Structural identification of phosphatidylcholines having an oxidatively shortened linoleate residue generated through its oxygenation with soybean or rabbit reticulocyte lipoxygenase

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Abstract **Phosphatidylcholines (PCs) with platelet-activating factor (PAF)-like biological activities are known to be generated by fragmentation of the *sn*-2-esterified polyunsaturated fatty acyl group. The reaction is free radical-mediated and triggered by oxidants such as metal ions, oxyhemoglobin, and organic hydroperoxides. In this study, we characterized the PAF-like phospholipids produced on reaction of PC having a linoleate group with lipoxygenase enzymes at low oxygen concentrations. When the oxidized PCs were analyzed by gas chromatography–mass spectrometry, two types of oxidatively fragmented PC were detected. One PC had an *sn*-2-short chain saturated or unsaturated acyl group (C_8–C_{13}) with an aldehydic terminal; the abundant species were PCs with C_9 and C_{13}. The other PC had a short chain saturated acyl group (C_6–C_9) with a methyl terminal, and the most predominant species was PC with C_8. When the extracts of oxidation products were subjected to catalytic hydrogenation, PCs having saturated acyl groups (C_6–C_{14}) were detected; the most abundant was C_{12} species. The less regiospecific formation of PAF-like lipids suggests that they were generated by oxidative fragmentation of PC hydroperoxides formed by non-stereoselective oxygenation of the alkyl radical of esterified linoleate that escaped from the active centers of lipoxygenases. One of the PAF-like PC with an aldehydic terminal was found to be bioactive; it inhibited the production of nitric oxide induced by lipopolysaccharide and interferon-γ in vascular smooth muscle cells from rat aorta.**—Tokumura, A., T. Sumida, M. Toujima, K. Kogure, K. Fukuzawa, Y. Takahashi, and S. Yamamoto. **Structural identification of phosphatidylcholines having an oxidatively shortened linoleate residue generated through its oxygenation with soybean or rabbit reticulocyte lipoxygenase.** ***J. Lipid Res.*** **2000.** 41: **953–962.**

Supplementary key words platelet-activating factor • phosphatidylcholine hydroperoxide • free radical • lipid peroxidation • gas chromatography–mass spectrometry • nitric oxide production

Evidence is accumulating that oxidized lipids in biological membranes and lipoproteins are involved in the pathogeneses of various diseases including atherosclerosis (1, 2). Much attention has recently been paid to phosphatidylcholines (PCs) with an *sn*-2-short acyl chain that are generated during oxygenation of phospholipids induced by the catalysis by metal ion (3–7), oxyhemoglobin (8), or organic hydroperoxide (9, 10). These compounds with structures that resemble that of platelet-activating factor (PAF) are biologically active (11). The PAF-like lipids have been reported to aggregate platelets (5), activate neutrophils (9), stimulate proliferation of vascular smooth muscle cells (7), and induce attachment of monocytes to vascular endothelial cells (12). PCs having a short acyl chain with a terminal aldehyde group form adducts with proteins in membranes and lipoproteins and disturb their functions (3, 13).

15-Lipoxygenase has been suggested to induce oxidative stress in the artery wall (14, 15), and purified mammalian 15-lipoxygenase and soybean lipoxygenase oxygenate low density lipoproteins in vitro (16–18). These lipoxygenases oxidize esterified fatty acids in phospholipids (18–21). Thus we attempted to examine whether lipoxyge-

Abbreviations: FAB–MS, fast atom bombardment–mass spectrometry; GC–MS, gas chromatography–mass spectrometry; PAF, platelet-activating factor; PC, phosphatidylcholine; tBDMS, *tert*-butyldimethylsilyl; TLC, thin-layer chromatography. The nomenclatures used to designate various oxidized phosphatidylcholines (1-palmitoyl) are monocarboxylate-PC, dicarboxylate-PC, dicarboxylate semialdehyde-PC, and dicarboxylate seminitrile-PC for phosphatidylcholines containing an *sn*-2-monocarboxylate, dicarboxylate, dicarboxylate semialdehyde, and dicarboxylate seminitrile residues, respectively. The molecular species of these PCs are indicated by the total number of carbons in the acyl chain at an *sn*-2-position and the degree of unsaturation, e.g., 8:0-monocarboxylate-PC for PC having an octanoyl group (1-palmitoyl-2-octanoyl-*sn*-glycero-3-phosphocholine) and 9:0-dicarboxylate semialdehyde-PC for PC having a 9-oxononanoyl group [1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphocholine].

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nase-catalyzed reactions could generate PAF-like lipids. Here, we report production of novel molecular species of PAF-like lipids produced by enzymatic oxygenation of linoleate-containing PC with lipoxygenases, and discuss possible mechanisms of generation of the oxidatively modified PCs.

MATERIALS AND METHODS

Materials

l-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine, type-I-B soybean lipoxygenase (specific activity, 12 μmol of linoleic acid oxygenated/min/mg protein at 25°C), phospholipase C from *Bacillus cereus* and 2-(*p*-toluidino)naphthalenesulfonic acid potassium salt were purchased from Sigma Chemical Co. (St. Louis, MO). Purified 15-lipoxygenase from rabbit reticulocytes (2.5 μmol of linoleic acid oxygenated/min/mg protein at room temperature) was supplied by Biomol Research Laboratories (Plymouth Meeting, PA). Sodium deoxycholic acid and PtO_2 were from Wako Pure Chemical Industries (Osaka, Japan). *O*-Methylhydroxylamine hydrochloride, *tert*-butyldimethylchlorosilane and triphenylphosphine were from Kanto Chemical Co. (Tokyo, Japan), Tokyo Kasei Kogyo Co. (Tokyo), and Nacalai Tesque (Kyoto, Japan), respectively. Various 1-palmitoyl-2-short chain acyl-*sn*-glycero-3-phosphocholines were synthesized by the reaction of 1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine with deuterated acetic anhydride, hexanoic anhydride, heptanoic anhydride, octanoic anhydride, nonanoic anhydride, decanoic anhydride or lauric anhydride in the presence of perchloric acid as described previously (22).

1-Palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphochline was prepared from 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine essentially as described by Kamido et al. (23) for preparation of cholesteryl 9-oxononanoate. The aldehydic phospholipid was purified by thin-layer chromatography (TLC) on Silica gel 60 TLC plates (Merck, Darmstadt, Germany) developed with chloroform–methanol–water 65:35:5 (by vol) and analyzed by fast atom bombardment–mass spectrometry (FAB–MS) using thioglycerol as a matrix. FAB–MS was performed on a JEOL JMS-SX102A mass spectrometer under the following conditions: gun high voltage, 3 kV; filament current, 1 A; emission current, 5 mA; accelerating voltage, 10 kV. Mass number calibration was carried out with perfluorokerosene as a standard.

Peroxidation of PC by lipoxygenase

Oxygenation of PC was conducted with type I-B soybean lipoxygenase, essentially as described by Brash et al. (24). Briefly, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (1 μmol) was suspended in 14 ml of 50 mm borate buffer (pH 9.0) and mixed with 200 μl of a 10% solution of sodium deoxycholic acid. The mixture was bubbled with a stream of N_2 gas for 30 min and incubated with 0.5 ml of a solution of soybean lipoxygenase (2.5 mg protein) dissolved in 50 mm borate buffer at 24°C for 30 min under hypoxic conditions.

Oxygenation of PC with reticulocyte lipoxygenase was performed essentially as described previously (25). Linoleate-containing PC (1 μmol) was dispersed in 2 ml of 50 mm Tris-HCl buffer (pH 7.4) containing 0.25% sodium deoxycholic acid. The suspension was bubbled with N_2 gas for 30 min and then incubated with 70 μl of a solution of purified reticulocyte lipoxygenase (0.4 mg protein) at 24°C for 60 min under hypoxic conditions.

Fractionation by TLC of oxygenated phospholipids before and after derivatization for gas chromatography–mass spectrometry

Lipids were extracted from the mixtures after reaction with lipoxygenase by the method of Bligh and Dyer (26). In some experiments, an amount of triphenylphosphine equivalent to that of the parent PC was dissolved in the organic solvent to reduce the produced hydroperoxide promptly to the corresponding hydroxyl group. In other experiments, lipid extracts of samples untreated with triphenylphosphine were dissolved in a mixture of methanol and ethylacetate 1:1, and subjected to catalytic hydrogenation with H_2 gas in the presence of 10 mg PtO_2 at 80°C for 30 min, as described previously (27). Alternatively, the oxygenated lipids were treated with *O*-methylhydroxylamine hydrochloride in phosphate-buffered saline at 37°C for 30 min (12) or with *O*-methylhydroxylamine hydrochloride in pyridine at room temperature for 24 h (4).

Lipid extracts were then fractionated by TLC on Merck Silica gel 60 TLC plates in a solvent system of chloroform–methanol–water 65:35:5 (by vol). Bands of oxygenated phospholipids separated from deoxycholic acid were scraped off the plate and were extracted from the silica gel suspension with water by the method of Bligh and Dyer (26). Samples of oxygenated phospholipids were dissolved in chloroform–methanol 2:1, and 1-μl aliquots were mixed with 1 μl of thioglycerol for FAB–MS.

Analysis of PAF-like lipids by gas chromatography–mass spectrometry

Samples containing oxygenated phospholipids were hydrolyzed with *Bacillus cereus* phospholipase C, and the resultant glycerides were converted to *tert*-butyldimethylsilyl (tBDMS) derivatives, as described previously (4–6). Gas chromatography–mass spectrometry (GC–MS) was performed on a JEOL JMS-AM 150 coupled with a gas chromatograph with an attached DB-1 capillary column (30 m × 0.25 mm I.D., 0.25 μm thickness; J & W Scientific). The column temperature was programmed to rise from 200 to 320°C at 30°C/min. The temperatures of the injection port, interface, and ion source were 300, 280, and 280°C, respectively. The filament emission was 300 μA, and He was used as a carrier gas at 44 ml/min.

Measurement of nitric oxide production

Culture of vascular smooth muscle cells from rat aorta was performed as previously described (28). Nitric oxide production at passages 4–9 was estimated from the accumulation of nitrite converted from nitric oxide in the culture media as described below. The cells were plated at a density of 10^6 cells per 35-mm dish with 1 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37°C for 24 h under 5% CO_2 in air. The media were replaced with fresh serum-free Dulbecco's modified Eagle's media, and 10 μl each of solutions of PC with an *sn*-2-9:0-dicarboxylate semialdehyde (dicarboxylate semialdehyde-PC) and lysophosphatidylcholine in phosphate-buffered saline containing 0.1% bovine serum albumin was added to the culture media. The cells were then incubated at 37°C for 24 h and exposed to lipopolysaccharide and interferon-γ at final concentrations of 1 μg/ml and 25 units/ml, respectively, or vehicle alone for 48 h. The nitrite concentration was measured with the Greiss regent as described (29).

RESULTS

Analysis of PCs containing fragmented acyl groups produced by oxygenation of linoleate-containing PC with soybean lipoxygenase

Under hypoxic conditions, linoleate-containing PC was incubated with soybean lipoxygenase and the oxidation

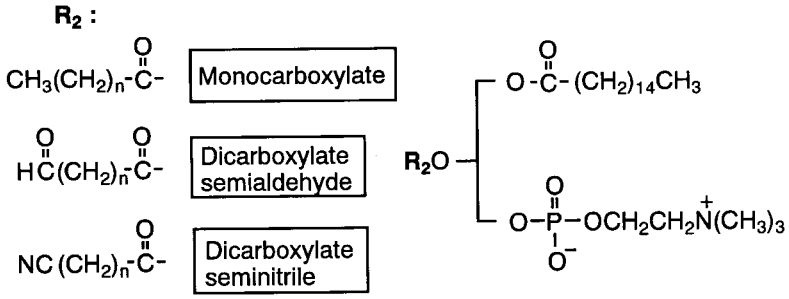

Fig. 1. Structures of 1-palmitoyl-PCs containing a short chain acyl group with a methyl, aldehyde, or nitrile terminal.

products were analyzed by TLC. Two major broad bands (R_f; 0.45–0.6 and 0.1–0.25) were detected. The upper band migrating close to a standard PC contained components that gave a positive reaction with 2,4-dinitrophenylhydrazine spray reagent. The lower band migrated below a standard lysophosphatidylcholine and did not react with the reagent. The lower band was not detected when incubated with the lipoxygenase under normoxic conditions. FAB–MS gave no diagnostic information on the components of the lower band. Therefore, we did not analyze this fraction further.

In the current study, we found that various PCs having an *sn*-2-short chain monocarboxylate group (monocarboxylate-PC) and dicarboxylate semialdehyde group (dicarboxylate semialdehyde-PC) were formed through the lipoxygenase reaction. Their general structures are shown in **Fig. 1**. **Figure 2** shows a typical result of GC–MS of the upper major band as a tBDMS derivative after hydrolysis with phospholipase C. An intense ion peak at 6.86 min in a mass chromatogram monitoring ions at *m/z* 513 was identified to be $[M-57]^+$ of the tBDMS derivative of 8:0-monocarboxylate-PC (Fig. 2A), as both its retention time and mass spectrum measured at the tip of the peak were identical with those of the standard compound. Peaks at 5.96 and 6.37 min monitored at *m/z* 485 and 499, respectively, were assignable to $[M-57]^+$ of 6:0- and 7:0-monocarboxylate-PCs (Fig. 2A) as compared with authentic monocarboxylate-PCs. A small peak of $[M-57]^+$ of 9:0-monocarboxylate-PC with a retention time of 7.37 min was also observed in some experiments (Fig. 2A). The relative amounts of monocarboxylate-PCs calculated from their peak areas of $[M-57]^+$ were as follows: 6:0, 5.7 ± 2.9%; 7:0, 6.9 ± 3.4%; 8:0, 86.5 ± 7.9%; and 9:0, 0.86 ± 0.86% (n = 3).

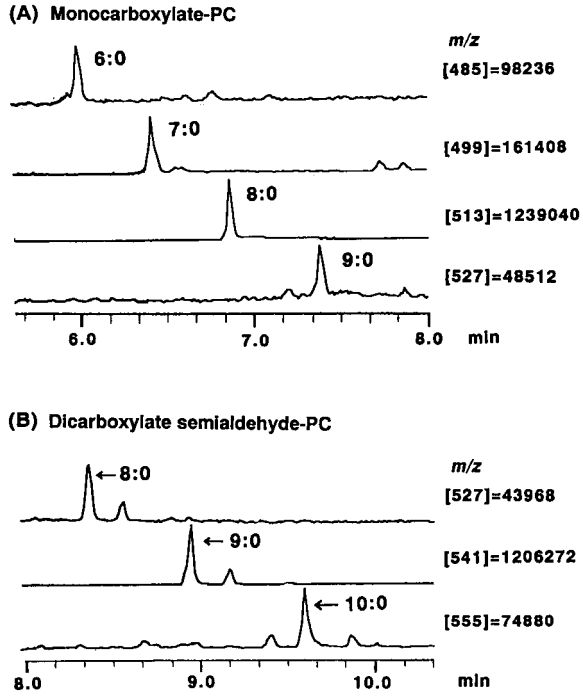

Fig. 2. Ion-current profiles of various ions corresponding to $[M-57]^+$ due to tBDMS derivatives of glycerides of monocarboxylate-PC (A) and dicarboxylate semialdehyde-PC (B) formed during soybean lipoxygenase-catalyzed oxygenation of PC.

In addition to these peaks of monocarboxylate-PCs, there was an intense peak at 8.95 min (*m/z* 541), as seen in Fig. 2B. Its typical electron impact ionization–mass spectrum is shown in **Fig. 3A**. There were diagnostic ions of $[M-57]^+$ (*m/z* 541), $[R_1CO$ (palmitoyl) + $74]^+$ (*m/z* 313), $[R_2CO + 74]^+$ (*m/z* 229), and $[R_2CO]^+$ (*m/z* 155). R_1CO and R_2CO are acyl residues attached at the *sn*-1 and 2 positions of the glycerol backbone, respectively. High resolution mass spectrometric analyses of characteristic ions assignable to $[M-57]^+$, $[R_2CO + 74]^+$ and $[R_2CO]^+$ indicated that R_2 had an oxygen atom. Moreover, the pattern of distribution of fragmented ions in the mass spectrum was similar to that of the tBDMS derivative of 5:0-dicarboxylate semialdehyde-PC, but not to those of PCs having an *sn*-2-acyl group (C_5) with an epoxy or keto group (4). These results suggest that this intense peak is due to $[M-57]^+$ of the tBDMS derivative of 9:0-dicarboxylate semialdehyde-PC. Furthermore, moderate ions at 8.35 and 9.60 min (*m/z* 527 and 555) were identified to be $[M-57]^+$ of tBDMS derivatives of 8:0- and 10:0-dicarboxylate semialdehyde-PCs, respectively (Fig. 2B), based on their retention times and mass spectra. The relative amounts of dicarboxylate semialdehyde-PCs were assessed based on the peak areas of their $[M-57]^+$ as follows: 7:0, 0.48 ± 0.48%; 8:0, 18.7 ± 2.0%; 9:0, 78.6 ± 2.2%; and 10:0, 1.8 ± 0.9 (n = 3).

In order to examine whether the PAF-like lipids are artificially generated by the decomposition of PC hydroperoxides during the extraction of oxidized lipids, we added triphenylphosphine to the organic solvent for lipid extraction to reduce the PC hydroperoxides to the corresponding PC hydroxides. Upon FAB–MS a significant ion attributable to the protonated molecular ion of PC hydroperoxide was seen at *m/z* 790 in the triphenylphosphine-untreated sample. It was not found in the triphenylphosphine-treated

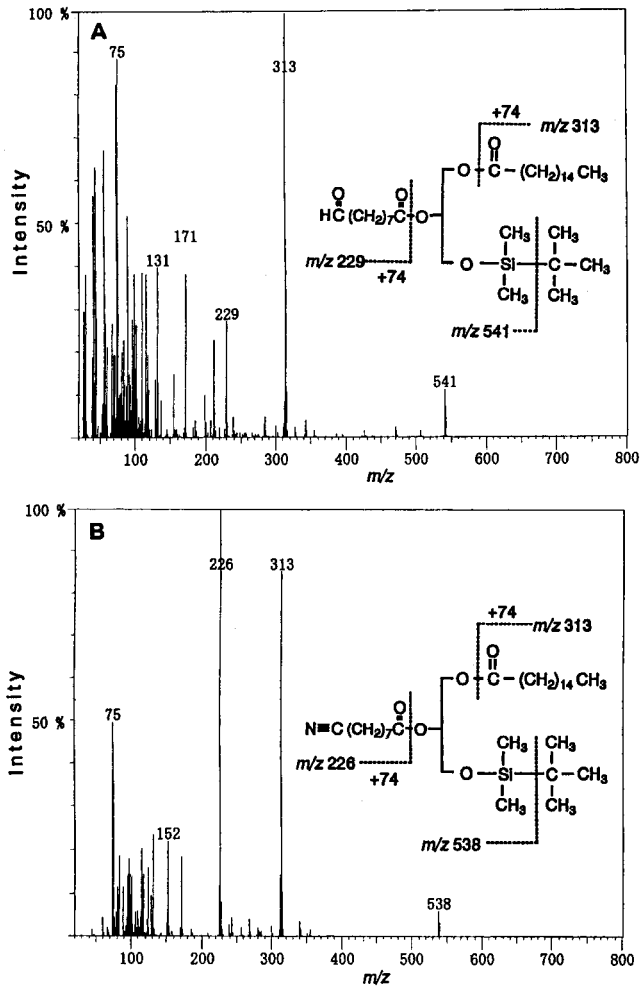

Fig. 3. Electron impact ionization–mass spectra of tBDMS derivatives of 9:0-dicarboxylate semialdehyde-PC (A) and 9:0-dicarboxylate seminitrile-PC (B).

sample. We observed a significant ion at *m/z* 772 that is assignable to the molecular ion of PC hydroxide. Thus, the PC hydroperoxide was reduced almost completely by triphenylphosphine under our conditions. Results by GC–MS showed that the triphenylphosphine-treated samples prepared from the lipoxygenase-oxygenated PC contained four species of monocarboxylate-PC (6:0, 0.75 ± 0.75%; 7:0, 15.2 ± 3.7%; 8:0, 83.6 ± 2.9% ; 9:0, 0.43 ± 0.43%, n = 3) and three species of dicarboxylate semialdehyde-PC (8:0, 1.4 ± 1.4%; 9:0, 92.6 ± 3.9%; 10:0, 6.0 ± 3.0%). Similar results were obtained with the samples not treated with triphenylphosphine, excluding the possibility that the PAF-like lipids are artifacts formed during lipid extraction.

Identification of PC containing an unsaturated acyl group with an aldehydic terminal generated by soybean lipoxygenase reactions

As described above, we identified saturated but not unsaturated species of dicarboxylate semialdehyde-PC in the lipoxygenase-treated sample. The unsaturated dicarboxylate semialdehyde-PCs may be less stable than saturated compounds. Therefore, we attempted to analyze the unsaturated derivatives by GC–MS after their treatment with methylhydroxylamine. The methylhydroxylamine-treated samples were hydrolyzed with phospholipase C and the resultant glycerides were converted to tBDMS derivatives. Typical ion-current profiles of various selected ions are shown in **Fig. 4**. We detected new peaks (d–h) in the chromatograms together with those of the tBDMS derivatives of 6:0-, 7:0-, 8:0-, and 9:0-monocarboxylate-PCs (data not shown) and 8:0-, 9:0-, and 10:0-dicarboxylate semialdehyde-PCs (peaks a, b, and c, respectively). Figure 3B shows an electron impact ionization mass spectrum of the unknown peak d eluted at 9.8 min. Characteristic ions at *m/z* 538, 313, 226, and 152 were assigned to $[M-57]^+$, $[R_1CO(palm-$

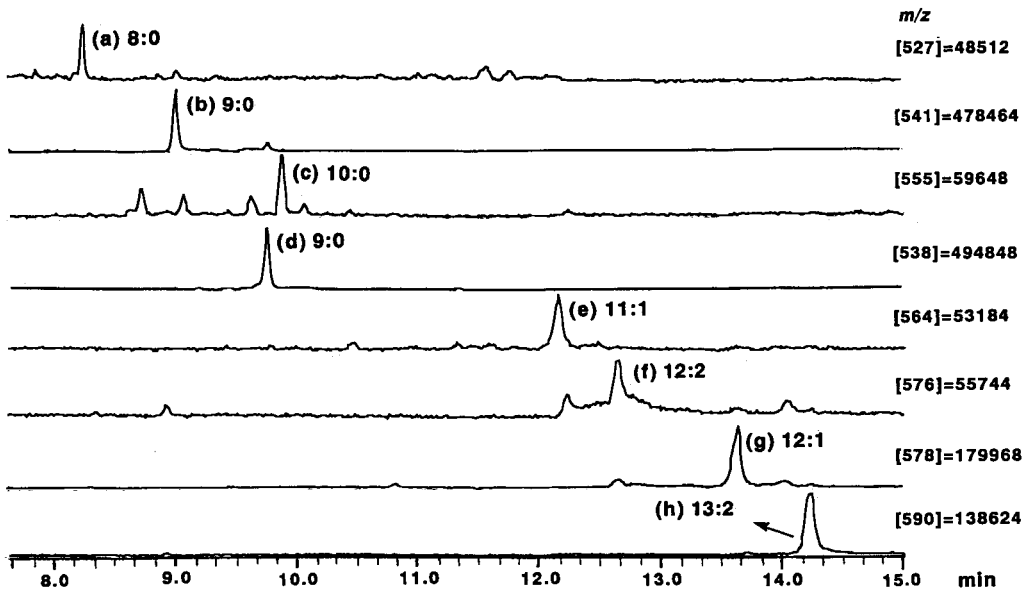

Fig. 4. Ion-current profiles of various ions corresponding to $[M-57]^+$ due to tBDMS derivatives of glycerides with a short chain dicarboxylate semialdehyde or dicarboxylate seminitrile formed during soybean lipoxygenase-catalyzed oxygenation of PC treated with *O*-methylhydroxylamine hydrochloride.

TABLE 1. Dicarboxylate semialdehyde-PCs and dicarboxylate seminitrile-PCs detected by GC–MS of linoleate-containing PC oxygenated with soybean lipoxygenase

	Dicarboxylate Semialdehyde-PC	Dicarboxylate Seminitrile-PC
	%	
8:0	3.7 ± 0.4	—
9:0	23.5 ± 5.4	32.9 ± 1.5
10:0	4.8 ± 0.6	—
11:1	—	5.6 ± 1.1
12:1	—	10.3 ± 0.8
12:2	—	3.4 ± 1.2
13:2	—	15.7 ± 3.8

Values are percentages of the sum of dicarboxylate semialdehyde-PCs and dicarboxylate seminitrile-PC calculated from peak areas of $[M-57]^+$ (means ± SEM, n = 4).

itoyl) + 74]$^+$, [R_2CO + 74]$^+$, and [R_2CO]$^+$, respectively. High resolution mass spectrometric analyses showed that $[M-57]^+$ had an elemental composition of $C_{30}H_{56}O_5NSi$ (observed mass 538.3946, calculated mass 538.3928), and that the observed mass of [R_2CO + 74]$^+$ was 226.1273, which was very similar to the calculated mass of 226.1263 for $C_{11}H_{20}O_2NSi$. In addition, the mass spectra of methyloxime derivatives of dicarboxylate semialdehyde-PC previously reported (4, 30) were different from that of the unknown peak d. These results suggested that R_2CO was an acyl group ($C_{9:0}$) with a nitrile terminal, but not with a terminal aldehyde that had been converted to a methyloxime derivative. Possibly, dicarboxylate seminitrile-containing PC (dicarboxylate seminitrile-PC) was produced by degradation of the methyloxime derivative of dicarboxylate semialdehyde-PC with loss of methanol during its hydrolysis with phospholipase C and derivatization with *tert*-butyldimethylchlorosilane. The general structure of dicarboxylate seminitrile-PC is shown in Fig. 1.

Similarly, unknown peaks e (12.2 min), f (12.7 min), g (13.7 min), and h (14.3 min) could be ascribed to $[M-57]^+$ of 11:1, 12:2, 12:1, and 13:2 species of tBDMS derivative of dicarboxylate seminitrile-PC, on the basis of their high resolution mass spectrometric analyses (data not shown). The most abundant species of dicarboxylate seminitrile-PC was 9:0, followed by 13:2 and then 12:1, as seen in **Table 1**, which shows percentages of the sum of dicarboxylate semialdehyde-PC and dicarboxylate seminitrile-PC calculated from their peak areas of $[M-57]^+$.

Analysis of PCs containing a short unsaturated monocarboxylate group generated by reactions with soybean lipoxygenase

Soybean lipoxygenase is known to catalyze stereospecific abstraction of a hydrogen atom attached to the carbon atom 11 of the linoleic acid moiety of PC and to produce the 13-hydroperoxide of PC (31). The 13-hydroperoxide of PC could then be decomposed to 13:2-dicarboxylate semialdehyde-PC and 12:2-monocarboxylate-PC via an alkoxyl radical of PC. As described above, we detected the unsaturated species of dicarboxylate semialdehyde-PC as tBDMS derivatives of dicarboxylate seminitrile-PC after the treatment with methylhydroxylamine, but not unsaturated monocarboxylate-PCs. We therefore conducted catalytic hydrogenation of the soybean lipoxygenase products of PC to convert unsaturated monocarboxylate-PC to heat-stable saturated forms. As analyzed by GC–MS, they contained many monocarboxylate-PCs with various chain lengths, although we could not completely exclude the possibility that the newly detected moncarboxylate-PCs were formed by elimination of functional groups of oxygenated products during the hydrogenation. The percentages of various saturated monocarboxylate-PCs were calculated from the peak areas of their $[M-57]^+$, as shown in **Fig. 5A**. The most abundant species was 8:0 followed by 12:0.

PAF-like lipids generated by oxygenation of PC with reticulocyte lipoxygenase

Linoleate-containing PC was incubated with rabbit reticulocyte lipoxygenase and its oxygenation products were analyzed by GC–MS as described with soybean lipoxygenase. The following monocarboxylate-PCs were detected in four separate experiments: 8:0 (94.2 ± 2.5%), 6:0 (2.2 ± 1.4%), and 7:0 (3.7 ± 1.0%). The predominant dicarboxylate semialdehyde-PC was 9:0 (73.1 ± 4.4%, n = 4), and two

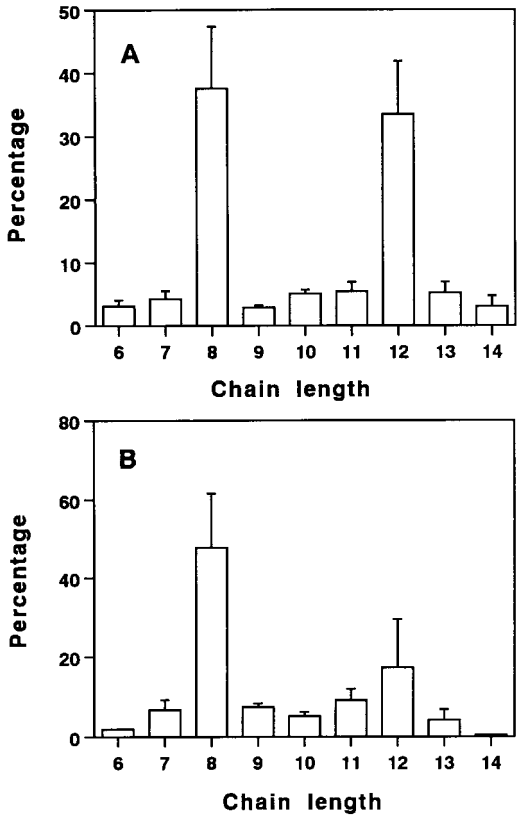

Fig. 5. Monocarboxylate-PCs detected by GC–MS after catalytic hydrogenation. PCs oxygenated with soybean (A) and reticulocyte (B) lipoxygenases were subjected to catalytic hydrogenation followed by phospholipase C hydrolysis and tBDMS derivatization. Values are expressed as means ± SEM (n = 5 for soybean and n = 3 for reticulocyte lipoxygenase) of percentages of the sum of dicarboxylate semialdehyde-PCs and dicarboxylate seminitrile-PCs calculated from peak areas of $[M-57]^+$.

TABLE 2. Dicarboxylate semialdehyde-PCs and dicarboxylate seminitrile-PCs detected by GC–MS of linoleate-containing PC oxygenated with reticulocyte lipoxygenase

	Dicarboxylate Semialdehyde-PC	Dicarboxylate Seminitrile-PC
	%	
8:0	3.8 ± 1.0	—
9:0	41.6 ± 0.7	31.4 ± 4.1
10:0	9.5 ± 3.5	—
11:1	—	0.4 ± 0.2
12:1	—	—
12:2	—	0.9 ± 0.7
13:2	—	10.9 ± 8.3

Values are percentages of the sum of dicarboxylate semialdehyde-PCs and dicarboxylate seminitrile-PCs calculated from peak areas of $[M-57]^+$ (means ± SEM, n = 3).

minor species (10:0, 19.9 ± 3.1% and 8:0, 2.3 ± 0.2%) were also identified. As shown in Fig. 5B, saturated monocarboxylate-PCs of various carbon chain lengths were detected in oxygenation products of PC subjected to catalytic hydrogenation, as in the incubation with soybean lipoxygenase. **Table 2** shows the results by GC–MS of dicarboxylate semialdehyde-PCs and dicarboxylate seminitrile-PCs upon methylhydroxylamine treatment of reticulocyte lipoxygenase products. These results were similar to those obtained in the experiments with soybean lipoxygenase.

Inhibitory effect of PAF-like PC with an oxononanoyl group on nitric oxide production by vascular smooth muscle cells

We selected 9:0-dicarboxylate semialdehyde (9-oxononanoyl)-PC for the evaluation of biological activity of PAF-like lipid because this aldehydic phospholipid was found to be a predominant PAF-like lipid generated through the lipoxygenase-catalyzed reactions of PC having an *sn*-2-linoleoyl residue. The structure of the standard 9:0-dicarboxylate semialdehyde-PC was determined by FAB–MS. The protonated molecular ion was observed at *m/z* 650 as well as the base peak of [phosphocholine + H]$^+$ at *m/z* 184. The *sn*-2-oxononanoyl group in the standard was confirmed by GC–MS after its conversion to a volatile tBDMS derivative by the method described above (data not shown).

As shown in **Fig. 6**, nitrite accumulation by rat aorta vascular smooth muscle cells stimulated with both lipopolysaccharide and interferon-γ was prevented by pretreatment with 9:0-dicarboxylate semialdehyde-PC in a concentration-dependent manner. This phospholipid failed to decrease the basal accumulation of nitrite even at 50 μm. In contrast to 9:0-dicarboxylate semialdehyde-PC, 50 μm lysophosphatidylcholine (1-palmitoyl), another proinflammatory phospholipid, augmented the nitrite accumulation induced by lipopolysaccharide and interferon-γ, but it did not affect the basal level of nitrite in the unstimulated cells (Fig. 6).

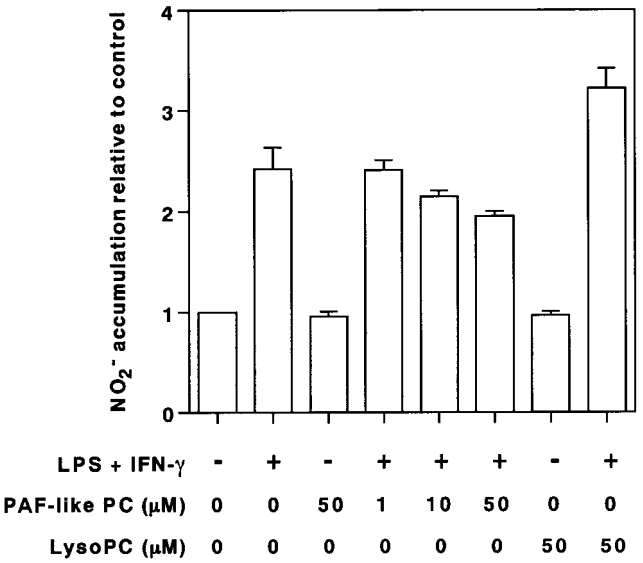

Fig. 6. Inhibition by 9:0-dicarboxylate semialdehyde-PC of the production of nitric oxide in vascular smooth muscle cells from rat aorta. Vascular smooth muscle cells were treated with 9:0-dicarboxylate semialdehyde-PC (PAF-like PC), lysophosphatidylcholine (LysoPC) or vehicle alone for 24 h, and then incubated with or without lipopolysaccharide (LPS, 1 μg/ml) and interferon-γ (IFN-γ, 25 units/ml) for 48 h. The accumulation of nitrite in the culture media was measured with the Griess reagent as described in Materials and Methods. Values are means ± SEM of three separate experiments done in triplicate.

DISCUSSION

Lipid hydroperoxide is known to be degraded to secondary products under various conditions (32, 33). β-Scission of carbon–carbon bonds of lipid hydroperoxides is thought to be formed via an alkoxyl radical (32–35). Previous reports showed that both pentane and fatty dienal (C_{13}) were produced by a homolytic cleavage mechanism when linoleate or its 13-hydroperoxide was incubated with soybean lipoxygenase or reticulocyte lipoxygenase under anaerobic conditions (36, 37). Indeed, the oxygen concentration was reported to determine the shares of dioxygenase and hydroperoxidase activities of soybean and reticulocytes lipoxygenases (38, 39).

Previously, we reported that four types of PAF-like lipids were generated from various PCs having an *sn*-2-polyunsaturated acyl group (4–6, 40). They were PCs having an *sn*-2-shortened fatty acyl group, the end of which was a methyl, hydroxyl, carboxyl, or aldehydic residue. These previous experiments were conducted under aerobic conditions, but we used hypoxic conditions in the present study with lipoxygenases because of the intracellular origin of lipoxygenases and their effects in cellular membranes where the oxygen tension appears to be low. We found that various monocarboxylate-PCs and dicarboxylate semialdehyde-PCs were formed through the reaction of linoleate-containing PC with only very high concentrations of lipoxygenases under hypoxic conditions. Their composition of the oxidatively fragmented PCs was more complicated than that of the low molecular weight oxidation products of linoleate or its 13-hydroperoxide incubated with lipoxygenase under anaerobic conditions (36, 37),

while the exact mechanism for the formation of the PAF-like lipids remains unknown. When the oxygen supply into the tissue is not sufficient, oxygen concentration may decrease below 20 μm and, in severe hypoxia, to 5–10 μm (41–43). Therefore, it is likely that the linoleate-containing PC is degraded to PAF-like lipids by lipoxygenase reactions in vivo under hypoxic conditions. Conversion of a ferrous ion in the catalytic center of lipoxygenase to a ferric ion may be coupled with the formation of an alkoxyl radical as suggested for soybean lipoxygenase-catalyzed degradation of fatty acid hydroperoxides (44), leading to a homolytic–heterolytic β-scission of C–C bonds (33). In addition to 13(*Z*,*E*)-hydroperoxide as a major product, small amounts of both 13(*E*,*E*)- and 9(*E*,*Z*; *E*,*E*)-hydroperoxides were produced from linoleate by soybean lipoxygenase under aerobic conditions (45). Non-enzymatic oxygenation of linoleate may be triggered by the alkyl radical of linoleate escaping from the catalytic center of the lipoxygenase. Previously, soybean lipoxygenase was reported to produce almost equal amounts of 13- and 9-hydroperoxides of linoleic acid at low oxygen concentrations (38). Similar observations were made when esterified unsaturated fatty acids in biomembranes and lipoproteins were treated with rabbit reticulocyte lipoxygenase (18, 46). The oxygenation products of esterified unsaturated fatty acids were reported to be less stereospecific and regiospecific than those of free fatty acids, and this tendency was prominent when the concentration of substrate was high (18, 46). Consistent with these previous studies, our results showed that the PAF-like products from linoleate-containing PC induced by soybean and reticulocyte lipoxygenases under hypoxic conditions were also less regiospecific. It is possible that the alkyl radicals of esterified unsaturated fatty acids were released easily from the active centers of the lipoxygenases, and reacted non-enzymatically with molecular oxygen to yield their peroxides and then hydroperoxides that can be decomposed to various PAF-like lipids by a homolytic and hetrolytic cleavage via the formation of an alkoxyl radical.

Previously we reported the formation of PAF-like lipids during the oxygenations of different PCs in an Fe^{2+}/EDTA/ascorbate system, and showed that the chain lengths of the *sn*-2-acyl groups in major saturated species of PAF-like lipids were dependent on the position of a double bond vicinal to the ester linkage to the glycerol backbone in the parent PC molecules. The chain length of the *sn*-2-acyl group in the major saturated monocarboxylate-PC was one methylene unit shorter than that in dicarboxylate semialdehyde-PC (4, 5, 40). Upon autoxidation by the Fenton reaction or ultraviolet irradiation, Passi et al. (47) reported a similar relationship between the chain lengths and the position of the double bond vicinal to the ester linkage of parent fatty acids. They also analyzed dicarboxylates after hydrolysis of mitochondrial phospholipids treated with oxyhemoglobin or ADP/Fe^{2+}/NADPH, and detected minor dicarboxylates with two methylene unit shorter chain lengths than those of major dicarboxylates generated with these induction systems of oxygenation. It was suggested that a 7-alkyl radical formed by the intramolecular hydrogen transfer of the 9-peroxide of PC was oxygenated to the 7-peroxide of PC and then converted to the 7-hydroperoxide of PC. The latter compound was further degraded to the 7-alkoxyl radical of PC, and yielded 7:0-dicarboxylate-containing PC via the formation of 7:0-dicarboxylate semialdehyde-PC. Itabe et al. (13) reported that a minor amount of 8:0-dicarboxylate semialdehyde-PC was formed together with 9:0-dicarboxylate semialdehyde-PC as the major species on oxygenation of linoleate-containing PC with Fe^{2+}/ascorbate (13). We also detected minor PAF-like lipids having an *sn*-2-acyl chain by one methylene unit shorter (monocarboxylate- and dicarboxylate-containing PCs) than those of the major species after oxygenation with Fe^{2+}/EDTA/ascorbate (6). Consistent with these results, our current study showed that there were both minor monocarboxylate-PCs and dicarboxylate semialdehyde-PCs having an *sn*-2-acyl group one or two methylene units shorter than those of major species upon the oxygenation of linoleate-containing PC with soybean and rabbit reticulocyte lipoxygenases. In preliminary experiments we found 2:0- and 3:0-monocarboxylate-PCs as minor species generated by oxygenation of arachidonate-containing PC with rabbit reticulocyte lipoxygenase. In this context, it should be mentioned that a small amount of 16:0-PAF itself was produced (1–8.6 pmol/mg protein) on oxygenation of different subspecies of low density lipoprotein with Cu^{2+} (48). These findings are potentially important, because PAF-like lipids containing an *sn*-2-acyl group with a shorter chain length have stronger biological activities (11). The exact mechanisms of formation of these minor PAF-like lipids require further studies, but we speculate that their formations involve intramolecular abstraction of a hydrogen atom by a peroxyl radical via an intermediate with 5- or 6-membered cyclic structure; possible pathways of the formation of 7:0-monocarboxylate-PC and 8:0-dicarboxylate semialdehyde-PC are shown in **Fig. 7**.

Oxidized low density lipoprotein is known as a key substance in the pathogenesis of atherosclerosis (1, 2). Recently, low density lipoprotein minimally oxidized by incubation with fibroblasts overexpressing 15-lipoxygenase was found to stimulate chemotaxis of monocytes in a PAF receptor-dependent manner (49). By electrospray ionization–mass spectrometry of purified lipids of minimally oxidized low density lipoprotein, Watson et al. (12) identified 5:0-dicarboxylate-containing PC and 5:0-dicarboxylate semialdehyde-PC as active components that activate endothelial cells to bind with monocytes, and suggested that they were generated by oxidative fragmentation of the arachidonate moiety of PC in the low density lipoprotein. These PAF-like lipids were found in atherosclerotic lesions of cholesterol-fed rabbits, and were also recognized by autoantibodies that reacted with oxidized low density lipoprotein epitope and immunostained atherosclerotic lesions in apoE null mice (12). Similarly, dicarboxylate semialdehyde-PC was recognized by a monoclonal antibody prepared with a human atherosclerotic plaque as an antigen source (50). The effect of oxidized low density lipoprotein on atherogenesis may be mediated, in part, by alteration in the production of nitric oxide by vascular cells (51).

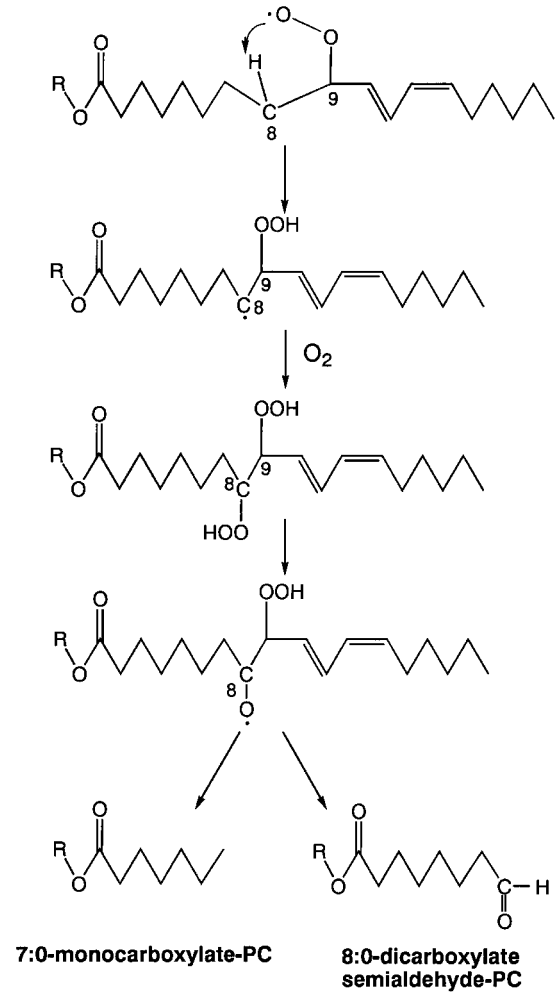

Fig. 7. Possible pathways of formation of 7:0-monocarboxylate-PC and 8:0-dicarboxylate semialdehyde-PC.

Nitric oxide exerts anti-atherogenic effects by inhibiting leukocyte adehesion to endothelial cells, platelet aggregation, and smooth muscle cell proliferation under conditions where local concentration of O_2^- is low (52). In mouse macrophages, the cooperative effect of lipopolysaccharide and interferon-γ on the expression of inducible nitric oxide synthase gene was suppressed by oxidized low density lipoprotein (53). The inhibitory effect of oxidized low density lipoprotein was attributed to the extractable lipid component, while its structure remained characterized. The present investigation clearly showed that 9:0-dicarboxylate semialdehyde-PC reduced the nitric oxide production by rat vascular smooth muscle cells activated by lipopolysaccharide and interferon-γ. The finding suggests that this oxidatively fragmented PC is one of possible active compounds in oxidized low density lipoprotein inhibiting the activity of inducible nitric oxide synthase. Our study revealed that lysophosphatidylcholine, another active component in oxidized low density lipoprotein, potentiated the generation of nitric oxide stimulated with both lipopolysaccharide and interferon-γ. This is consistent with a recent report of a similar effect of lysophosphatidylcholine on expression of inducible nitric oxide synthase by interleukin-1β in human glomerular mesangial cell line HMCL (54). The opposite effect of bioactive phospholipids potentially present in oxidized low density lipoprotein indicated that apparent biological effects of low density lipoprotein were dependent on not only cell type but also on the degree of its oxidation and, therefore, the composition of oxidized lipids. Thus, all the potentially active lipids in oxidized low density lipoprotein must be identified and their biological activities should be examined for evaluation of their contribution to the pathogenesis of atherosclerosis.

Both extracellular and intracellular PAF acetylhydrolases are known to hydrolyze oxidized phospholipids (21, 55) like paraoxonase (56) and lecithin:cholesterol acyltransferase (57) associated with high density lipoproteins. For initiation and maintenance of atherosclerosis, the imbalance is critical between the generation of bioactive oxidized phospholipids by various pro-oxidants including lipoxygenase and the capabilities of enzymes to remove the active phospholipids.

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