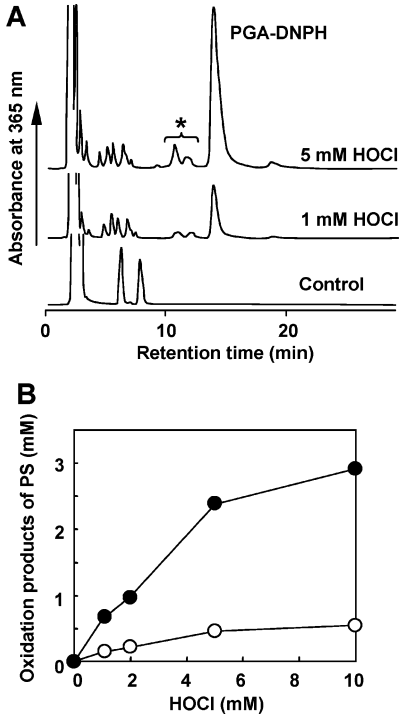


FIGURE 9: Formation of PGA in HOCl-treated porcine brain PS. (A) HPLC profiles of the reaction mixtures of porcine brain PS and HOCl after derivatization with DNPH. Porcine brain PS (5 mg/mL) was emulsified in phosphate buffer (pH 7.4) and incubated with HOCl at 37 °C for 1 h. After incubation, the reaction mixtures were derivatized with DNPH and analyzed by HPLC. The asterisk indicates the chlorohydrin derivatives of PGA. (B) Formation of PGA (●) and the chlorohydrin derivatives (○) upon reaction of porcine brain PS with HOCl.

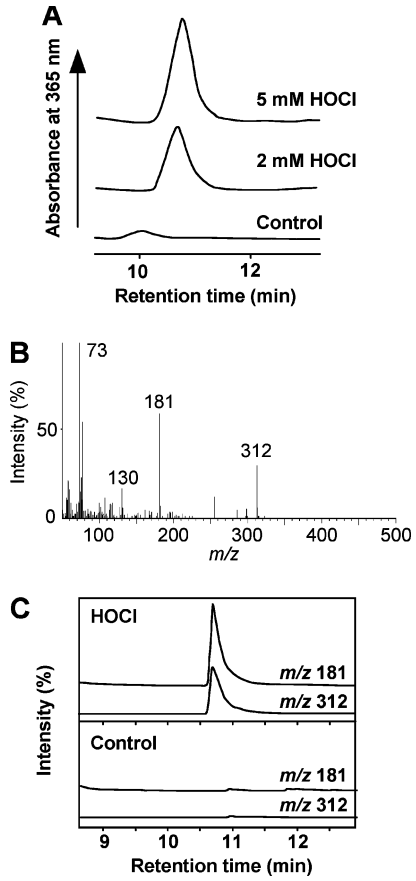
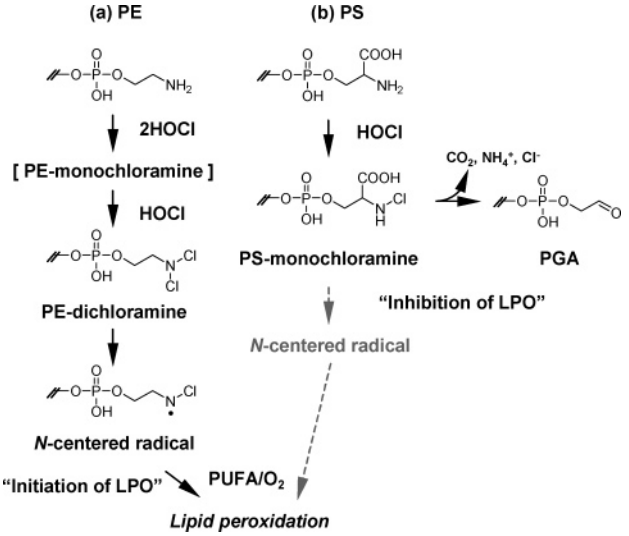


FIGURE 10: Formation of PGA in the HOCl-treated erythrocyte membrane ghost. (A) HPLC profiles of the reaction mixtures of the rabbit erythrocyte membrane ghost and HOCl after derivatization with DNPH. The ghost (2.7 mg of protein/mL of phosphate buffer, pH 7.4) was incubated with HOCl at 37 °C for 1 h. After incubation, the reaction mixtures were derivatized with DNPH and analyzed by HPLC. (B) Mass spectrometric characterization of PGA formed in the reaction of the ghost with HOCl. The HOCl-treated ghost obtained from the reaction mixture (5 mM HOCl) in panel A was hydrolyzed with phospholipase D followed by derivatization with PFBHA and BSTFA and subjected to GC−MS. (C) Selected ion monitoring of the PFB/TMS derivative of glycolaldehyde at *m*/*z* 181 and 312: (top) HOCl-treated ghost and (bottom) untreated ghost.

generally known that amino groups of biomolecules are the primary targets of hypohalous acids and that the haloamines are generated as the major products. For example, the formation of protein-derived (*33*−*35*) and nucleic acid-derived (*36*−*38*) chloramines has previously been reported. Indeed, we could observe the formation of chloramines in HOCl-treated PE and PS (Figures 4 and 6). It is of interest that *N*,*N*-dichloramine is a major product of HOCl-modified PE but the monochloramine was scarcely detected (Figure 4). A similar observation has been reported; dichloramine was specifically generated at the ϵ-amino group of lysine in a peptide exposed to HOCl (*29*). Preferential formation of dichloramines may be explained by a hypothesis that monochloramine is a transient intermediate and is rapidly converted to dichloramine during the reaction with HOCl. We found that the presence of PE enhances the HOCl-induced lipid peroxidation of EYPC (Figure 2). Using EPR experiments with spin trapping, we showed the generation of a nitrogen-centered radical in the HOCl-modified PE (Figure 5) and then concluded that the PE-dichloramine form is a plausible initiator of lipid peroxidation (Scheme 1).

Although several lines of evidence have suggested that HOCl or the MPO/H_2O_2/Cl^- system induces lipid peroxidation in model phospholipid systems (*15*, *16*) and in lipoproteins, especially low-density lipoproteins (*17*, *18*), the mechanism by which HOCl induces lipid peroxidation in vivo remains controversial. Unsaturated fatty acids in lipoproteins exposed to HOCl are consumed to a much greater extent than when thiobarbituric acid-reactive substances were formed (*39*). Upon reaction with unsaturated fatty acids, HOCl binds to the olefinic double bonds of lipids to yield chlorohydrins (*5*, *6*, *40*). In addition, the amount of lipid peroxidation products in HOCl-treated LDL was substantially lower than in Cu^{2+}-treated LDL (*18*). On the basis of these observations, it could be concluded that HOCl itself is not a good initiator of lipid peroxidation. However, it has been reported that HOCl-mediated secondary products can initiate lipid peroxidation. During incubation with LDL, HOCl preferentially reacts with the protein moiety to generate amino acid-derived chloramines, which subsequently produce radicals that initiate lipid peroxidation (*41*). In addition, the presence of lipid hydroperoxides enhances the HOCl-induced lipid peroxidation (*16*). Although the precise reaction mechanism remains unproven, it has recently been reported that the reaction between HOCl and lipid hydroperoxide generates a peroxyl radical and singlet molecular oxygen (*42*). These observations suggest that secondarily formed reactive species may initiate the lipid peroxidation reaction. It has been reported that lipopolysaccharide-induced inflammation with the infiltration of neutrophils initiates lipid peroxidation in the target tissues, including the liver and lungs (*43*−*45*). We

Scheme 1: Reaction of Aminophospholipids with HOCl[a]



[a] (a) PE-derived pathway that initiates lipid peroxidation and (b) PS-derived pathway that generates PGA. Abbreviations: PUFA, polyunsaturated fatty acids; PGA, phosphatidylglycolaldehyde; LPO, lipid peroxidation. The [PE-monochloramine] is a transient product that may be rapidly chlorinated to PE-dichloramine. The pathway in gray could not be observed under our experimental conditions.

found the formation of a nitrogen-centered radical in vitro from the PE−dichloramine derivative as an alternative pathway of HOCl-induced lipid peroxidation reaction in the membrane. In phospholipids, HOCl preferentially reacts with amines compared with double bonds in unsaturated fatty acids (refs *20* and *46* and Figure 9). In addition, PE is one of the major phospholipid molecules following PC in human liver and lungs (*47*, *48*). These observations suggest that HOCl-modified PE, at least in part, may contribute to the initiation of the phospholipid peroxidation reaction in inflammatory tissues.

In contrast to the enhancement of HOCl-induced peroxidation by PE, PS significantly inhibited the lipid peroxidation reaction (Figures 2 and 3). Although the preferential formation of dichloramine upon reaction of PE was observed, monochloramine was determined to be the major product in the HOCl-treated PS. In addition, we could not detect the nitrogen-centered radical in the HOCl-modified PS under our experimental conditions (Figure 5). Therefore, it is presumed that the radical-generating activity of PS-derived chloramine may be relatively lower than that of PE-derived dichloramine. The lower yield of the PS−dichloramine derivative than of the PE−dichloramine derivative may be due to steric hindrance and/or negative charge by the α-substitution with a carboxyl group of PS. Furthermore, we found that the PS−monochloramine derivative, once formed, is rapidly decomposed to an aldehyde PGA, a novel class of aldehydic phospholipids (Figure 6). The formation of PGA is thought to take place thorough a pathway similar to the formation of amino acid-derived aldehydes (refs *9* and *32* and Scheme 1). We confirmed that PGA itself could not induce lipid peroxidation in vitro. These observations indicate that PS scavenges HOCl and then "detoxifies" the potential reactivities of HOCl and/or chloramines as lipid peroxidation initiators.

It has been reported that the lipid peroxidation reaction in phospholipids generates a series of aldehydes at the *sn*-2 position of phospholipids (termed "core-ester aldehydes"), such as 9-oxononanoyl (linoleic acid ester-derived) and 5-oxovaleroyl (arachidonic acid ester-derived) phosphatidylcholines (*49*). The biological activities of these core-ester aldehydes, such as smooth muscle cell proliferation (*50*), neutrophil activation (*51*), and platelet aggregation (*52*), have been reported. Our current study is the first report about the identification of the phospholipid head group aldehyde, as far as we know, although the biological activity of PGA remains unknown.

We here demonstrate that PS may act as an endogenous antioxidant molecule against HOCl-induced injury and that the formation of PGA is a "footprint" of the antioxidative action of PS. It was found that PS is a good scavenger of HOCl for stably attenuating the reactivity. In the human tissues, especially in brain and erythrocytes, PS is the major phospholipid molecule (*19*, *53*). Epidemiological evidence has suggested an elevated incidence of Alzheimer's disease associated with an increased level of MPO expression and a polymorphism in the MPO gene promoter (*54*, *55*). Elevated levels of catalytically active MPO protein in the temporal and frontal cortex of Alzheimer's disease brains colocalize with β-amyloid deposits and neurofibrillary tangles, suggesting that plaque components induce MPO expression and HOCl formation (*54*, *56*). Furthermore, hippocampal levels of 3-chlorotyrosine, a biomarker of HOCl formation in vivo, were shown to be elevated in Alzheimer's disease subjects compared to nondemented subjects (*56*). Because formation of HOCl from MPO results in the oxidation of many important biomolecules, it is highly plausible that HOCl contributes to the extensive oxidative stress and oxidative damage observed in human neurodegenerative diseases such as Alzheimer's disease. Therefore, it is important to identify the pathways for HOCl-induced toxicity and its inhibition in vivo. Although the formation of HOCl-modified PS, including the novel product PGA, in neurodegenerative diseases remains unproven, the results in this study indicate that PS could act as an endogenous antioxidant in brain and that the oxidation products of PS may be a plausible biomarker for the antioxidative action of PS in vivo.

In summary, we have investigated the reaction mechanism for the HOCl-mediated phospholipid peroxidation and demonstrated the novel pathways involving mono- or dichlorinated aminophospholipids, which may chemically regulate the lipid peroxidation reactions in cell membranes. These results may provide a novel mechanism for tissue damage derived from phagocyte-derived oxidants such as HOCl and the endogenous protection in vivo.

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SUPPORTING INFORMATION AVAILABLE

Carbonyl formation in HOCl-treated DPPS determined by TLC analysis (Figure S1) and formation of PGA and the chlorohydrin derivative in HOCl-treated 1-palmitoyl-2-oleoyl-PS (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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