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Oxidized Phospholipids in Oxidized Low-Density Lipoprotein Reduce the Activity of Tissue Factor Pathway Inhibitor Through Association with Its Carboxy-Terminal Region

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ABSTRACT

Tissue factor pathway inhibitor (TFPI) is a Kunitz-type protease inhibitor that inhibits the initial reactions of blood coagulation. In this study, we explored the nature of active components that reduce the anticoagulant activity of TFPI in oxidized low-density lipoprotein (ox-LDL). The organic solvent-soluble fraction obtained from ox-LDL was fractionated by normal-phase HPLC. The binding profile of each fraction to TFPI showed a single peak eluting near purified oxidized phospholipid. To explore further the components in oxidized phospholipid that inhibit TFPI activity, we used oxidized phospholipids that mimic the biological activity of ox-LDL. The oxidation products of 1- and/or 2-oleoyl phosphatidylcholine or phosphatidylethanolamine were the most potent inhibitors of TFPI activity, whereas those of arachidonyl phosphatidylcholine possessed only a weak inhibitory effect on the TFPI activity. These oxidized phospholipids mainly associated with the C-terminal basic region of the TFPI molecule. The results indicate that oxidation products of δ-9 unsaturated phospholipids are candidate active components of ox-LDL that impair the function of TFPI through specific association with its C-terminal basic region. *Antioxid. Redox Signal.* 6, 705–712.

INTRODUCTION

TISSUE FACTOR PATHWAY INHIBITOR (TFPI) is a Kunitz-type protease inhibitor that inhibits the initial reactions of blood coagulation. A major pool of TFPI is associated with the surface of endothelial cells, and is speculated to play an important role in regulating the functions of vascular wall cells, as well as thrombus formation in atherosclerotic plaque. Many reviews on TFPI have been published (5–8, 20). TFPI consists of three tandem Kunitz inhibitor domains, the first and second of which inhibit the tissue factor (TF)–factor VIIa (FVIIa) complex and factor Xa (FXa), respectively. Heparin-binding sites are located in the third Kunitz domain (11, 23) and the C-terminal basic region (31), and are important for the inhibitory effect of TFPI on the initiation of blood coagulation and the enhancement by heparin. In the bloodstream, TFPI exists in a free form and also in lipoprotein-associated forms (25).

Atherosclerosis is a process of chronic inflammation initiated and sustained in response to injury of the vascular wall. Several reports have demonstrated the colocalization of TFPI and TF in atherosclerotic plaques, suggesting a significant role for TFPI in the regulation of TF activity in the atherosclerotic region and in the attenuation of the thrombogenicity of the atherosclerotic plaque (9, 10).

Oxidized (ox-) low-density lipoprotein (LDL) has been shown to exist not only in the plasma, but also in atherosclerotic lesions, and the formation of ox-LDL in subendothelial space of the artery wall has been implicated in the pathogenesis of atherosclerosis (21, 22, 23). In addition, ox-LDL might

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serve to initiate thrombus formation at atherosclerotic lesions through the perturbation of the anticoagulant environment on the luminal surface of the endothelium. It has been reported that ox-LDL induces the expression of TF (30) and a reduction of thrombomodulin (16), which are key pro- and anticoagulant factors, respectively, in the blood coagulation system. Further, the expression of TF has been found in ruptured atherosclerotic plaques (32). Thus, it is important to investigate whether the ox-LDL-dependent reduction of the TFPI activity participates in thrombus formation in atherosclerotic lesions.

We have already shown that TFPI associates rapidly with ox-LDL via its C-terminal basic region, with a reduction of its anticoagulant activity (14, 15). We have also shown that components that inhibit the function of TFPI are present in the phospholipid fraction of ox-LDL (13). These findings indicate that ox-phospholipids in ox-LDL might be the key to the impairment of the anticoagulant activity of TFPI. The present work was undertaken to appreciate the common feature of the active phospholipids in ox-LDL that inhibit the anticoagulant function of TFPI.

MATERIALS AND METHODS

Materials

Full-length recombinant TFPI (rTFPI) or C-terminal-deleted rTFPI (rTFPI-C) and monoclonal and polyclonal antibodies against rTFPI were prepared as described previously (1, 11). Bovine FXa was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine factor X (FX), FXa, and FVIIa were obtained from the sources described previously (14). Phospholipids were purchased from Sigma.

Isolation and oxidation of LDL

LDL was prepared from human plasma by stepwise density-gradient centrifugation (22). Ox-LDL was prepared by the incubation of LDL (200 μg/ml protein) with 5 μ*M* $CuSO_4$ in phosphate-buffered saline (PBS) at 37°C for 16 h. The reaction was stopped by the addition of 0.25 m*M* EDTA. The oxidation of LDL was confirmed as described previously (15).

Lipid extraction and separation

Total lipids of native LDL or ox-LDL separated with chloroform/methanol (1:2) according to the method described previously (16) or the phospholipid fraction from thin-layer chromatography (TLC) was subjected to normal-phase HPLC. An amount of lipids corresponding to 0.6 mg of ox-LDL protein was injected into a Cosmosil Silica column (250 × 10 mm) (Nacalai Tesque, Kyoto, Japan) as a solution in chloroform/methanol (1:1). The lipids were eluted isocratically with a mobile solvent of acetonitrile/methanol/water (79:8:13, by volume) at a flow rate of 0.8 ml/min and detected at 205 nm. Fractions were collected at 0.8-min intervals. The solvent was evaporated under a nitrogen stream, and the dried lipid residue was then dispersed in 50 m*M* Tris-HCl (pH 7.5) containing 150 m*M* NaCl by sonication.

Nondenaturing agarose gel electrophoresis

Twenty nanograms of rTFPI or rTFPI lacking the C-terminal basic region was incubated for 3 h at 37°C with lipids, and the mixture was subjected to agarose gel electrophoresis as described previously (15).

Microplate binding assay

Lipid fractions obtained from ox-LDL or LDL by HPLC were dissolved in 100 μl of ethanol per well on a plain plate, and the solvent was evaporated off. The plate was blocked with 1% bovine serum albumin and then incubated with TFPI for 2 h at 4°C. TFPI binding to lipid fractions was detected with rabbit anti-TFPI antibody followed by horseradish peroxidase-labeled anti-rabbit IgG. The color was developed with hydrogen peroxide and *o*-phenylenediamine, and absorbance was measured at 490 nm.

Measurement of antigenicity of TFPI

The antigenicity of TFPI was determined by measuring the TFPI antigen level using a sandwich enzyme-linked immunosorbent assay (ELISA) with polyclonal antibody and monoclonal antibody specific for the conformation between Kunitz domains 1 and 2 of TFPI (15). rTFPI was incubated for 2 h at 37°C with ox-LDL or lipid fractions and subjected to the ELISA. A reduction of antigenicity is considered to reflect a conformational alteration of the TFPI molecule due to interaction between the C-terminal part of TFPI and ox-LDL (15).

Measurement of TFPI activity

TFPI activity was determined by measuring the inhibitory activity on FX activation through formation of the quaternary complex FVIIa/TF/FXa/TFPI as described previously (14).

Preparations of ox-phospholipids

Commercially available phospholipids, which had been dried under argon gas, were suspended in PBS. Ascorbic acid (0.4 m*M*) and $FeSO_4$ (40 μ*M*) were added to the reaction mixture containing 0.4 n*M* phospholipid, and the mixture was incubated for 3 h at 37°C under air with mild agitation. Unoxidized phospholipid and phospholipid remaining in the original spot were removed after separation by TLC on silica gel plate with a solvent of chloroform/methanol/water (130:60:11, by volume). The concentration of each phospholipid was estimated by measuring phosphorus content (14), and then the amount equivalent to the content of total ox-phospholipid in the ox-LDL (50 μg/ml protein) was used. 1-Palmitoyl/stearoyl-2-nonyl aldehyde phosphatidylcholine (PC) (9CHO-PC), 1-palmitoyl/stearoyl-2-nonoic acid PC (9COOH-PC), 1-palmitoyl-2-pentyl aldehyde PC (5CHO-PC), and 1-stearoyl-2-pentanoic acid PC (5COOH-PC) were prepared as described previously (13).

Statistics

Data are expressed as means ± SD from three independent experiments.

RESULTS

Normal-phase HPLC of organic solvent-soluble components in ox-LDL

We have already shown that the phospholipid fraction obtained from ox-LDL inhibits TFPI activity (13). In the present study, an organic solvent-soluble fraction from ox-LDL was analyzed by normal-phase HPLC, and then the TFPI binding activity to each fraction was measured. Figure 1 shows a typical binding profile of TFPI to lipid fractions obtained by HPLC. A single major peak was observed in ox-LDL lipids, but not in native LDL lipids, eluting near the top fraction, which might contain more short-chain fatty acid phospholipid than representative phospholipids that contain palmitic or stearic acid. In fact, 9CHO-PC, a major oxidation product of 1-palmitoyl-2-oleoyl PC (PO-PC), was recovered in the area of the top fraction (see comments below about 9CHO-PC). When the phospholipid fraction obtained from ox-LDL by TLC was subjected to HPLC and the TFPI binding activity was analyzed, almost the same binding of TFPI to each fraction was seen (data not shown). Therefore, it was suggested that only oxidation products of phospholipid showing altered hydrophobicity or affinity for a silica column bound to TFPI, and other lipid components of ox-LDL, such as free fatty acid, 7-ketocholesterol, cholesterol, triacylglycerol, and others, had little affinity for the TFPI molecule.

Effects of ox-phospholipid derivatives on TFPI activity and antigenicity

To appreciate the general profile of the active components in ox-phospholipid that inhibit the function of TFPI, we oxidized commercially available phospholipids that mimic the biological activity of ox-LDL (17, 29), and examined the effects of each ox-phospholipid on the activity and antigenicity of TFPI (Fig. 2). The oxidized products from phospholipid derived from PO-PC, 1-stearoyl-2-oleoyl PC (SO-PC), and 1-oleoyl-2-palmitoyl PC (OP-PC), designated as ox-PO-PC, ox-SO-PC, and ox-OP-PC, respectively, all markedly decreased

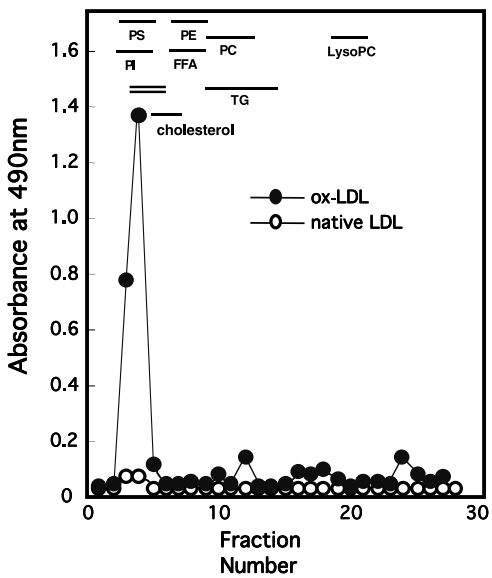

FIG. 1. Normal-phase HPLC of organic solvent-soluble components of ox-LDL. The organic solvent-soluble fraction was separated by a silica column, and the TFPI binding activity of each fraction was measured using a microplate binding assay as described in Materials and Methods. The solid lines at the top of figure show the elution positions of standard nonoxidized phospholipids. The double line indicates the elution position of purified 9CHO-PC. FFA, free fatty acid; PC, phosphatidylcholine; LysoPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TG, triacylglycerol.

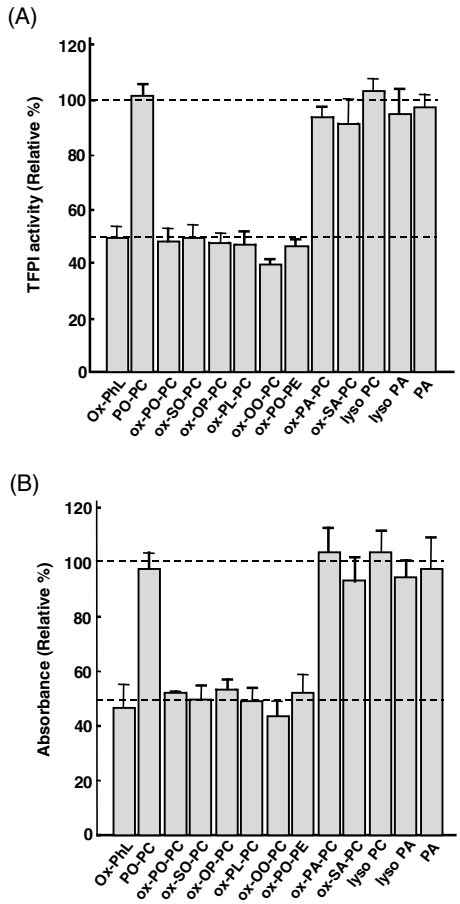

FIG. 2. Effects of ox-phospholipids of various compositions on the activity (A) and antigenicity (B) of TFPI. Commercially available phospholipids containing various fatty acids in the sn-1 and sn-2 positions were oxidized with ferrous ion, and ox-lipid fractions were recovered as described in Materials and Methods. The TFPI activity and the antigenicity of TFPI were determined after incubation of rTFPI (10 ng/ml) with each sample at a concentration equivalent to ox-LDL (50 μg/ml protein). Results are expressed as percentages of the control value (rTFPI only) and are the means ± SD from three independent experiments. See text for abbreviations.

the activity of TFPI, and that of 1-palmitoyl-2-linoleoyl PC (PL-PC) (ox-PL-PC) also had a similar effect (Fig. 2A). When TFPI was exposed to the oxidation products of 1,2-dioleoyl PC (OO-PC) (ox-OO-PC), a more prominent reduction of TFPI activity was observed, and the oxidation products of 1-palmitoyl-2-oleoyl phosphatidylethanolamine (PO-PE) (ox-PO-PE), which has a different head group, also inhibited the TFPI activity similarly to ox-PO-PC. On the other hand, the oxidation products of 1-palmitoyl-2-arachidonoyl PC (PA-PC) (ox-PA-PC) and 1-stearoyl-2-arachidonoyl PC (SA-PC) (ox-SA-PC) showed weaker inhibitory effects on the TFPI activity, and compounds known to be proatherogenic, such as 1-oleoyl PC (lysoPC) and 1-oleoyl phosphatidic acid (lysoPA), had almost no inhibitory effect. The effects of these ox-phospholipids on the antigenicity of TFPI were completely consistent with the effects on the TFPI activity (Fig. 2B). These results indicated that oxidation products of phospholipid containing oleic acid and linoleic acid, but not arachidonic acid, at the sn-1 and/or sn-2 positions specifically impair TFPI activity and antigenicity, irrespective of the composition of the head group. However, these phospholipids used in the above experiment are a mixture of oxidation products of phospholipids such as aldehyde phospholipids, carboxylic phospholipid, and hydroperoxide phospholipid. Therefore, the inhibitory effects of purified 9CHO-PC/9COOH-PC and 5CHO-PC/5COOH-PC, which are the major oxidation products of PO-PC (17) and SA-PC (29), respectively, were investigated further (Fig. 3). 9CHO-PC and its ultimate oxidation product (9COOH-PC), but not 5CHO-PC or 5COOH-PC, dose-dependently inhibited the TFPI activity, and the activity fell to become approximately one-third of that of TFPI alone at a concentration of 50 μg/ml.

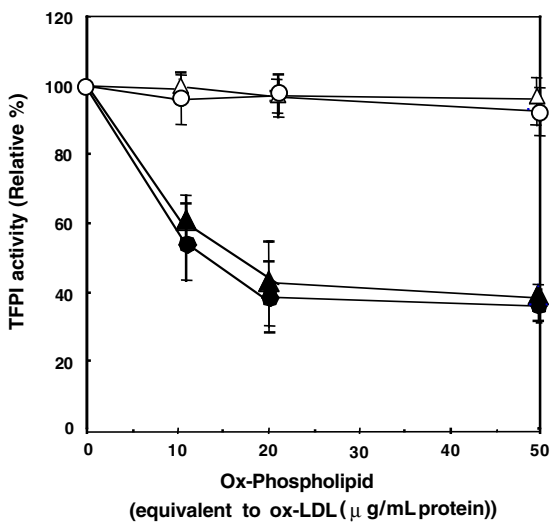

FIG. 3. Effects of oxidized products from PO-PC and SA-PC on the TFPI activity. rTFPI (10 ng/ml) was incubated with 9CHO-PC (●), 9COOH-PC (▲), 5CHO-PC (○), or 5COOH-PC (△) at a concentration equivalent to ox-LDL (0~50 μg/ml protein) for 3 h, and TFPI activity was determined as described in Materials and Methods. Results are expressed as percentages of the control value (rTFPI only) and are the means ± SD from three independent experiments.

Ox-phospholipid associates strongly with TFPI via the C-terminal basic region of TFPI

To examine the direct binding of phospholipids with TFPI, rTFPI was incubated for 1 h with vesicles of various ox-phospholipids, and each incubation mixture was subjected to nondenaturating agarose gel electrophoresis followed by immunoblotting (Fig. 4). As described previously, rTFPI migrated faster than ox-LDL to the phospholipid fraction obtained from ox-LDL (14). In the presence of ox-PO-PC, 9CHO-PC, or 9COOH-PC, which inhibit TFPI activity and antigenicity, almost all the rTFPI migrated with the same mobility as that of phospholipids obtained from ox-LDL, as confirmed by lipid staining of the gel with Sudan dye. On the other hand, no essential change in the migration of rTFPI was observed after the incubation of rTFPI with vesicles of PO-PC, ox-SA-PC, 5CHO-PC, or 5COOH-PC. When rTFPI-C, which lacks the C-terminal basic region of TFPI, was incubated with ox-PO-PC, 9CHO-PC, and 9COOH-PC instead of rTFPI, only small amounts of rTFPI-C comigrated with these phospholipid vesicles. Therefore, it was suggested that the ox-phospholipid-dependent decreases in the activity and antigenicity of TFPI are mainly due to the interaction between ox-phospholipids and the C-terminal basic region of TFPI.

DISCUSSION

In this study, we demonstrated that the ability of ox-LDL to inhibit the function of TFPI is due to oxidation products of phospholipids in ox-LDL. The oxidation product(s) of 1- and/or 2-oleoyl PC or phosphatidylethanolamine (PE) showed potent inhibitory activity, whereas those of arachidonyl PC showed only a weak effect on TFPI activity and antigenicity. The structural requirements for inhibitory activity of oxidation products of phospholipid are summarized in Fig. 5. A key feature of the unsaturated fatty acid in preoxidation phospholipid is the position of the double bond, *i.e.*, δ-9, but not δ-5 fatty acid at the sn-1 and/or sn-2 positions. When rTFPI was incubated with purified 9CHO-PC, the functional

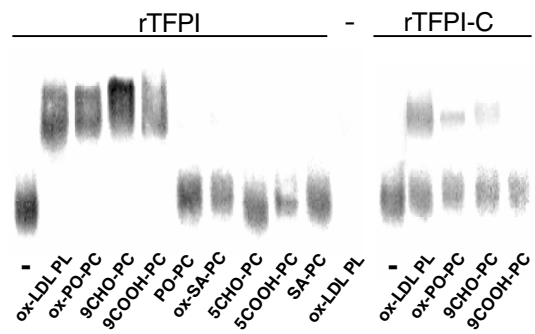

FIG. 4. Agarose gel electrophoresis of rTFPI and rTFPI-C in the presence of various phospholipids. rTFPI or rTFPI-C (20 ng) was incubated with ox-phospholipids (PL) (a concentration equivalent to 50 μg of protein of ox-LDL) for 3 h, and the incubation mixture was subjected to agarose gel electrophoresis. TFPI was detected with a polyclonal antibody after western blotting as described in Materials and Methods.

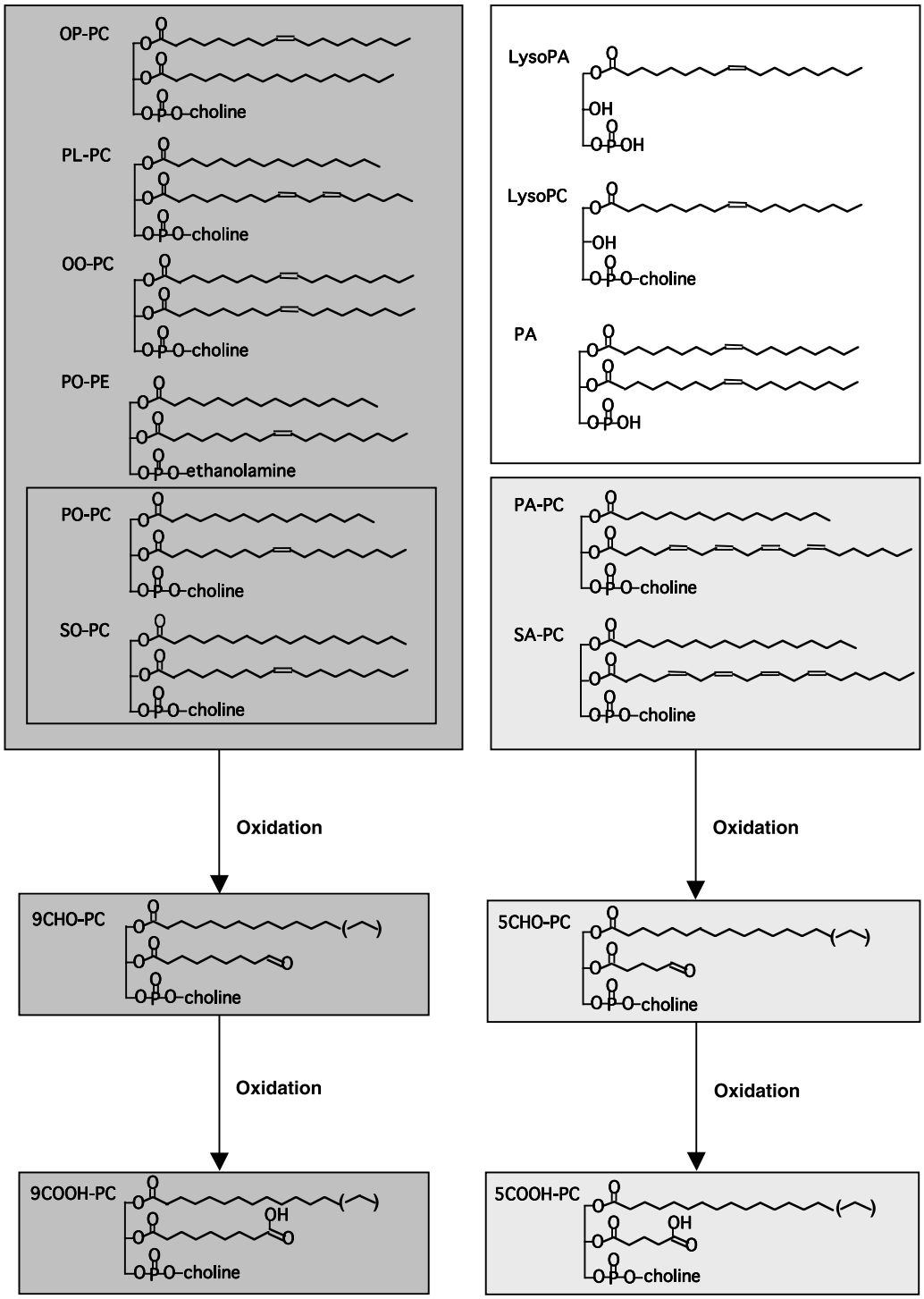

Fig. 5. Structures of phospholipids and the oxidation products. The structural requirements of oxidation products of phospholipid for inhibitory activity on TFPI are summarized. The common feature of active compounds is the position of the double bond of the unsaturated fatty acid in the preoxidation phospholipid, *i.e.,* δ-9, but not δ-5, fatty acid at the sn-1 and/or sn-2 positions. 9CHO-PC and 5CHO-PC were prepared from PO-PC and SA-PC, respectively. 9COOH-PC and 5COOH-PC were prepared by further oxidation of 9CHO-PC and 5CHO-PC, respectively.

activity of TFPI was specifically impaired. It has been reported that 5CHO-PC forms Schiff base adducts with lysine residues of apolipoprotein B and presumably other proteins (29). Friedman *et al.* (12) reported that, whether oxidized or not at the sn-2 position, certain monomeric phospholipids containing short-chain fatty acids had antigenicity for an IgM monoclonal autoantibody to ox-phospholipid that blocks the uptake of ox-LDL by macrophages. However, 5CHO-PC

showed only a weak inhibitory effect on TFPI activity. 9COOH-PC, the ultimate oxidation product of 9CHO-PC, had a similar inhibitory effect to that of 9CHO-PC. When rTFPI was incubated with a chemically reduced form of 9CHO-PC, *i.e.,* its alcohol, no decrease in TFPI activity was observed (unpublished observation). Although the reason for such a chain length-dependent and functional group-dependent difference of ox-phospholipid association with TFPI is not clear, one possibility is that a specific structural conformation of ox-phospholipid is important for the interaction with TFPI.

Other lipid components of ox-LDL, such as free fatty acid, lysoPC, cholesterol, and triacylglycerol, did not show binding affinity to TFPI (Fig. 1). This result supports our previous finding that, among the TLC fractions, only the phospholipid fraction inhibited the TFPI activity (13). As oxidized sterols such as 7β-hydroxycholesterol and 25-hydroxycholesterol are reported to be biologically active (26), the effect of these components on the TFPI activity was examined using commercially available chemicals; neither of them affected the activity or the antigenicity (data not shown). Further, the degradation fragments of apolipoprotein B-100, which acquire net negative charge (27), are not candidates, because the aqueous extract of ox-LDL was inactive.

Our results show that the decrease in TFPI activity by ox-phospholipid was well correlated with the ox-phospholipid-dependent reduction of the antigenicity of TFPI, which was measured with an ELISA system consisting of a polyclonal antibody and a monoclonal antibody specific for conformation between Kunitz domains 1 and 2 of TFPI. By means of the direct binding assay, the importance of the C-terminal basic part for the association of ox-phospholipid with TFPI was confirmed. However, a weak association of ox-phospholipid with TFPI-C was also observed. The C-terminal part of TFPI, which is known to be important for the activity (31), contains eight lysine and four arginine residues in the 31 amino acids from the end (34). The basic region of the Kunitz 3 domain between Gly212 and Phe243, which is involved in the interaction of TFPI with proteoglycans (11, 23), may also contribute to the interaction of TFPI with ox-phospholipids. Therefore, it is likely that the TFPI activity would be impaired by the formation of adducts between these basic amino acid residues of TFPI and the oxidation products of δ-9 unsaturated phospholipids in ox-LDL. These interactions are likely to induce a conformational alteration in the TFPI molecule, and may result in the inhibition of FXa binding to the Kunitz 1 domain and TF/FVIIa binding to the Kunitz 2 domain of TFPI.

Recently, Itabe *et al.* (18) reported that minimally oxidized LDL, which was produced by dialyzing LDL with $FeSO_4$, contains a more than fourfold greater amount of aldehyde PCs, in which the concentration of 9CHO-PC is much higher than that of 5CHO-PC, compared with heavily oxidized LDL prepared with a copper catalyst. In vessel walls, the physiological location of LDL modification, tissue fluid surrounding the LDL particles is likely to circulate continuously, and it can be speculated that minimally oxidized LDL prepared using dialysis bags is a reasonable model of oxidatively modified LDL present *in vivo*. As compared with 5CHO-PC, little attention has been given to the question of whether or not 9CHO-PC affects the biological activity. However, our findings suggest that specific oxidized derivatives of δ-9 unsaturated fatty acid-containing phospholipids, such as 9CHO-PC, may be important initiators of atherogenesis through impairment of anticoagulant molecules on the subendothelial vasculature, giving rise to atherosclerotic plaques.

The inhibition of LDL oxidation by antioxidants has been reported (30). In addition to the antioxidizing effect, these drugs inhibit the expression of adhesion molecules and the proliferation of endothelial cells and smooth muscle cells (3, 4). Many basic studies have offered insights into potential positive effects of antioxidants in atherosclerosis both in experimental animals and in humans (19, 27). These results suggest that antioxidants, which suppress the oxidative modification of LDL, should be effective in preventing atherogenesis. The antioxidation effects of such drugs on LDL might contribute to the maintenance of effective anticoagulant activity of TFPI in atherosclerotic regions.

ABBREVIATIONS

ELISA, enzyme-linked immunosorbent assay; FVIIa, activated factor VII; FX, factor X; FXa, activated factor X; LDL, low-density lipoprotein; lysoPA, 1-oleoyl phosphatidic acid; lysoPC, lysophosphatidylcholine; OO-PC, 1,2-dioleoyl PC; OP-PC, 1-oleoyl-2-palmitoyl PC; ox-, oxidized; PA, 1,2-dioleoyl phosphatidic acid; PA-PC, 1-palmitoyl-2-arachidonoyl PC; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL-PC, 1-palmitoyl-2-linoleoyl PC; PO-PC, 1-palmitoyl-2-oleoyl PC; PO-PE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine; rTFPI, recombinant TFPI; rTFPI-C, rTFPI lacking C-terminal basic part; SA-PC, 1-stearoyl-2-arachidonoyl PC; SO-PC, 1-stearoyl-2-oleoyl PC; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TLC, thin-layer chromatography; 5CHO-PC, PC containing a 5-carbon chain with a terminal aldehyde in the sn-2 position (oxovaleroyl PC, 1-palmitoyl-2-pentyl aldehyde PC); 5COOH-PC, PC containing a 5-carbon chain with a terminal carboxylic acid in the sn-2 position (1-stearoyl-2-pentanoic acid PC); 9CHO-PC, PC containing a 9-carbon chain with a terminal aldehyde in the sn-2 position (oxononanoyl PC, 1-palmitoyl/stearoyl-2-nonyl aldehyde PC); 9COOH-PC, PC containing a 9-carbon chain with a terminal carboxylic acid in the sn-2 position (1-palmitoyl/stearoyl-2-nonoic acid PC).

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