# Identification of short-chain oxidized phosphatidylcholine in human plasma

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**Abstract** Oxidized phospholipids have been recognized as potentially important compounds that carry biological activities similar to the platelet-activating factor, but their presence in biological tissue has not been firmly established. We developed a novel technique for the quantitative analysis of phospholipids with oxidized acyl chains. The method involves *1*) lipid extraction, *2*) chromatographic enrichment of phospholipids with short acyl chains, *3*) derivatization with 9-(chloromethyl)anthracene, *4*) solid-phase extraction of the derivatives, and *5*) reversed-phase HPLC with fluorescence detection. The technique was capable of measuring dicarboxylate-containing phosphatidylcholines (PCs) at the picomole level. The method was suited to monitor the generation of oxidized phospholipids from 1-palmitoyl-2-arachidonoyl-PC in the presence of $Fe^{2+}$/ascorbate. The new procedure was used to isolate lipids from human plasma that were identified as anthracene derivatives of short-chain oxidized PC on the basis of chromatographic, enzymatic, and spectroscopic evidence. The plasma concentration, determined with an internal standard (1-palmitoyl-2-suberoyl-PC), was 0.6 ± 0.2 μM (n = 11). The analytical method did not produce oxidation artifacts in significant amount. We concluded that human blood contains oxidatively fragmented PC in submicromolar concentration.—**Schlame, M., R. Haupt, I. Wiswedel, W.J. Kox, and B. Rüstow.** Identification of short-chain oxidized phosphatidylcholine in human plasma. *J. Lipid Res.* 1996. **37:** 2608–2615.

**Supplementary key words** anthracene • fluorescence • free radical • high performance liquid chromatography • peroxidation • phospholipid • platelet activating factor

Oxidative fragmentation of unsaturated PC generated new PC species, of which some have biological activity (1–3). The activity resembled that of 1-O-alkyl-2-acetyl-PC, the platelet-activating factor (1–3), which is a potent pro-inflammatory mediator (4). Recently, it was demonstrated that acute inflammation, induced by the platelet-activating factor, can be reversed by recombinant platelet-activating factor acetylhydrolase (5), an enzyme that degrades both the authentic platelet-activating factor and oxidatively fragmented PC (6, 7).

When PC is peroxidized, a myriad of species is produced (7, 8). Some of the products could be identified as short-chain PCs with a hydroxyl, aldehyde, or carboxyl group in the ω-position (9–11). Those species are formed by radical-mediated degradation of unsaturated acyl groups, which are usually located in the *sn*-2 position of mammalian phospholipids. Due to the heterogeneity of the PC oxidation products, it has not been possible to identify the molecular species responsible for biological activities like neutrophil activation (1) or platelet aggregation (10). A systematic study of synthetic PCs with short *sn*-2 chains revealed that the length of the *sn*-2 chain was inversely proportional to the platelet aggregating activity and that 1-O-alkyl derivatives were more potent than 1-acyl derivatives (10).

Lipid peroxidation has been implicated in human diseases such as inflammation, atherosclerosis, or ischemia, and has mainly been documented by the measurement of the end products of lipid peroxidation, i.e., thiobarbituric acid reactive substances (12). However, in light of the potential activities of oxidatively fragmented PC, it seems indispensable to search for the occurrence of intact phospholipids that are intermediates of the peroxidation pathway. Tsukatani et al. (13) isolated a hypotensive lipid fraction from bovine brain that was later characterized as *sn*-2-short-chain PC, a species that can be generated by PC peroxidation (for a review see ref. 14). While this was the first account of oxidatively fragmented PC in vivo, the lengthy procedure used for lipid preparation raises doubt whether the novel PC species were authentic brain substances or peroxidation products generated during the isolation. Other evidence of the occurrence of oxidatively fragmented PC

Abbreviations: HPLC, high performance liquid chromatography; PC, 3-*sn*-phosphatidylcholine (1,2-diacyl-*sn*-glycero-3-phosphorylcholine).

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in biological material was obtained in endothelial cell culture, where a membrane PC-derived, neutrophil-stimulating activity was generated upon hydroperoxide treatment (2). Moreover, immunohistochemical data suggested that oxidized PC is present in foam cells of human atherosclerotic lesions (15). Despite such circumstantial evidence, a direct determination of oxidatively fragmented PC in human tissue has not been accomplished. We developed a new analytical method to identify oxidized PC species by fluorescence-HPLC, and demonstrated that short-chain oxidized PCs are present in the plasma of human blood.

## METHODS

### Lipid syntheses and derivatization techniques

We synthesized three PC species with short chain dicarboxylates in *sn*-2 position and a number of derivatives containing anthryl-9-methyl and silyl residues. The structures of the synthetic lipids are defined in Fig. 1 and Table 1. 1-Palmitoyl-2-succinyl-PC (compound I) and 1-palmitoyl-2-glutaroyl-PC (compound II) were synthesized by esterification of 1-palmitoyl-2-lyso-PC with the symmetric anhydride of the respective dicarbonic acid. Ten mg of 1-palmitoyl-2-lyso-PC was stirred with 20 mg anhydride in 1 ml of dry pyridine for 24 h at room temperature. The reaction mixture contained catalytic amounts of 4-(dimethylamino)pyridine. For the synthesis of 1-palmitoyl-2-suberoyl-PC (compound III), 50 mg of suberic acid was activated by some grains of 1,1′-carbonyldiimidazole in 2.5 ml benzene–methanol 5:1 (v/v) for 30 min. This solution was added to 5 mg of dried 1-palmitoyl-2-lyso-PC and incubated at 37°C for 1 h. The synthetic compounds were purified by a protocol comprising *i*) Bligh-Dyer extraction (16), *ii*) HPLC on $C_{18}$-Nucleosil (10 μm) eluted by methanol–water–acetonitrile 905:70:25 (v/v/v) containing 25 mM $HCOONH_4$, and *iii*) thin-layer chromatography on silica gel 60 plates developed by chloroform–methanol–32% ammonia 65:25:8 (v/v/v). The presence of a free carboxyl group in I–III conferred characteristic migration behavior on thin layers. With acidic solvents, the compounds migrated between PC and lyso-PC, whereas with alkaline solvents, they migrated below lyso-PC.

The fluorescence-labeled derivatives Ia–IIIa were obtained by esterification of the terminal carboxyl groups with 9-(chloromethyl)anthracene. Two hundred μl of a reagent, containing 0.1 M 9-(chloromethyl)anthracene in benzene–triethylamine 4:1 (v/v), was added to the dried phospholipids (I, II, or III) and the mixture was heated to 70°C for 2 h in a sealed microreaction vessel. The products were purified by solid-phase extraction in 1-ml columns of silica gel Si60 (Lichroprep, 15–25 μm, Merck) developed first by 4 ml of chloroform–triethylamine 90:10 (v/v) and then by 4 ml of methanol–32% ammonia 95:5 (v/v). Compounds Ia–IIIa were obtained in the second eluate. Further purification was achieved by gradient HPLC on reversed-phase silica as described below.

Compounds Ib–IIIb were generated by phospholipase C hydrolysis of Ia–IIIa. The substrates were dissolved in 2 ml of diethylether–ethanol 98:2 (v/v) and stirred for 24 h in the presence of 1 ml buffer containing 0.45 M HEPES (pH 7.2), 0.02 M $CaCl_2$ and 2 units of phospholipase C (*Clostridium perfringens*, Sigma Chemical Corp.). Compounds Ib–IIIb were purified by chloroform extraction and gradient HPLC (see below).

The silyl derivatives Ic–IIIc were generated from dried compounds Ib–IIIb by addition of a reagent containing 1 M imidazole and 1 M *tert*-butyldimethylsilylchloride in dimethylformamide. The reaction was performed at 37°C for 5 h and the products were purified by extraction into n-hexane followed by gradient HPLC (see below).

### Mass spectroscopy

A Finnigan MAT SSQ 710 quadruple mass spectrometer was used for both electron impact mass spectroscopy and negative ion chemical ionization mass spectroscopy. The mass spectrometer was linked to a DEC-station 5000/33 for data processing. Samples were applied by direct exposure probe (DEP). The DEP used a detachable rhenium filament that was programmed as follows: initial filament temperature 20°C, held for 1 min, followed by an increase to 1200°C at a rate of 500°C/min. The final temperature was held for 1 min. Negative ion chemical ionization was performed in the presence of methane. The ion source temperature was 150°C and the electron energy 70 eV.

### Processing of blood samples

Venous blood from healthy volunteers was drawn into heparin-coated syringes and immediately spun to sediment cells. A volume of 0.5 ml plasma was withdrawn and supplemented by the internal standard (compound III) to a final concentration of 1.0 μM. Lipids were extracted from the plasma sample (16) and subsequently separated on a $C_{18}$-reversed-phase column (Nucleosil, 10 μm, 4.6 × 200 mm) with the mobile phase methanol–water–acetonitrile 905:70:25 (v/v/v) containing 25 mM $HCOONH_4$. This procedure separated short-chain PC, like the platelet-activating factor and related compounds, from long-chain PC. Short-chain PC eluted in the first 15 ml and was collected and reextracted into chloroform. The lipids of this fraction were derivatized

with 9-(chloromethyl)anthracene and the reaction mixture was purified by solid phase extraction exactly as described above. The derivatized lipids were finally dissolved in 0.25 ml methanol.

### Gradient-HPLC with fluorescence detection

Fifty- to 100-µl aliquots of the derivatized lipids were separated on a $C_{18}$-reversed-phase HPLC column (Spherisorb, 5 µm, 4.6 × 250 mm) developed by gradient elution. The solvents contained 20 mM choline chloride in methanol–water 90:10 (v/v) (solvent A) or methanol–acetonitrile 90:10 (v/v) (solvent B), respectively. A Merck/Hitachi integrated HPLC system was used and controlled by the interface D-6000. A linear gradient was run from 100% A to 100% B in 60 min by the pump L-6200A operated at a flow rate of 1.0 ml/min. The solvents passed a degasser (Degasys DG-1310) before entering the pump. Samples were injected by the autosampler AS-2000A and the column temperature was kept at 40°C by the thermostat L-5025. The HPLC system was connected to the fluorescence monitor RF-551 (Shimadzu) operated in high sensitivity mode at an excitation wavelength of 360 nm and an emission wavelength of 460 nm, with a response time of 0.5 sec.

### Straight-phase HPLC

Phospholipid class separation was performed on a tandem column eluted by a water gradient in acetonitrile. The first column (8 × 60 mm) contained Lichrosorb Si-100 (5 µm) and the second column (4 × 250 mm) contained Lichrosorb Diol (5 µm). Solvent A was acetonitrile and solvent B was acetonitrile–water 4:1 (v/v). The gradient was raised to 15% B from 0 min to 5 min, to 23% B from 5 min to 8 min, to 70% B from 8 min to 12 min, and to 75% B from 12 min to 13 min. The proportion of 75% B was maintained until 27 min, after which the gradient was increased to 95% B within 3 min. The flow rate was 1.5 ml/min.

### Phospholipase experiment

Anthracene-derivatized lipids from human plasma were subjected to phospholipase treatment as follows. The lipids were dried under a stream of nitrogen and redissolved in 0.7 ml diethylether–ethanol 98:2 (v/v). Then, 0.5 ml of buffer, containing the phospholipase, was added and the mixture was stirred overnight in a tightly sealed tube. The buffer contained 0.45 M HEPES (pH 7.2), 0.02 M $CaCl_2$, and either 0.5 mg of phospholipase C from *Clostridium perfringens* or 70 µg of phospholipase C from *Bacillus cereus*. In another experiment, the buffer contained 0.1 M boric acid, 0.05 M HEPES, 0.05 M NaCl, and 2.5 mM $CaCl_2$ (pH 7.4) with 150 units phospholipase $A_2$ from *Naja mocambique mocambique*. All phospholipases were purchased from Sigma Chemical Company. After phospholipase treatment, the lipids were extracted into chloroform–methanol and analyzed by gradient-HPLC with fluorescence detection.

### Treatment of 1-palmitoyl-2-arachidonoyl-PC

The polyunsaturated lipid 1-palmitoyl-2-arachidonoyl-PC was obtained from a commercial source (Sigma Chemical Corporation, product number P 0319) and either oxidized by $Fe^{2+}$/ascorbate/EDTA or chromatographed for further purification. For the oxidation, 3 µmol of the lipid was dispersed in 8 ml of water (adjusted to pH 4 by HCl) by sonication. After the addition of 25 µM $FeSO_4$, 50 µM ascorbic acid, and 25 µM EDTA, the mixture was incubated for 3 h at room temperature. Then, 0.2 ml of 0.1 M HCl was added and the lipids were extracted into chloroform–methanol (16). HPLC purification of commercial 1-palmitoyl-2-arachidonoyl-PC was achieved on a $C_{18}$-reversed-phase Nucleosil column (10 µm, 4.6 × 200 mm) developed by methanol–water–acetonitrile 905:70:25 (v/v/v) containing 25 mM $HCOONH_4$. 1-Palmitoyl-2-arachidonoyl-PC eluted after a solvent volume of 42 ml.

### Miscellaneous methods

Yields of synthetic lipids were measured by quantitation of the phosphate content after ashing (17) and by gas chromatographic determination of the palmitoyl groups. Anthracene-labeled lipids were visualized on thin-layer plates under UV light of 254 nm (DESAGA UVIS lamp, Heidelberg, Germany). Phospholipids were stained on thin-layer plates by a molybdate spray reagent (18). Absorption spectroscopy of the lipid derivative, isolated from human plasma, was performed in methanolic solution, using the automated UV-visible recording spectrophotometer UV-160A (Shimadzu).

## RESULTS

### Quantitative analysis of dicarboxylate-PC by fluorescence-HPLC

Dicarboxylate-PC was identified among the oxidation products of unsaturated PC, and some evidence suggested it has certain biological activities (10, 19). We synthesized three dicarboxylate-PCs (I, II, and III) with different chain lengths in the dicarboxylate residue, and formed derivatives containing the anthryl-9-methyl group and the *tert*-butyldimethylsilyl group, respectively **(Fig. 1, Table 1).** The identities of the compounds were confirmed by thin-layer chromatographic migration behavior (see Methods section), phosphate staining, and negative ion chemical ionization mass spectroscopy of the silylated anthracene-9-methyl esters (compounds Ic, IIc, and IIIc). In the mass spectrum of each silylated

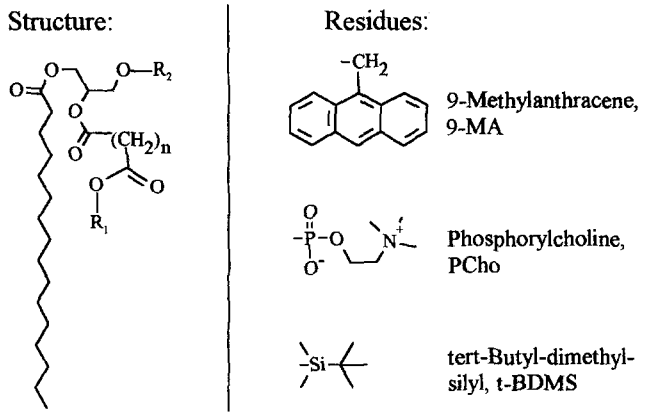


**Fig. 1.** Structure of the synthetic lipids. Individual compounds are listed in Table 1.

compound, we found the $[M + 32]^-$ ion (Ic: $m/z$ 767; IIc: $m/z$ 781, IIIc: $m/z$ 823) which was most likely caused by $O_2^-$ addition to the anthracene moieties. The base peaks of the spectra were the $[M-192]^-$ fragments generated by deletion of the anthracene-9-methyl residue.

To study the occurrence of dicarboxylate-PC in tissue samples, we aimed to develop a sensitive method of quantitation. The presence of a non-esterified ω-carboxyl group confers specific reactivity to dicarboxylate-PCs. Novel fluorescence derivatives were produced by reaction of the free carboxyls with 9-(chloromethyl)anthracene, forming compounds Ia, IIa, and IIIa (Fig. 1, Table 1). After purification by solid-phase extraction, the derivatives were applied to reversed-phase HPLC with gradient elution and fluorescence detection. **Figure 2A** shows superimposed chromatographic traces of compounds Ia, IIa, and IIIa. As anticipated, retention times increased with the number of carbon atoms. Ia and IIa eluted between 15 and 17 min in close proximity, while IIIa was better separated (retention time about 21 min). The peaks recovered between 2 and 7 min were byproducts of the derivatization reaction. Fluorescence was recorded at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. There was a linear relation between the fluorescence yield and the molar amount (Fig. 2B), so the technique was well suited to quantify the anthracene-labeled phospholipids. As demonstrated in Fig. 2C, the fluorescence-HPLC method was able to detect quantities as low as 1 picomole.

TABLE 1. Structure of the synthetic lipids

| Compound | n | $R_1$ | $R_2$ | RRT |
|---|---|---|---|---|
| I | 2 | | | n.d. |
| II | 3 | H | PCho | n.d. |
| III | 6 | | | n.d. |
| Ia | 2 | | | 0.76 |
| IIa | 3 | 9-MA | PCho | 0.78 |
| IIIa | 6 | | | 1.00 |
| Ib | 2 | | | 0.83 |
| IIb | 3 | 9-MA | H | 0.86 |
| IIIb | 6 | | | 1.12 |
| Ic | 2 | | | 2.02 |
| IIc | 3 | 9-MA | *t*-BDMS | 2.07 |
| IIIc | 6 | | | 2.40 |

For explanation see Fig. 1. Relative retention times (RRT) of the compounds were measured by reversed-phase HPLC with gradient elution and fluorescence detection. The RRTs were related to the retention time of compound IIIa; n.d., not determined.

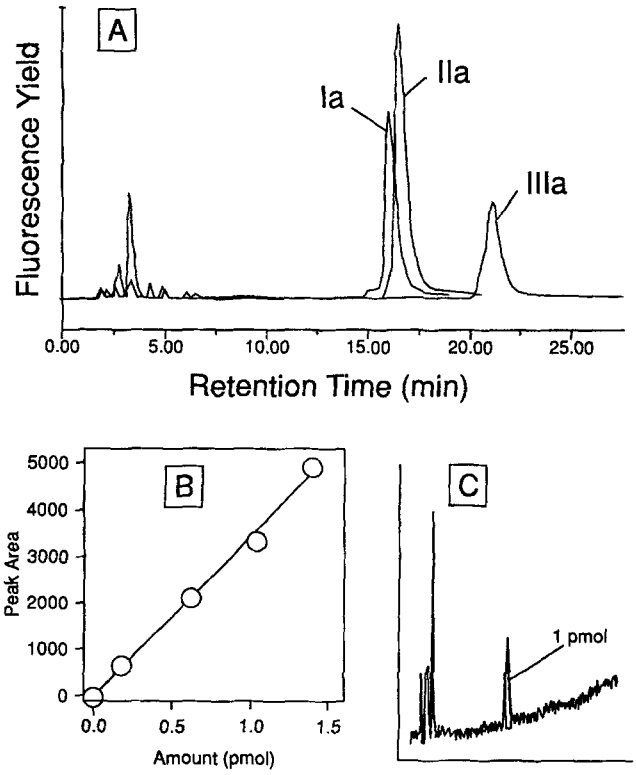


**Fig. 2.** Determination of dicarboxylate-PC by fluorescence-HPLC. A: Superimposed chromatographic traces of Ia (5.9 nmol), IIa (9.8 nmol), and IIIa (4.8 nmol). B: Relation between the molar amount and the area of the fluorescence peak of compound Ia. The peak area is given in arbitrary units. C: Chromatographic trace of 1 picomole of compound Ia.

### Oxidative fragmentation of unsaturated PC in vitro

The polyunsaturated lipid 1-palmitoyl-2-arachidonoyl-PC was shown to be degraded to dicarboxylate-PC and other short-chain PCs upon exposure to free radicals (1, 10). 1-Palmitoyl-2-arachidonoyl-PC was treated with $Fe^{2+}$/ascorbate/EDTA and subsequently analyzed by fluorescence labeling and HPLC as described above. Oxidation by $Fe^{2+}$/ascorbate generated products whose anthracene-9-methyl derivatives eluted in several peaks between 11 and 19 min **(Fig. 3A).** The largest peak had exactly the same retention time as compound IIa, consistent with the formation of compound II from 1-palmi-

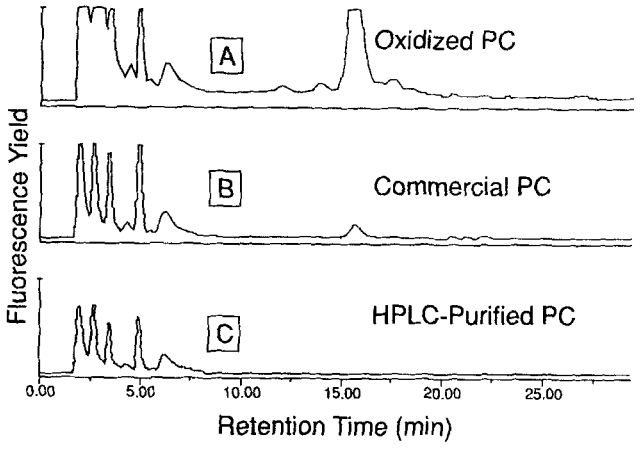


**Fig. 3.** Analysis of oxidation products of 1-palmitoyl-2-arachidonoyl-PC. Different preparations of 1-palmitoyl-2-arachidonoyl-PC (300 nmol each) were analyzed for lipid oxidation products. The analytical procedure included the enrichment of short-chain PCs, derivatization with 9-(chloromethyl)anthracene, purification of the derivatives by solid phase extraction, and fluorescence-HPLC. A: 1-palmitoyl-2-arachidonoyl-PC after $Fe^{2+}$/ascorbate oxidation. B: 1-Palmitoyl-2-arachidonoyl-PC as supplied by Sigma Chemical Corporation. C: 1-Palmitoyl-2-arachidonoyl-PC purified by HPLC.

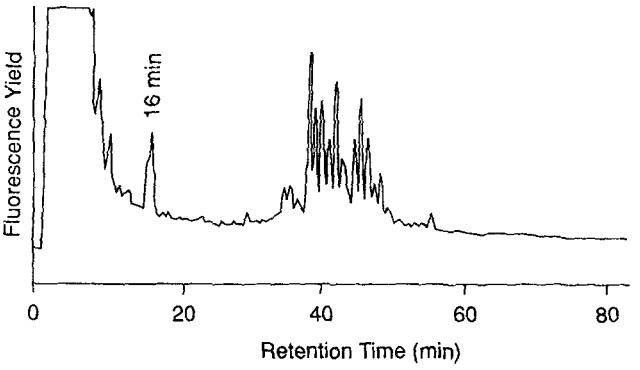


**Fig. 4.** Fluorescence-HPLC analysis of human plasma. Short-chain lipids were isolated from human plasma and derivatized with 9-(chloromethyl)anthracene. The derivatives were purified by solid phase extraction and separated by reversed-phase HPLC with fluorescence detection.

toyl-2-arachidonoyl-PC. When the untreated substrate, the commercial 1-palmitoyl-2-arachidonoyl-PC, was subjected to the analysis, small amounts of a fluorescence-labeled product were recovered at a retention time of 16 min (Fig. 3B). Again, this product had the same retention time as compound IIa. However, HPLC purification of 1-palmitoyl-2-arachidonoyl-PC removed the byproduct (Fig. 3C), suggesting that it had accumulated during handling, shipment, and storage of the sample.

### Identification of short-chain oxidized PC in human plasma

Lipids from human plasma were extracted into chloroform–methanol, and a phospholipid subclass was isolated by HPLC. This subclass contained the short-chain PCs, a fact that was demonstrated by separate chromatographic runs of short-chain standard lipids, like the *acetyl*-$^3$H-labeled platelet-activating factor and the synthetic compounds I, II, and III. The isolated lipids were derivatized with 9-(chloromethyl)anthracene, purified by solid phase extraction, and subsequently analyzed by fluorescence-HPLC. One fluorescence peak appeared at a retention time of about 16 min while a number of other peaks were observed between 35 and 55 min **(Fig. 4).** The latter group represented anthryl-9-methyl esters of contaminating free fatty acids, as shown by the use of standard fatty acids and gas chromatographic analysis, respectively. However, attention was directed to the 16-min peak because its retention time was similar to that of compound IIa. Thus, the peak was isolated and subjected to further studies. Absorption spectroscopy of the material, dissolved in methanol, revealed maxima at 385, 366, 347, and 329 nm, consistent with the presence of an anthracene moiety. The electron impact mass spectrum of the isolated material was rather complex. However, significant mass signals at $m/z$ 313 ($C_{15}H_{31}$–CO–O–$CH_2$–CHOH–$CH_2^+$), $m/z$ 299 ($C_{15}H_{31}$–CO–O–$CH_2$–$CH_2O^+$) and $m/z$ 239 ($C_{15}H_{31}$–$CO^+$) were indicative of the occurrence of a palmitoyl-glycerol moiety. A similar pattern was found in the mass spectra of the standard compounds I, II, III, Ia, IIa, and IIIa.

The 16-min peaks from several blood samples were pooled and loaded on a silica gel 60 high performance thin-layer plate. The plate was developed by chloroform–methanol–32% ammonia 65:35:8 (v/v/v), dried and visualized under UV light, revealing a fluorescing material that comigrated with standard PC from egg yolk ($R_f$ = 0.43). Although this material ran as a single spot, it contained at least two components which were incompletely resolved by rechromatography on a $C_{18}$-silica column (**Fig. 5A,** upper trace). When compound IIa was added to the material, it precisely coeluted with the second component of the peak (Fig. 5A, lower trace). Further evidence of the chromatographic identity with PC came from a straight-phase HPLC method for phospholipid class separation (Fig. 5B). The isolated material eluted close to long-chain PC, nearly identical to compound IIa, and well separated from other phospholipids, such as phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, and sphingomyelin.

To prove its phospholipid nature, the 16-min peak was treated with phospholipases. All phospholipases used, including phospholipase $A_2$ from *Naja mocambique mocambique,* phospholipase C from *Clostridium perfringens,* and phospholipase C from *Bacillus cereus,* hy-

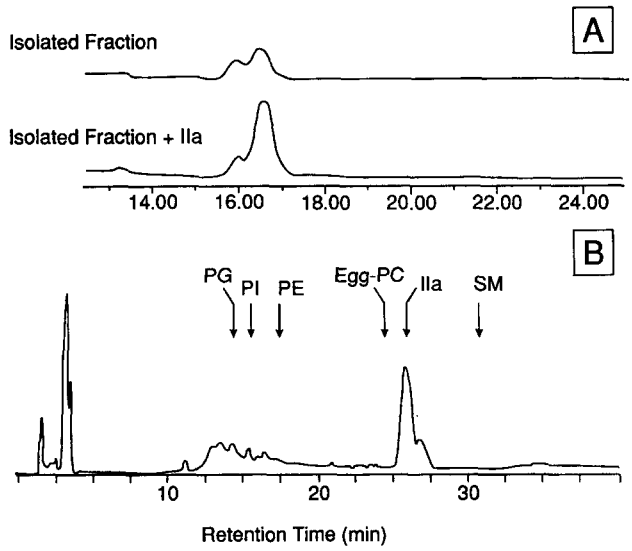


**Fig. 5.** Chromatographic behavior of the isolated plasma lipid derivatives. The 16-min peak from Fig. 4 was isolated and subjected to further chromatography. A: Rechromatography on $C_{18}$ reversed-phase. The lower trace shows a run in the presence of compound IIa. B: Rechromatography by straight-phase HPLC on two consecutive columns containing Lichrosorb Si-100 and Lichrosorb Diol, respectively. The method is suitable for phospholipid class separation. The position of standard phospholipids in the chromatogram is shown. PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; SM, sphingomyelin.

drolyzed the isolated 16-min peak to a considerable extent **(Table 2).** In the phospholipase C incubations, the diacylglycerol product could be identified by fluorescence-HPLC. Its retention time was shifted 1–2 min after the substrate peak. A similar shift of retention time was observed for compounds Ib–IIIb, the diacylglycerol products of Ia–IIIa (Table 1). The susceptibility to the PC-specific phospholipase C from *Clostridium perfringens* corroborated that the isolated material was, in fact, PC.

### Quantification of short-chain oxidized PC in human plasma

To estimate the concentration of short-chain oxidized PC from the fluorescence data, an internal standard was required. Compound III proved to be a suitable standard for the measurement of plasma samples because: it was a short-chain PC; it reacted with 9-(chloromethyl)anthracene; it was not an endogenous component of human blood; and the derivative IIIa was well separated from other fluorescence peaks in the HPLC chromatogram. The recovery of IIIa was only 70–80%, and the estimation of the concentration of endogenous short-chain PCs was based on the assumption that their anthracene derivatives had the same recovery as IIIa. In support of this assumption, we obtained similar recoveries for the fluorescence derivatives Ia, IIa, and IIIa, suggesting that various short-chain PCs show identical behavior.

Plasma was obtained from freshly drawn human blood and compound III was added to a final concentration of 1.0 μM prior to lipid extraction. To determine the concentration of the endogenous lipids, their peak area was referred to the peak area of IIIa in the analytical fluorescence chromatogram. Eleven healthy adults were included in this study, and the characteristic peak for oxidized PC was found in each plasma sample **(Table 3).** The mean concentration was estimated to be 0.6 ± 0.2 μM. Relative retention times, with respect to the standard compound IIIa, were in a narrow range (0.78–0.82, Table 3). The consistency of relative retention times in 11 blood samples suggested that similar, or perhaps identical, oxidized lipids were present in the individuals studied.

Because lipids may undergo oxidative degradation, it is essential to process the blood samples as quickly as possible. We limited the time between drawing the blood sample and lipid extraction to 7 min. To assess the impact of delayed sample processing, we incubated three blood samples at room temperature for an addi-

TABLE 2. Phospholipase susceptibility of the isolated plasma lipids

| Treatment | Lipid Recovery | Hydrolysis |
|---|---|---|
| | *pmol* | % |
| No enzyme (Control) | 324 ± 30 | 0 |
| Phospholipase $A_2$ (*Naja mocambique m.*) | 155 ± 21 | 52 |
| Phospholipase C (*Clostridium perfringens*) | 159 ± 24 | 51 |
| Phospholipase C (*Bacillus cereus*) | 54 ± 8 | 83 |

The 16-min peak from Fig. 4 was isolated and subsequently treated by phospholipase. After the reaction, lipid recovery was measured by HPLC with fluorescence detection. In the control experiment, lipids were incubated in the absence of enzyme.

TABLE 3. Concentration of short-chain oxidized PC in human blood plasma

| Volunteer No. | Sex | Age | Concentration | Relative Retention Time |
|---|---|---|---|---|
| | | *yr* | μ*M* | |
| 1 | M | 36 | 0.325 | 0.798 |
| 2 | M | 37 | 0.537 | 0.781 |
| 3 | M | 38 | 0.947 | 0.783 |
| 4 | M | 37 | 0.372 | 0.780 |
| 5 | M | 38 | 0.570 | 0.796 |
| 6 | F | 23 | 0.689 | 0.806 |
| 7 | M | 21 | 0.323 | 0.808 |
| 8 | F | 40 | 0.631 | 0.820 |
| 9 | M | 60 | 0.503 | 0.783 |
| 10 | F | 33 | 0.714 | 0.780 |
| 11 | M | 38 | 0.527 | 0.805 |
| Mean ± SD | | 36 ± 10 | 0.56 ± 0.19 | 0.79 ± 0.01 |

The concentrations were determined with compound III as internal standard. The relative retention times are related to compound IIIa; SD, standard deviation.

tional 40 min before extraction. After this incubation, the concentration of short-chain oxidized PC was increased by 52 ± 30%.

## DISCUSSION

In this paper, we report for the first time the chemical determination of oxidatively fragmented PC in human blood. The presence of oxidized phospholipids in human tissue has been suspected, because they are the missing link between free radical initiated degradation of unsaturated phospholipids and the appearance of end stage oxidation products. However, it has not been clear at what concentration the putative phospholipid intermediates accumulate in vivo. This question is of foremost importance considering the potential biological activity of oxidized phospholipids.

We developed a novel analytical technique for the detection of oxidized phospholipids that is based on the chromatographic selection of short-chain lipids and the subsequent derivatization of oxidized functional groups with 9-methylanthracene. The method permits quantitation of anthracene-labeled lipids by fluorescence-HPLC and has a lower detection limit of about 1 picomole (Fig. 2). Due to the high sensitivity of the technique, it may potentially be used for the diagnostic assessment of lipid oxidation in various diseases or for characterization of lipid degradation in food and lipid-containing pharmaceutical products.

The method was first used to monitor the accumulation of oxidized PC from a single species of unsaturated PC (Fig. 3). After treatment of 1-palmitoyl-2-arachidonoyl-PC by $Fe^{2+}$ ascorbate, new products appeared in the fluorescence-HPLC chromatogram, one of which had the same retention time as compound IIa. Tanaka et al. (10) already reported the formation of compound II from 1-palmitoyl-2-arachidonoyl-PC, measured by gas chromatography–mass spectroscopy, in a similar oxidation system. However, we discovered that even the untreated, commercial preparation of 1-palmitoyl-2- arachidonoyl-PC contained detectable amounts of oxidized phospholipids. The fact that no oxidation products were found in the HPLC-purified 1-palmitoyl-2-arachidonoyl-PC demonstrates that it is possible to obtain a pure preparation of this lipid. A pure preparation of the unsaturated PC was an important negative control, because the manipulations involved in the analytical procedure can potentially result in lipid oxidation. As no oxidized phospholipids were recovered from the pure preparation, it is clear that the analytical technique did not generate oxidation artifacts from previously non-oxidized lipids.

The analytical technique was used to isolate a specific class of lipids from human blood. The lipids were identified as anthryl-labeled short-chain oxidized PC based on the following observations. *i*) The material was degradable by various phospholipases (Table 2), indicating its phospholipid nature. *ii*) The head group was shown to be choline, because one of the phospholipases was PC-specific, and because of the migration patterns in thin-layer chromatography, straight-phase HPLC, and reversed-phase HPLC (Fig. 5). *iii*) At least one acyl chain of the isolated PC fraction was short because of the retention time in fluorescence-HPLC, and because short-chain PCs were selected in the initial chromatographic step. *iv*) The presence of oxidized groups was a requirement for the reaction with 9-(chloromethyl)anthracene. The existence of an anthryl moiety was shown by characteristic absorption maxima.

The 16-min peak from human blood (Fig. 4) had a similar retention time as the PC species generated by peroxidation of 1-palmitoyl-2-arachidonoyl-PC (Fig. 3), corroborating the notion that the 16-min peak represented oxidized PC. The shape of the 16-min peak (Fig. 5A) indicated a heterogeneous composition of this fraction, comprising at least two components. This may reflect chain heterogeneity, including different chain lengths or oxidized groups, respectively. The incomplete degradation of the isolated PC by phospholipases (Table 2) may have been caused by certain acyl chains incompatible with phospholipase hydrolysis. On the other hand, it is a common observation that enzyme-catalyzed phospholipid degradation provides low yields in vitro.

The plasma concentration of short-chain oxidized PC, calculated from the fluorescence yield, was between 0.3 and 1.0 μM (Table 3). The calculation was based on the assumption that the anthryl/phospholipid ratio of the blood-derived lipids was identical to that of the internal standard (1:1). While this is the most likely case, the presence of more than one anthryl group per PC is possible. Thus, the actual phospholipid concentration might be lower than that shown in Table 3.

The presence of short-chain oxidized PC in submicromolar concentration is evidence for ongoing fragmentation of blood PC in healthy subjects. Neither the chemistry of this fragmentation nor the precise structures of the fragments are known with certainty. Most likely, the fragmentation pathway is initiated by hydrogen abstraction from methylene groups that are sandwiched between double bonds (12, 20, 21). Several free radicals are sufficiently reactive to attack methylene groups, most notably the hydroxyl radicals. As a result, the methylene groups turn into radicals themselves giv-

ing rise to double bond rearrangements and further reactions with oxygen or other free radicals. The oxidized intermediates are unstable and prone to chain fragmentation.

It is not clear whether the accumulation of oxidized PC has any biological significance, although there is evidence supporting a cellular mediator function of oxidized PC similar to that of the platelet-activating factor (1–3). While the concentration of oxidized PC required to induce cellular effects is much higher than the biologically active concentration of the platelet-activating factor, it is nevertheless below 1 μM. This was demonstrated for both neutrophil adhesion induced by 1-palmitoyl-2-(5-oxovaleroyl)-PC (1) and platelet aggregation induced by 1-O-hexadecyl-2-succinyl-PC (10). Thus, the plasma concentrations of oxidized phospholipids reported in this paper are in a range consistent with biological activity. However, before the impact of oxidized phospholipids can be assessed, it is necessary to identify the molecular species generated in vivo. Future work must establish the biological potency of individual compounds and their role in human disease.

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## REFERENCES

1. Smiley, P. L., K. E. Stremler, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1991. Oxidatively fragmented phosphatidylcholines activate human neutrophils through the receptor for platelet-activating factor. *J. Biol. Chem.* **266:** 11104–11110.
2. Patel, K. D., G. A. Zimmerman, S. M. Prescott, and T. M. McIntyre. 1992. Novel leukocyte agonists are released by endothelial cells exposed to peroxide. *J. Biol. Chem.* **267:** 15168–15175.
3. Watson, A. D., M. Navab, S. Y. Hama, A. Sevanian, S. M. Prescott, D. M. Stafforini, T. M. McIntyre, B. N. Du, A. M. Fogelman, and J. A. Berliner. 1995. Effect of platelet activating factor–acetylhydrolase on the formation and action of minimally oxidized low density lipoprotein. *J. Clin. Invest.* **95:** 774–782.
4. Hanahan, D. J. 1986. Platelet activating factor: a biologically active phosphoglyceride. *Annu. Rev. Biochem.* **55:** 483–509.
5. Tjoelker, L. W., C. Wilder, C. Eberhardt, D. M. Stafforini, G. Dietsch, B. Schimpf, S. Hooper, H. Le Trong, L. S. Cousens, G. A. Zimmerman, Y. Yamada, T. M. McIntyre, S. M. Prescott, and P. W. Gray. 1995. Anti-inflammatory properties of a platelet-activating factor acetylhydrolase. *Nature* **374:** 549–553.
6. Stremler, K. E., D. M. Stafforini, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1989. An oxidized derivative of phosphatidylcholine is a substrate for the platelet-activating factor acetylhydrolase from human plasma. *J. Biol. Chem.* **264:** 5331–5334.
7. Stremler, K. E., D. M. Stafforini, S. M. Prescott, and T. M. McIntyre. 1991. Human plasma platelet-activating factor acetylhydrolase. Oxidatively fragmented phospholipids as substrates. *J. Biol. Chem.* **266:** 11095–11103.
8. Tokumura, A. 1995. A family of phospholipid autacoids: occurrence, metabolism and bioactions. *Prog. Lipid Res.* **34:** 151–184.
9. Tanaka, T., H. Minamino, S. Unezaki, A. Tsukatani, and A. Tokumura. 1993. Formation of platelet-activating factor-like phospholipids by $Fe^{2+}$/ascorbate/EDTA-induced lipid peroxidation. *Biochim. Biophys. Acta.* **1166:** 264–274.
10. Tanaka, T., M. Iimori, H. Tsukatani, and A. Tokumura. 1994. Platelet-aggregating effects of platelet-activating factor-like phospholipids formed by oxidation of phosphatidylcholines containing an *sn*-2-polyunsaturated fatty acyl group. *Biochim. Biophys. Acta.* **1210:** 202–208.
11. Tanaka, T., A. Tokumura, and H. Tsukatani. 1995. Platelet-activating factor (PAF)-like phospholipids formed during peroxidation of phosphatidylcholines from different foodstuffs. *Biosci. Biotech. Biochem.* **59:** 1389–1393.
12. Gutteridge, J. M. C., and B. Halliwell. 1990. The measurement and mechanism of lipid peroxidation in biological systems. *Trends Biochem. Sci.* **15:** 129–135.
13. Tsukatani, H., S. Yamada, A. Tokumura, T. Miyamoto, and K. Takauchi. 1976. Isolation of an acute hypotensive substance from bovine brain lipid fraction. *Chem. Pharm. Bull.* **24:** 2294–2300.
14. Tokumura, A., T. Tanaka, T. Yotsumoto, and H. Tsukatani. 1992. Formation of PAF-like compounds by peroxidation of phospholipids from bovine brain. *J. Lipid Med.* **5:** 127–130.
15. Itabe, H., E. Takeshima, H. Iwasaki, J. Kimura, Y. Yoshida, T. Imanaka, and T. Takano. 1994. A monoclonal antibody against oxidized lipoprotein recognizes foam cells in atherosclerotic lesions. Complex formation of oxidized phosphatidylcholines and polypeptides. *J. Biol. Chem.* **269:** 15274–15279.
16. Hamilton, S., R. J. Hamilton, and P. A. Sewell. 1992. Extraction of lipids and derivative formation. *In* Lipid Analysis. A Practical Approach. R. J. Hamilton and S. Hamilton, editors. IRL Press, Oxford. 13–64.
17. Hallermayer, G., and W. Neupert. 1974. Lipid composition of mitochondrial outer and inner membranes of *Neurospora crassa*. *Hoppe-Seyler's Z. Physiol. Chem.* **355:** 279–288.
18. Vaskovsky, V. E., and E. Y. Kostetsky. 1968. Modified spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.* **9:** 396.
19. Itabe, H., Y. Kushi, S. Handa, and K. Inoue. 1988. Identification of 2-azelaoylphosphatidylcholine as one of the cytotoxic products generated during oxyhemoglobin-induced peroxidation of phosphatidylcholine. *Biochim. Biophys. Acta.* **962:** 8–15.
20. Halliwell, B., and J. M. C. Gutteridge. 1990. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol.* **186:** 1–85.
21. Morrow, J. D., T. A. Minton, C. R. Mukundan, M. D. Campbell, W. E. Zackert, V. C. Daniel, K. F. Badr, I. A. Blair, and L. J. Roberts. 1994. Free radical-induced generation of isoprostanes in vivo. Evidence for the formation of d-ring and e-ring isoprostanes. *J. Biol. Chem.* **269:** 4317–4326.