Increase in fragmented phosphatidylcholine in blood plasma by oxidative stress

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Abstract Oxidatively modified phospholipids with fragmented acyl chains have attracted much interest because of their proinflammatory activity and their potential involvement in atherosclerosis. They can be formed in vitro by free radical treatment of unsaturated phospholipids but it is not known under which conditions they accumulate in vivo. We assayed one species of fragmented phosphatidylcholine (PC) in human blood plasma by high performance liquid chromatography after precolumn derivatization with chloromethylanthracene. Structural analysis suggested that fragmented PC was a diacyl species with a palmitoyl group and a short oxidized residue, which most likely had four carbons. The concentration of fragmented PC was higher in elderly individuals with coronary heart disease than in young healthy controls. Smoking one cigarette acutely increased the concentration of fragmented PC in healthy adults. Fragmented PC also increased in the reperfusion period after treatment with cardiopulmonary bypass. The increase coincided with a surge of circulating neutrophils. In rats, the plasma concentration of fragmented PC was elevated by vitamin E deficiency and exposure to high oxygen. The data demonstrate that fragmented PC increases in blood plasma in response to various forms of oxidative stress.—Frey, B., R. Haupt, S. Alms, G. Holzmann, T. Konig, H. Kern, W. Kox, B. Rustow, and M. Schlame. **Increase in fragmented phosphatidylcholine in blood plasma by oxidative stress. *J. Lipid Res.* 2000.** 41: **1145–1153.**

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Reactive oxygen species can induce peroxidation of lipids, a process that has been implicated in atherosclerosis (1–3), inflammation (1, 4), aging (5) and other diseases associated with oxidative stress (6, 7). Most studies in the field have focused on the end products of lipid oxidation, such as aldehydes and volatile hydrocarbons (8). These compounds are good indicators of the extent of lipid degradation but they have only limited toxicity, if any. More recent studies have begun to unravel the early intermediates of lipid peroxidation, which still retain the complex structure of the parent lipid (4, 9–11). Oxidatively modified lipids are interesting, because they may have significant biological activities (1, 4, 12, 13) as well as provoke autoimmune reactions (14, 15).

One example of oxidative modification is acyl chain fragmentation. Fragmented phospholipids can be produced in vitro by oxidation of unsaturated phosphatidylcholine (PC) and were shown to activate neutrophils via the receptor for platelet-activating factor (PAF) (16). Moreover, peroxidation in vitro of isolated lipoproteins (17) and endothelial cell cultures (18) produced fragmented phospholipids that carried biological activities similar to PAF. The chemical structure of this activity is not known; however, several synthetic PCs with short acyl chains had stimulatory effects on neutrophils (16), platelets (19, 20), and monocytes (20).

Despite this evidence, it has been difficult to establish the presence of fragmented phospholipids in vivo. Fragmented phospholipids have been detected by bioassay in the blood plasma of hamsters exposed to cigarette smoke (21). However, it is problematic to quantify lipids that are defined by biological activity. As a result, it is not known at what level fragmented phospholipids accumulate in biological tissues and whether their concentration actually responds to various treatments in vivo. We developed a technique to quantify fragmented PC in blood plasma by high performance liquid chromatography (HPLC) with precolumn derivatization (22). Although this technique prevents assessment of bioactivity, it enabled us to determine the chemical concentration of one fragmented phospholipid species. Now we use this method to measure fragmented PC in humans and rats under conditions of oxidative stress.

Abbreviations: ANOVA, analysis of variance; CPB, cardiopulmonary bypass; HPLC, high performance liquid chromatography; PAF, platelet-activating factor; PC, phosphatidylcholine.

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MATERIALS AND METHODS

Materials

Standard fragmented PCs, including 1-palmitoyl-2-succinyl-PC, 1-palmitoyl-2-glutaroyl-PC, and 1-palmitoyl-2-suberoyl-PC, were synthesized by acylation of 1-palmitoyl-2-lyso-PC and were purified by extraction, HPLC, and thin-layer chromatography as described previously (22). Alkyl-PC was synthesized in a similar fashion with 1-hexadecyl-2-lyso-PC as substrate. Individual molecular species of unsaturated long-chain PCs were obtained from Sigma (St. Louis, MO). 1-Hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphorylcholine was purchased from DuPont-New England Nuclear (Boston, MA).

Analysis of fragmented PC from blood plasma

Blood was drawn into syringes containing EDTA and immediately spun to obtain plasma. An aliquot of 0.5 mL of plasma was supplemented by 0.5 nmol of the internal standard 1-palmitoyl-2-suberoyl-PC. Protein was immediately precipitated by addition of 2 mL of methanol. This was followed by adding 1.5 mL of water and 2 mL of dichloromethane containing 0.01% butylated hydroxytoluene. The mixture was vortexed and centrifuged to achieve phase separation. The organic phase was removed and the upper phase was reextracted with dichloromethane. The organic extract was then dried under a stream of nitrogen and finally redissolved in 0.25 mL of benzene–triethylamine 4:1 (v/v) containing 0.1 M 9-(chloromethyl)anthracene. Derivatization occurred in sealed microreaction vessels at 70°C for 2 h. After that, the reaction mixture was loaded into Extract-Clean Silica solid-phase extraction tubes (200 mg of silica per tube; Alltech, Deerfield, IL) equilibrated with chloroform–triethylamine 9:1 (v/v). The columns were washed with 4 mL of chloroform–triethylamine 9:1 (v/v) and PC was eluted with 4 mL of methanol–32% ammonia 95:5 (v/v). The solvent was removed under a stream of nitrogen and the residue was redissolved in 0.1 mL of methanol. This solution was loaded on a Lichrospher Si 100 HPLC column (5 μm, 60 × 8 mm), which was eluted isocratically with acetonitrile–water 88:12 (v/v) at a flow rate of 2.5 mL/min at 50°C. The eluent was passed consecutively through an ultraviolet detector (205 nm) and a fluorescence detector (excitation, 360 nm; emission, 460 nm). The fluorescing anthracene derivative of fragmented PC eluted at the end of the large UV peak generated by nonderivatized PC. The fluorescing PC was collected, dried under nitrogen, redissolved in 0.1 mL of methanol, and chromatographed on a reversed-phase HPLC column (C_{18}-Nucleosil 100, 10 μm, 250 × 4 mm). The column was eluted with a gradient running from methanol–water 9:1 (v/v) to methanol–acetonitrile 9:1 (v/v) in 60 min. The solvents contained 0.02 M choline chloride to increase resolution of PC molecular species. The flow rate was 1 mL/min and the column temperature was 50°C. Fragmented PC was quantified by its fluorescence yield relative to the internal standard.

HPLC of nonderivatized PAF-like lipids

Two milliliters of plasma from EDTA-anticoagulated human blood was extracted with chloroform–methanol. The extract was dried under a stream of nitrogen and subsequently redissolved in 2 mL of chloroform plus 0.4 mL of ethanol. This mixture was loaded on a solid-phase extraction column filled with 500 mg of silica (Supelclean LC-Si SPE, 3 mL; Supelco, Bellefonte, PA). The column was first eluted with 3 mL of ethanol and then with 4 mL of methanol–water 3:1 (v/v). The methanol–water solution was loaded on another solid-phase extraction column filled with 100 mg of C_{18}-bonded silica (Supelclean LC-18 SPE, 1 mL; Supelco). Polar phospholipids were eluted from this column with 3 mL of methanol–water 9:1 (v/v), dried under nitrogen, and redissolved in 1 mL of HPLC solvent (methanol–acetonitrile–water 84:15:1 (v/v/v), containing 1 mM ammonium acetate). The solution was injected into a reversed-phase HPLC column (C_{18}-Nucleosil 100, 10 μm, 250 × 4 mm) developed isocratically at a flow rate of 1 mL/min. Fractions of 1 mL were collected and assayed for fragmented PC or PAF-like bioactivity. To measure bioactivity, the lipid fractions were dried under a stream of nitrogen and redissolved in 0.1 mL of buffer (pH 7.4) containing 103 mM NaCl, 40 mM NaH_2PO_4, 4.7 mM KH_2PO_4, 4.5 mM glucose, 12.9 mM sodium citrate, and fatty acid-free bovine serum albumin (2.5 g/L; Sigma). This solution was added to [^{3}H]serotonin-labeled rabbit platelets and release of [^{3}H]serotonin was measured exactly as described previously (20). PAF-like lipids were typically recovered in fractions 4–6.

Treatment of lipids with PAF acetylhydrolase

PAF acetylhydrolase was partially purified from human plasma by lipoprotein precipitation, detergent extraction, and ion-exchange chromatography, similar to the initial steps of a protocol described by Stafforini, Prescott, and McIntyre (23). The activity of the final preparation was 8.7 ± 0.5 U/mL and the purification factor was 28 ± 2 (n = 3). Polar lipids from human plasma were partially purified by solid-phase extraction using Extract-Clean Hi-Load C_{18} cartridges (200 mg of C_{18}-bonded silica per column; Alltech). The cartridges were eluted with 5.5 mL of methanol–water–acetonitrile 905:70:25 (v/v/v) containing 25 mM $HCOONH_4$. The eluate was dried and reconstituted in aqueous buffer (0.1 M HEPES [pH 7.2], 1 mM EGTA) by sonication. PAF acetylhydrolase (1.75 U) was added and the tubes were incubated at 37°C for 30 min. The reaction was stopped by addition of methanol and chloroform followed by lipid extraction. Fragmented PC was assayed in the lipid extract as described above.

Mass spectroscopy

Before mass spectroscopy, samples were derivatized with methylanthracene and purified by HPLC (see above). For measurement of matrix-assisted laser desorption ionization time of flight (MALDI-TOF), samples were dissolved in acetonitrile–water 4:1 (v/v), using 2,5-dihydroxybenzoic acid as the laser-sensitive component. Portions of these solutions were applied to distinct regions of the target, the solvent was removed by vacuum, and the sample was placed in the laser beam of the ionization region. The MALDI-TOF-Reflex mass spectrometer (Bruker, Billerica, MA) was operated with a laser ionization pulse rate of 10 Hz. Three hundred shots were applied per measurement. The target was optically monitored by a video system. Positive ions were accelerated at 28.5 kV and masses were separated in a linear drift region. Sensitivity and resolution of the system were enhanced by an additional reflector system (30 kV). Fast atom bombardment-mass spectroscopy (FAB-MS) was performed in the positive mode. Lipids were dissolved in either 3,5-dinitrobenzylalcohol or glycerol matrices and attached as droplets on an adjustable target. The adjustable neutral particle FAB gun (Iontech [Cincinnati, OH], modified by AMD-Intectra [Harpstedt, Germany]) was operated with xenon accelerated at 6 kV (0.6-mA emission at 4×10^{-5} torr). FAB-induced ions were accelerated at 3 kV in a commercial EI source of a double-focusing high-resolution mass spectrometer with reverse Nier–Johnson geometry (CH 5 DF; Finnigan MAT, Bremen, Germany) and a resolution of R > 3,000. The sensitivity of the commercial mass spectrometer was enhanced by an adjustable conversion dynode allowing the reacceleration and refocusing of ions before registration by a conventional secondary electron multiplier connected to the data system DP 10 (technical modifications installed by AMD-Intectra).

Animal model

Young Wistar rats (100–120 g) were fed a vitamin E-free diet (Altromin, Lage, Germany) for 5 weeks followed by a 24-h period of

food deprivation. Subsequently, vitamin E was refed to some rats for 24 h; this resulted in high plasma levels of the vitamin. Animals entered the experiment either in the vitamin E-deficient or in the vitamin E-supplemented state. During the experiment, they were kept in glass chambers that were either purged with oxygen (1 L/min) or left to equilibrate with the atmosphere. The oxygen concentration inside the chamber was determined with a Clark electrode. After 20 h, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (0.3 mg per g body weight). Subsequently, blood was obtained by cardiac puncture and the animals were killed. Concentrations of vitamin E and fragmented PC were determined in blood plasma. The protocol was approved by the animal welfare commission of Humboldt University (Berlin, Germany).

Human subjects

Several healthy individuals (nine females and eight males; age range, 21–64 years), all of whom were hospital employees, volunteered to donate blood for this study. Further, we studied patients (five females and eight males; age range, 52–79 years) with coronary atherosclerosis, who had double- or triple-vessel disease as determined by angiography. These patients were scheduled for aortocoronary bypass grafting. All patients donated blood after informed consent and with institutional approval.

Smokers (two females and three males; age range, 24–57 years) who participated in this study had no medical condition other than the habit of smoking. They had not smoked or eaten for at least 8 h before participating in the experiment. A venous catheter was placed in the upper limb and the volunteer was instructed to smoke one cigarette within 5 min. All participants were free to choose their brand of cigarette. According to supplier information, the cigarettes contained 0.9–1.0 mg of nicotine and 12 mg of tar.

Five patients (one female and four males; age range, 61–77 years) were studied during coronary surgery with cardiopulmonary bypass (CPB). Anesthesia was induced with etomidate (0.3 mg/kg), pancuronium (0.1 mg/kg), and sufentanil (0.5–1.0 μg/kg) and was maintained with isoflurane and sufentanil. Before institution of CPB, patients received 35 to 50 units of heparin per kilogram for anticoagulation. Nonpulsatile CPB was performed with a Biomedicus centrifugal pump (Metronic, Minneapolis, MN) under near-normothermic conditions (34°C). The pump flow rate was 2.5 L/min/m^2. Membrane oxygenators (D 703; Dideco, Mirandola, Italy) were used and 40-μm pore size filters were positioned in the arterial line. Pump oxygenators were loaded with 1.4–1.6 L of saline. The cardioplegic solution contained 15 mM NaCl, 9 mM KCl, 4 mM $MgCl_2$, and 0.015 mM $CaCl_2$, as well as mannitol and amino acids. Bypass times ranged from 40 to 65 min. After separation from CPB, dopamine (up to 7 μg/kg/min) was infused to improve cardiovascular stability.

Other assays

Vitamin E was measured in human plasma by HPLC with UV detection at 295 nm (24). The total phospholipid concentration was determined in lipid extracts from 0.1 mL of plasma. Extracts were dried and digested with perchloric acid, and the liberated phosphate was measured by colorimetric assay (25). Cell counts and hematocrits were measured by flow cytometry.

Statistical analysis

Values are presented as means ± the standard error of the mean. Treatment groups were compared by the *t*-test, the paired *t*-test, and by two-way analysis of variance (ANOVA) as indicated. Correlation was analyzed by linear regression and the correlation coefficient (R) was derived. A *P* value less than 0.05 was regarded as statistically significant. All analyses were performed with the program SigmaStat 2.0 (Jandel Scientific, San Rafael, CA).

RESULTS

Assay of fragmented PC

Previously, we had developed a method for the measurement of oxidatively fragmented PC by attaching a fluorescence label to the oxidized side chain (22). In the present work, an improved version of the original analytical strategy is used (**Fig. 1**). Previously, we had used reversed-phase HPLC before chemical derivatization, whereas the new method uses normal-phase HPLC after derivatization, greatly enhancing selectivity because of the attached fluorescent label. Accordingly, chromatograms looked cleaner than before and the derivative of fragmented PC was obtained in a symmetrical peak (Fig. 1). Plasma concentrations of fragmented PC were lower when the old protocol was used, first, because recovery of fragmented PC was lower because of losses during the initial chromatography, which was done before derivatization, and second, because samples were less pure, such that contaminating peaks sometimes obscured the anthracene-labeled PC derivative.

Validation of the assay technique included *i*) evidence that fragmented PC was indigenous to blood plasma, *ii*) assessment of assay specificity, and *iii*) assessment of the method of quantification. *i*) It was demonstrated that frag-

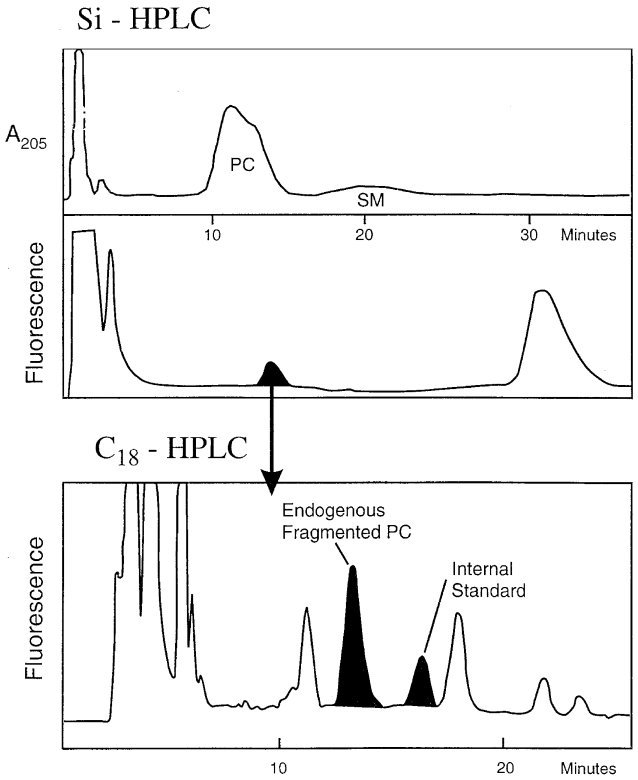

Fig. 1. Quantification of fragmented PC in blood plasma. Lipids were extracted, pretreated with 9-(chloromethyl)anthracene, and chromatographed on normal-phase silica (Si-HPLC). The fluorescent component of the PC peak was collected and rechromatographed on reversed-phase silica (C_{18}-HPLC). The derivative of endogenous fragmented PC eluted shortly before the derivative of the internal standard (1-palmitoyl-2-suberoyl-PC). Further details of the technique are given in text. A_{205}, absorbance at 205 nm; SM, sphingomyelin.

mented PC was not an artifact generated by sample workup in vitro, because treatment of nonoxidized PC did not yield a fluorescence derivative (22). In support of this idea, we demonstrated an increase in the fluorescence signal in proportion to the volume of extracted plasma, such that extrapolation to zero plasma yielded zero fragmented PC (data not shown); *ii*) assay specificity was based on highly reproducible HPLC retention times (relative retention times, with respect to the internal standard, varied between 0.79 and 0.80), suggesting that the method consistently picked up the same compound. The specific HPLC peak was found not only in blood plasma of humans and rats, but also in extracts from rat liver and lung (B. Frey, R. Haupt, S Alms, G. Holzmann, T. König, H. Kern, W. Kox, B. Rüstow, and M. Schlame, unpublished data); *iii*) quantification with internal standard is valid only if the standard compound has the same extraction–derivatization recovery as the target compound. Recovery analyses were done with several synthetic species of fragmented PC, yielding 81, 72, and 76% for 1-palmitoyl-2-succinyl-PC, 1-palmitoyl-2-glutaroyl-PC, and 1-palmitoyl-2-suberoyl-PC, respectively. For biological samples, the lower limit of detection was 0.1–0.2 μM. In four repeated analyses of the same plasma sample, the coefficient of variation was 19%.

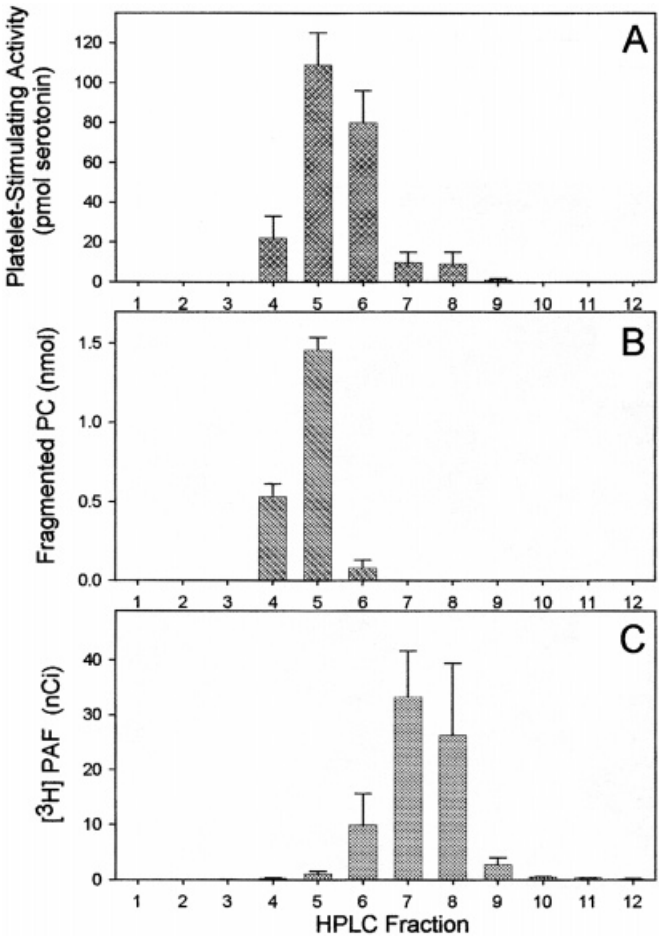

Fig. 2. Plasma lipids with platelet-stimulating activity copurify with fragmented PC. Polar lipids from 2 mL of human blood plasma were purified by a four-step protocol consisting of chloroform–methanol extraction, silica-solid-phase extraction, reversed-solid-phase extraction, and reversed-phase HPLC. HPLC fractions (1 mL) were collected and assayed for (A) platelet-stimulating activity (expressed as picomoles of serotonin released per 2 min and 3×10^8 platelets), (B) fragmented PC, and (C) radioactivity from exogenous [^{3}H]PAF. Means and standard errors of mean of three independent experiments are shown.

Characterization of fragmented PC

Nonderivatized fragmented PC from human plasma showed HPLC characteristics similar to those of platelet-stimulating lipids (**Fig. 2**). Furthermore, fragmented PC was susceptible to hydrolysis by PAF acetylhydrolase (**Fig. 3**), suggesting that fragmented PC was a PAF-like compound. However, the retention time of fragmented PC was shorter than that of authentic [^{3}H]PAF (Fig. 2), which was consistent with the idea that fragmented PC contained a polar side chain. To characterize fragmented PC further, it was compared with synthetic short-chain PCs, using analytical HPLC after derivatization (**Table 1**). As expected, retention times were generally dependent on chain length and the type of bonding in the *sn*-1 position (ether or ester). The retention time of fragmented PC was closest to the retention times of 1-palmitoyl-2-succinyl-PC and 1-palmitoyl-2-glutaroyl-PC. Fragmented PC also ran similar to the oxidation products of 1-palmitoyl-2-docosahexaenoyl-PC and 1-palmitoyl-2-arachidonoyl-PC. We concluded that fragmented PC from human plasma contained a palmitoyl residue and a short fragment (C_4 or C_5) derived from either arachidonoyl or docosahexaenoyl, respectively. Finally, fragmented PC was compared with the internal standard, 1-palmitoyl-2-suberoyl-PC, by mass spectroscopy in MALDI mode. The ions [1-palmitoyl-2-lyso-PC + H]$^+$ (*m/z* 496), [MH]$^+$ (*m/z* 842), and [MH + choline]$^+$ (*m/z* 945) were found in the mass spectrum of the anthracenemethyl ester of 1-palmitoyl-2-suberoyl-PC. In contrast, the corresponding signals of the anthracenemethyl ester of frag-

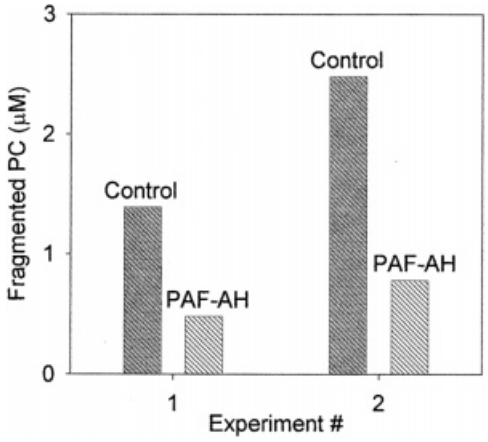

Fig. 3. Fragmented PC from human plasma is sensitive to PAF acetylhydrolase. Polar phospholipids were extracted from human plasma and reconstituted in aqueous buffer containing either PAF acetylhydrolase (PAF-AH) or no enzyme (Control). After incubation for 30 min at 37°C, lipids were reextracted and the amount of fragmented PC was determined. Two separate experiments are shown.

TABLE 1. HPLC retention times of the fluorescence derivatives of various short-chain PC's and oxidation products of long-chain PCs[a]

Compound	Chain Length *sn*-1	Chain Length *sn*-2	Linkage of *sn*-1 Chain	RT[b] (min)	RRT[b]
Synthetic PCs					
1-Palmitoyl-2-succinyl-PC	C_{16}	C_4	Ester	13.5	0.72
1-Palmitoyl-2-glutaroyl-PC	C_{16}	C_5	Ester	15.2	0.81
1-Palmitoyl-2-suberoyl-PC	C_{16}	C_8	Ester	18.8	1.00
1-Hexadecyl-2-succinyl-PC	C_{16}	C_4	Ether	16.2	0.86
1-Hexadecyl-2-glutaroyl-PC	C_{16}	C_5	Ether	18.4	0.98
PC oxidation products[c]					
Oxidized 1-palmityol-2-docosahexaenoyl-PC	C_{16}		Ester	15.0	0.80
Oxidized 1-palmitoyl-2-arachidonoyl-PC	C_{16}		Ester	15.2	0.81
Oxidized 1-palmitoyl-2-linoleoyl-PC	C_{16}		Ester	20.7	1.10
Oxidized 1-stearoyl-2-docosahexaenoyl-PC	C_{18}		Ester	20.3	1.08
Oxidized 1-stearoyl-2-arachidonoyl-PC	C_{18}		Ester	20.5	1.09
Oxidized 1-stearoyl-2-linoleoyl-PC	C_{18}		Ester	26.9	1.43
Fragmented PC from human plasma				14.9	0.79

[a] The indicated compounds were derivatized with chloromethylanthracene and analyzed by HPLC with fluorescence detection as described in text.

[b] Retention times (RT) and relative retention times (RRT) with respect to the internal standard (1-palmitoyl-2-suberoyl-PC) are presented. On repeated analyses RRTs varied by less than 0.01.

[c] Oxidized lipids were produced by treatment of the indicated long-chain PCs with Fe^{2+}–ascorbate–EDTA as described (19).

mented PC were *m/z* 496, *m/z* 772, and *m/z* 875. The comparison suggested that plasma-derived PC was smaller than 1-palmitoyl-2-suberoyl-PC by 70 mass units. Thus, the short oxidized residue could be $-COCH_2COOH$ (C_3) or $-COCH_2CH_2CH_2OH$ (C_4). This conclusion was confirmed by FAB mass spectroscopy, where the anthracenemethyl ester of fragmented PC produced *m/z* 496, *m/z* 564, and *m/z* 772, corresponding to $[1\text{-palmitoyl-2-lyso-PC} + H]^+$, $[M\text{-anthrylmethoxy}]^+$, and $[MH]^+$, respectively.

Animal study

Fragmented PC was measured in blood plasma of rats to investigate the effects of high oxygen and vitamin E deficiency (**Table 2**). Two groups of rats were studied with different vitamin E feeding status. The status was verified by determination of the vitamin E concentration in blood plasma. Both groups were kept for 20 h either in normal air or in 80% oxygen. The concentration of fragmented PC was higher in vitamin E-deficient rats than in vitamin E-fed rats. Hyperoxia also resulted in a small increase in fragmented PC. The combination of both hyperoxia and vitamin E deficiency increased the concentration of fragmented PC about 3-fold compared with normoxic, vitamin E-supplemented rats. The effects of vitamin E deficiency ($P = 0.003$) and high oxygen ($P = 0.040$) were statistically significant as shown by two-way ANOVA.

Human studies

We assayed fragmented PC in the plasma of healthy volunteers of different ages as well as in patients with coronary heart disease (**Fig. 4**). The subjects were divided into three groups: young and healthy individuals (age range, 21–42 years; n = 11), elderly and healthy individuals (age range, 52–64 years; n = 6), and elderly individuals with coronary heart disease (age range, 52–79 years; n = 13). There was no significant difference between the two age groups of healthy volunteers. However, elderly patients with coronary heart disease had a significantly higher concentration of fragmented PC than young and healthy individuals ($P = 0.025$, *t*-test). Two-way ANOVA did not reach statistical significance for either age or coronary disease, but the data must be interpreted with caution because

TABLE 2. Effects of hyperoxia and vitamin E on the plasma concentration of fragmented PC in rat

Oxygen Status[a]	Feeding Status[b]	n	Plasma Concentration: Vitamin E (μg/ml)	Plasma Concentration: Fragmented PC (μM)
Normoxia ($F_iO_2 = 0.21$)	Vitamin E supplemented	4	11.5 ± 3.3	0.92 ± 0.07
	Vitamin E deficient	4	1.2 ± 0.2	1.83 ± 0.31
Hyperoxia ($F_iO_2 = 0.80$)	Vitamin E supplemented	4	11.2 ± 0.8	1.41 ± 0.20
	Vitamin E deficient	4	2.7 ± 1.1	2.81 ± 0.50

[a] Significant effect of oxygen status on the plasma concentration of fragmented PC, $P = 0.04$ (two-way ANOVA).

[b] Significant effect of feeding status on the plasma concentration of fragmented PC, $P = 0.003$ (two-way ANOVA).

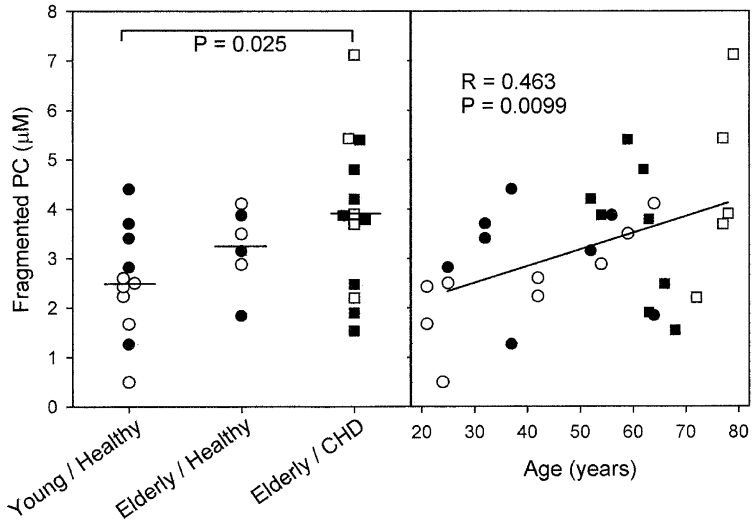

Fig. 4. Plasma concentration of fragmented PC in various study groups. Circles represent healthy individuals and squares represent patients with coronary heart disease (CHD). Open symbols represent females and closed symbols represent males. Left: A scatter plot of three different study groups: young/healthy (age range, 21–42 years), elderly/healthy (age range, 52–64 years), and elderly/CHD (age range, 52–79 years). Groups were compared by *t*-test. Right: Correlation between age and concentration. Linear regression analysis was performed.

there was no study group with coronary disease at a young age. There was a weak positive correlation between the concentration of fragmented PC and age when all data were analyzed regardless of the presence of coronary disease ($r = 0.463$; $P = 0.0099$).

The effect of smoking was studied in five individuals (age range, 24–57 years) who were apparently healthy except for the habit of smoking cigarettes (**Fig. 5**). After a period of abstinence from smoking for at least 8 h, the volunteers smoked one cigarette within 5 min. Blood was obtained before smoking and at several times after smoking. Acute smoking immediately increased the plasma concentration of fragmented PC in all individuals ($P = 0.0272$; paired *t*-test). About 1 h after smoking, the concentration of fragmented PC returned to nearly the pre-smoking level.

Fragmented PC was measured in surgical patients treated with CPB (**Fig. 6**). Plasma samples were obtained before and after the induction of anesthesia, 5 min after administration of heparin for anticoagulation, 25 min after institution of CPB, 10 min after weaning from CPB, and 3 h after surgery. At the same time points, polymorphonuclear leukocytes and platelets were measured. Dilution of plasma, due to infusion and pump fluid, was monitored by hematocrit. Fragmented PC was expressed as a percentage of total phospholipid to correct for dilution. During CPB, the concentration of fragmented PC was somewhat lower than before surgery. However, in the reperfusion period after CPB, fragmented PC increased in parallel with circulating neutrophils ($P < 0.05$, paired *t*-test). In contrast, the platelet count did not change significantly in the reperfusion period.

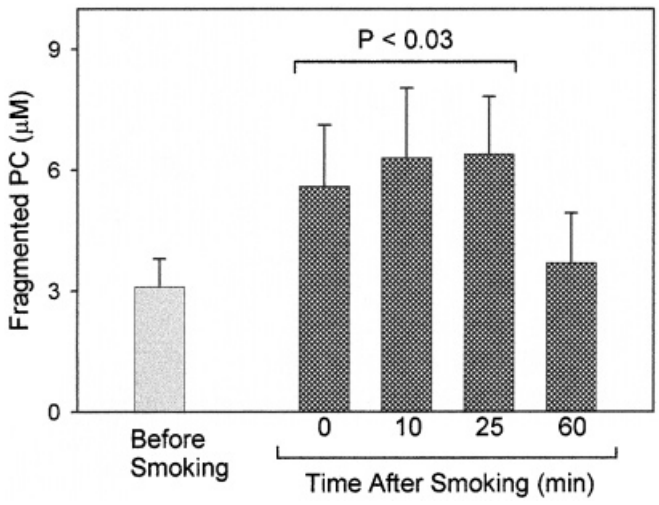

Fig. 5. Effect of acute smoking on the concentration of fragmented PC in blood plasma. Healthy individuals (two females and three males; age range, 24–57 years) smoked one cigarette (0.9–1 mg of nicotine, 12 mg of tar) within 5 min. The plasma concentration of fragmented PC was determined before smoking and at various times thereafter.

DISCUSSION

Evidence has accumulated that chemically modified phospholipids can be produced by reactions that involve free radicals (9, 16–18, 21, 26, 27). What remains to be defined is the chemical structure of the modified lipids, their concentration in vivo, as well as physiologic or pathologic states that favor oxidative modification of lipids. Previously, we identified an oxidatively fragmented PC in human blood plasma, using anthracene labeling in combination with fluorescence-HPLC (22). In the present article we show that the concentration of this phospholipid acutely increases in the presence of various forms of oxidative stress in vivo.

The assay quantitated a specific fluorescence-labeled lipid with a characteristic HPLC retention time. The retention time showed virtually no variation between different experiments. Previous analysis of this lipid identified the head group as phosphocholine and there was indirect evidence of the presence of a short, oxidized residue (22). Here we present further structural information about fragmented PC. The compound contained a palmitoyl chain because, first, it had a retention time similar to that of oxidized 1-palmitoyl-PC's (Table 1), and second, mass

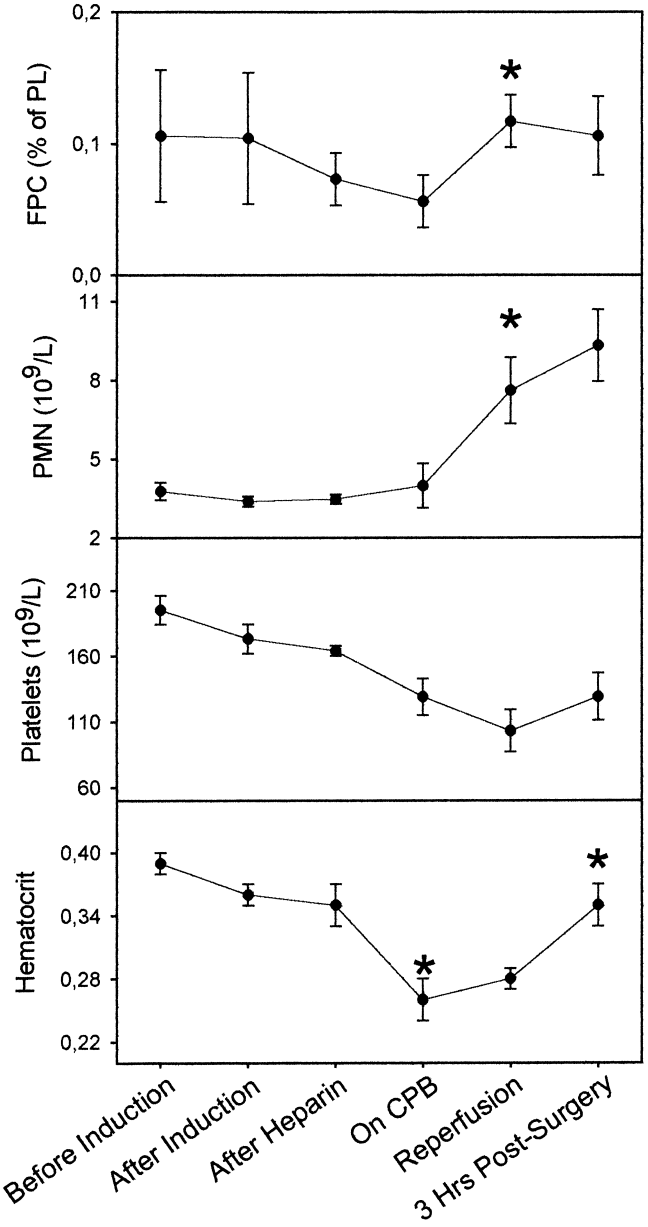

Fig. 6. Measurements during cardiac surgery. Five patients (one female, four males; age range, 61–77 years) underwent surgery for coronary artery bypass grafting. Blood samples were drawn before and after the induction of anesthesia, 5 min after administration of heparin for anticoagulation, 25 min after institution of CPB (On CPB), 10 min after weaning from CPB (Reperfusion), and 3 h after surgery. The asterisks indicate a significant difference from the previous point in time ($P < 0.05$, paired *t*-test). FPC, Fragmented PC; PL, total phospholipid; PMN, polymorphonuclear neutrophils.

spectra prominently displayed the *m/z* 496 fragment, corresponding to 1-palmitoyl-2-lyso-PC. Comparative HPLC excluded the possibility that fragmented PC was a 1-*O*-alkyl compound (Table 1). The presence of a short chain in fragmented PC can be deduced from both chromatographic data (Table 1) and the sensitivity to PAF acetylhydrolase (Fig. 3). Mass spectroscopic analysis is consistent with the presence of a C_3 residue or a C_4 residue, depending on the type of oxygen function that links the short chain to the anthracenemethyl moiety. HPLC data (Table 1) are consistent with the presence of a C_4 residue that is slightly less polar than the succinyl group, e.g., a C_4 residue with an ω-hydroxyl group. Fragmented PC was chromatographically similar to oxidation product(s) generated by Fe^{2+}–ascorbate treatment of 1-palmitoyl-2-arachidonoyl-PC and 1-palmitoyl-2-docosahexaenoyl-PC (Table 1). When 1-palmitoyl-2-arachidonoyl-PC and 1-palmitoyl-2-docosahexaenoyl-PC were exposed to the iron–ascorbate oxidation system, C_4 residues were found among the fragmentation products (19).

Fragmented PC copurified with PAF-like lipids from human plasma (Fig. 2). In fact, the elution profile of PAF-like activity matched fragmented PC much better than authentic [^{3}H]PAF. This experiment strongly suggested that the plasma-borne platelet-stimulating activity was not caused by authentic PAF but by other phospholipids related to fragmented PC. However, it is not known exactly which molecular species produced the platelet-stimulating activity. One study has suggested that most, if not all, biological activity recovered from oxidized lipoproteins was caused by fragmented alkyl-PCs rather than acyl-PCs (28). Because the present species of fragmented PC is acyl-PC, it may have little biological activity. Still, it is expected to reflect the general trend as to how oxidized phospholipids change in response to oxidative stress in vivo.

The involvement of free radicals in the formation of fragmented phospholipids has been well documented in vitro (16–18, 27), but there is little direct evidence that this is also true in vivo. The free radical hypothesis was supported by the observation that cigarette smoke acutely increased the concentration of fragmented PC (Fig. 5). It is well established that cigarette smoke contains free radicals and, in addition, causes secondary radical production (29). In hamsters, cigarette smoke was shown to induce the formation of neutrophil- and platelet-stimulating lipids that most likely correspond to fragmented PC (21). However, it was difficult to quantify these lipids by bioassay and they were virtually undetectable before smoke exposure. The low baseline level detected in hamsters via bioassay (21) was in contrast to the chemical assay (Fig. 5), which measured significant concentrations of fragmented PC even under resting conditions. This contradiction may be due to different sensitivities of the assay techniques, or to differences between humans and hamsters, or it may reflect the detection of different PC species.

Furthermore, the free radical hypothesis was supported by the finding that high levels of oxygen and vitamin E had opposite effects on the plasma concentration of fragmented PC (Table 2). Hyperoxia is known to increase the burden of free radicals (30) whereas vitamin E is a radical scavenger. For instance, some of the clinical side effects of hyperoxia can be attributed to free radicals (31). The pathology of oxygen toxicity is poorly understood, but inflammatory mediators such as PAF are potentially involved. Because fragmented phospholipids may have activities that resemble PAF (16–20), it can be hypothesized that they play a role in hyperoxic lung injury. Consistent with

that idea, increased lipid peroxidation was reported in patients with adult respiratory distress syndrome, in which both lung inflammation and hyperoxia are common (32). Oxidized phospholipids were found not only in blood, but also in lung tissue (33).

There is some evidence suggesting that aging is associated with the accumulation of lipid oxidation products (5, 34). The same applies to atherosclerosis, which was linked to oxidative modification of low density lipoprotein (2, 35). The present data suggest that fragmented PC is among the lipid oxidation products accumulating in elderly people with atherosclerosis (Fig. 4). However, the data do not establish an independent effect of either age or atherosclerosis because of the strong association between these two factors. Previously, atherosclerosis has been implicated in the enrichment of fragmented PC in rabbit aortic tissue (9). Specifically, two fragmented PCs with C_5 chains were quantified by mass spectroscopy. Increased amounts of these fragmented phospholipids were found in fatty streak lesions of the aorta of rabbits fed an atherogenic diet (9). Likewise, the amount of hydroxyeicosatetraenoic acids was higher in human atherosclerotic plaques than in normal arteries (36). Within atherosclerotic samples, unstable plaques, producing clinical symptoms, contained higher concentrations of hydroxyeicosatetraenoic acids than stable plaques.

CPB treatment of surgical patients is a model of cell-mediated oxidative stress because it leads to the activation of platelets and neutrophils (37). Neutrophil activation is primarily caused by endothelial interaction during ischemia. On reperfusion, activated neutrophils are flushed from pulmonary and cardiac capillaries into the systemic circulation (38). Accordingly, the reperfusion period was shown to entail an increase in circulating concentrations of free radicals (39, 40) and malondialdehyde (39). Because reperfusion also induced a rise in fragmented PC (Fig. 6), we propose that activation of neutrophils significantly enhances oxidative fragmentation of phospholipids in vivo.

The present data are consistent with several reports on F_2-isoprostanes, a well-established marker of lipid peroxidation (6, 7, 10, 26, 41–43). F_2-isoprostanes are prostaglandin-like acyl groups that are formed when free radicals attack arachidonoyl residues in phospholipids (10, 26). These acyl groups are released by hydrolysis and can be found in blood plasma and urine samples. Urinary levels of F_2-isoprostanes were increased in chronic smokers, and this increase was reversible by cessation of smoking or by antioxidant treatment (41, 42). Also, F_2-isoprostanes increased in the urine during the reperfusion period after CPB treatment (43). The emerging picture is that reperfusion produces several kinds of oxidative modifications in phospholipids, among them isoprostane formation and acyl chain fragmentation. Reperfusion is frequently associated with clinical problems, such as arrhythmia, low cardiac output, or loss of vascular resistance (38). Thus, the question is whether the generation of oxidatively modified lipids with biological activity is involved in the pathogenesis of these symptoms.

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