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Multiple Substrates for Paraoxonase-1 during Oxidation of Phosphatidylcholine by Peroxynitrite

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Paraoxonase (PON-1) is a high-density lipoprotein (HDL)-bound enzyme with activity toward multiple substrates. It hydrolyzes organic phosphate and aromatic carboxylic acid esters. It also inhibits accumulation of oxidized phospholipids in plasma lipoproteins by a mechanism yet to be determined. Therefore, we subjected apolipoprotein A-I proteoliposomes containing either 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine or 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine to oxidation by a peroxynitrite generator, SIN-1, in the presence and absence of purified PON-1. PON-1 modified the proportion of oxidation products without affecting the overall extent of PC oxidation. However, in the presence of PON-1, phosphatidylcholine isoprostanes were hydrolyzed to lysophosphatidylcholine. In addition, PON-1 hydrolyzed the phosphatidylcholine core aldehydes 1-palmitoyl-2-(9-oxo)nonanoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-(5-oxo)valeroyl-*sn*-glycero-3-phosphocholine to lysophosphatidylcholine. This hydrolysis was not affected by pefabloc, a serine esterase inhibitor. There was no detectable release of linoleate, arachidonate, or their hydroperoxy or hydroxy derivatives in the presence of PON-1. We conclude that PON-1 minimizes the accumulation of phosphatidylcholine oxidation products by the hydrolysis of phosphatidylcholine isoprostanes and core aldehydes to lysophosphatidylcholine with a serine esterase-independent mechanism.

Key Words: **paraoxonase-1; mass spectrometry; electrospray; hydroperoxides; core aldehydes; isoprostanes; apolipoprotein A-I; lysophosphatidylcholine.**

Abbreviations used: apoA-I, apolipoprotein A-I; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; ESI, electrospray ionization; HDL, high-density lipoproteins; LDL, low-density lipoproteins; SIN-1, 3-morpholinosydnonimine; lysoPC, lysophosphatidylcholine; PAF, platelet-activating factor; PAPC, palmitoyl, arachidonoyl glycerophosphocholine; PLPC, palmitoyl, linoleoyl glycerophosphocholine; PONPC, 1-palmitoyl-2-(9-oxo)nonanoyl-*sn*-glycero-3-phosphocholine; POVPC, 1-palmitoyl-2-(5-oxo)valeroyl-*sn*-glycero-3-phosphocholine; PON-1, paraoxonase-1.

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Paraoxonase-1 (PON-1) is exclusively associated with high-density lipoproteins (HDL) and is believed to protect against cardiovascular diseases by inhibiting oxidation of low density lipoproteins (LDL) (1). Thus, addition of HDL to LDL reduced the *in vitro* accumulation of oxidized phospholipids (2) and cholesteryl esters (3). Furthermore, HDL reduced the formation of low-molecular-weight aldehydes and subsequently reduced the uptake of LDL by macrophages (4). PON-1 is believed to be responsible for a major portion of the antioxidant activity of HDL (5). PON-1 deficient mice, fed an atherogenic diet, are more prone to develop atherosclerosis and PON-1 deficient HDL does not protect LDL against oxidation (6). Addition of purified PON-1 to LDL inhibited accumulation of oxidation products (7) and reduced the ability of LDL to induce expression of adhesion molecules by endothelial cells (2, 8). Platelet-activating factor (PAF) acetyl hydrolase and lecithin cholesterol acyltransferase have been reported to inhibit the accumulation of oxidized lipids (9, 10). The hydrolytic activity of PON-1over a wide range of substrates, including organophosphates, aromatic esters and more recently lactones, thiolactones and cyclic carbonate esters (11–14) has been well characterized. However, the mechanism by which PON-1 inhibits oxidation of phospholipids is not well studied.

Palmitoyl, arachidonoyl phosphatidylcholine (PAPC) and palmitoyl, linoleoyl phosphatidycholine (PLPC) constitute the major species of phospholipids in plasma lipoproteins and their oxidation produces a spectrum of

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PC derivatives including hydroperoxides, isoprostanes and core aldehydes. Recently, we reported that PON-1, in the absence of oxidant, hydrolyzed PC core aldehydes and produced lysophosphatidylcholine (lysoPC) (15). The present study demonstrates that PON-1 hydrolyzes PC isoprostanes, in addition to PC core aldehydes, to lysoPC. This hydrolysis was insensitive to pefabloc, a serine esterase inhibitor (16). Under these experimental conditions, PON-1 did not protect PLPC or PAPC against oxidation.

EXPERIMENTAL PROCEDURES

Materials. 3-Morpholinosydnonimine (SIN-1) and Pefabloc, 4-[2-aminoethyl] benzenesulfonyl fluoride were from Sigma–Aldrich (St. Louis, MO). 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine were from Avanti (Alabaster, AL). Choline phospholipid kit was from Roche Molecular Biochemicals. All solvents used in liquid chromatography (LC)/electrospray ionization (ESI)/mass spectrometry (MS) were HPLC grade. Other solvents and chemicals were of reagent grade, provided by local suppliers.

Serum HDL, from healthy subjects fasted for 12–14 h, was isolated by ultracentrifugation between densities 1.063 and 1.21 g/ml (17), and apoA-I was isolated from delipidated HDL protein by ion-exchange HPLC using an Aquapore AX-300 column (3 cm × 4.6 mm, Pierce, Rockford, IL) as described (15).

Preparation of proteoliposomes. Proteoliposomes were prepared by cholate dialysis as described by Sorci-Thomas *et al.* (18). Apo A-I, in 10 mM Tris–HCl buffer, pH 7.4, was added to a dried lipid film containing PLPC (or PAPC) in a molar ratio of 1:18:82:5 apoAI:PC: DMPC:cholesterol. The dispersed liposomes were dialyzed overnight against 10 mM Tris–HCl/0.15 M NaCl buffer, pH 7.4. PC isoprostanes were prepared by autoxidation of PAPC. Briefly, 10 mg PAPC were dissolved in chloroform/methanol (2:1) and dried in a number of tubes as a thin film. All tubes were exposed to air for 48–96 h at room temperature and the oxidation products were resolved by TLC (0.5 mm silica gel H) using chloroform/methanol/water/acetic acid (45:35: 12:6 v/v). Oxidation products were visualized using dichlorofluorescein. The individual bands were extracted, purified and identified by LC/ESI/MS as described previously (19, 20). Isoprostanes were prepared as a mixture of E_2/D_2 isoprostanes PC (*m/z* 830), F_2 isoprostanes PC (*m/z* 832), and epoxy isoprostane PC (*m/z* 828).

PC isoprostane proteoliposomes were prepared as described by Chen and Albers (21). ApoA-I in 10 mM Tris–HCl buffer, pH 7.4, was added to a dried lipid film containing a mixture of PC isoprostanes, DMPC and free cholesterol in a molar ratio of 0.8:60:190:12.5 apoAI:PC isoprostanes:DMPC:cholesterol. The dispersed liposomes were dialyzed overnight against 10 mM Tris–HCl buffer, pH 7.4.

Oxidation of proteoliposomes. PLPC (or PAPC) proteoliposomes were incubated with 1 mM SIN-1 in 1 mM Tris–HCl, pH 7.4, at 37°C in the presence or absence of purified PON-1 at 75 arylesterase units (final activity). The amount of PC used was measured using an enzymatic kit (Roche Molecular Biochemicals). At each time point, an aliquot was withdrawn and the lipids were extracted with chloroform/methanol 2:1 (v/v) (22).

Liquid chromatography–electrospray ionization–mass spectrometry (LC/ESI/MS). Analysis was performed using a normal-phase silica column (2.1 × 25 mm, Supelco, Bellefonte, PA), in a Hewlett–Packard (HP) Model 1050 liquid chromatograph, connected to an HP Model 5989A quadrupole mass spectrometer, equipped with a nebulizer-assisted electrospray ionization interface (HP 59987A). The column was eluted with a linear gradient of chloroform/methanol/30% ammonium hydroxide, 80:19.5:0.5 (v/v) to chloroform/methanol/water/30% ammonium hydroxide, 60:34:5.5:0.5 (v/v) in 14 min, and held for 10 min, at a flow rate of 0.25 ml/min and the effluent was admitted directly into the mass spectrometer (23). The capillary exit voltage was set at 150 V, with electron multiplier at 1795 V. Positive ESI spectra were examined in the mass range 450–1100 amu. Negative ESI spectra were examined in the mass range 250–1100 amu. The molecular species of the oxidation products were identified, based on the molecular mass provided by ESI/MS, the knowledge of the fatty acid (or oxo-fatty acids) composition of the phosphatidylcholine classes and the relative HPLC retention time as described (24). The amount of phosphatidylcholine and its oxidation products were measured using an internal standard as described (15).

PON-1 enzyme assay. PON-1 arylesterase was determined using 1 mM phenyl acetate dissolved in 1 mM $CaCl_2$ and 20 mM Tris–Cl, pH 8.0, and the product was monitored at 270 nm at room temperature. PON-1 arylesterase activity was calculated against blanks to correct for spontaneous hydrolysis of phenyl acetate (25). The effect of Pefabloc (0.1 or 0.2 mM final concentration) on PON-1 was studied during exposure of PLPC (or PAPC) proteoliposomes to SIN-1. Pefabloc was also added to PC core aldehydes or PC isoprostane proteoliposomes (oxidant free medium).

Serum PON-1 purification. PON-1 Q192 type was purified from human plasma through (pseudo) affinity chromatography using Cibacron Blue 3GA (Sigma Chemical Co.) and anion-exchange chromatography using two consecutive DEAE BioGel A (Bio-Rad, Richmond, CA) columns as described (26, 27). Confirmation of PON-1 purification was performed by microcapillary electrospray LC/MS/MS as described previously (15). Neither lecithin cholesterol acyltransferase nor PAF-acetyl hydrolase was detected in the PON-1 preparations.

Statistics. Statistical analysis was performed by repeated-measures analysis of variance (ANOVA) followed by Bonferroni corrected Student's *t* test for individual points using Graph Pad Software (San Diego, CA).

RESULTS

PON-1 Inhibits Accumulation of Oxidized Phosphatidylcholine

PLPC and PAPC were oxidized by exposure to SIN-1, in the presence and absence of PON-1. PON-1 decreased the concentration of monohydroperoxides (*m/z* 790) during oxidation of PLPC (Fig. 1, PLPC-OOH) and also decreased the concentration of monohydroperoxides (*m/z* 814) during oxidation of PAPC (Fig. 2, PAPC-OOH) (ANOVA and 0.0014, $P = 0.0129$ respectively). In the presence of PON-1, the increase of PLPC monohydroxides reached a plateau after 2 h of oxidation (Fig. 1, PLPC-OH). In contrast, in the presence of PON-1, the accumulation of monohydroxides of PAPC was linear over the 4 h of oxidation (Fig. 2, PAPC-OH). The total amount of monohydroxide was low at all time points, compared to the amount of oxidized PLPC and PAPC at 4 h. The lower concentration of monohydroperoxide in the presence of PON-1 appears to be due to conversion to PC core aldehydes, rather than monohydroxides.

PC core aldehydes are produced by beta-scission of the oxidized unsaturated fatty acid. Oxidation of PLPC produces primarily a nine-carbon aldehyde,

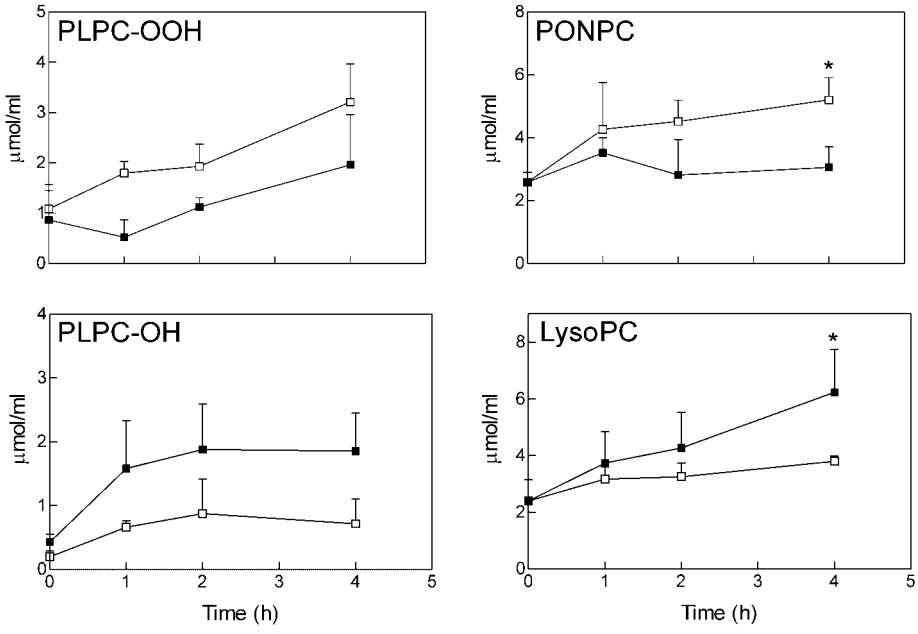


FIG. 1. Accumulation of PC monohydroperoxides (PLPC-OOH), PC monohydroxides (PLPC-OH), PC core aldehyde (PONPC), and palmitoyl lysophosphatidylcholine (lysoPC) during oxidation of PLPC in the presence (closed squares) or absence (open squares) of PON-1. Details of PLPC oxidation and LC/ESI/MS operating conditions were as described under Experimental Procedures. Values represent means ± SD of three separate experiments. *$P < 0.05$.

1-palmitoyl-2-(9-oxo)nonanoyl-*sn*-glycero-3-phosphocholine (PONPC) (*m/z* 650). Figure 1 shows the effect of PON-1 on the accumulation of PONPC during oxidation of PLPC proteoliposomes. In the presence of PON-1, there was a limited increase of PONPC ($P = 0.0031$) (Fig. 1, PONPC) and significant increase in lysoPC (Fig. 1, LysoPC).

The oxidation of PAPC produces PC isoprostanes, in addition to a five-carbon PC core aldehyde, 1-palmitoyl-2-(5-oxo)valeroyl-*sn*-glycero-3-phosphocholine (POVPC) (*m/z* 594). PC isoprostanes are produced as a mixture of regioisomers: E_2/D_2 isoprostanes (*m/z* 830), F_2 isoprostanes (*m/z* 832), and epoxy isoprostanes (*m/z* 828). Figure 2 shows the effect of PON-1 on the accumulation of POVPC and PC isoprostanes during oxidation by SIN-1. In the absence of PON-1, POVPC increased linearly with time of oxidation, while PON-1 significantly reduced the accumulation of POVPC (Fig. 2, POVPC) ($P = 0.0048$), and increased the accumulation of lysoPC (Fig. 2, LysoPC). The amount of lysoPC under these conditions exceeded the decrease in POVPC. PON-1 significantly decreased the accumulation of PC isoprostanes ($P = 0.0087$) during PAPC oxidation (Fig. 2, PAPC-isoP). In contrast, there was virtually no formation of lysoPC during oxidation of PAPC in the absence of PON-1. To determine whether hydrolysis of PC isoprostanes by PON-1 could account for the decreased accumulation of isoprostanes during oxidation, we carried out incubations of PON-1 with PC isoprostane proteoliposomes. In the absence of PON-1, the level of PC isoprostanes remained unchanged, whereas in the presence of PON-1, PC isoprostanes decreased ($P = 0.001$) (Fig. 3, PAPC-isoP) and, lysoPC increased ($P = 0.0002$) (Fig. 3, LysoPC).

There was no hydrolysis of PC monohydroxides in the presence of PON-1, as indicated by the absence of free monohydroxy fatty acids in the incubation medium.

PON-1 Does Not Spare Phosphatidylcholine from SIN-1 Oxidation

Figure 4 (upper panels) shows the effect of SIN-1 oxidation on PLPC or PAPC proteoliposomes, in the presence or absence of PON-1. PON-1 had no effect on the decrease in total diacyl PC concentrations during oxidation of proteoliposomes with SIN-1 (Figs. 1 and 2). We observed that, in the first 2 h, the loss of PAPC was more rapid compared to PLPC (data not shown), while by 4 h, there was no significant difference in the extent of oxidation between these two PCs. The loss of diacyl PC was accounted for by the accumulated oxidation products. These data show that PON-1 did not prevent the oxidation of phosphatidylcholine. There was no loss of DMPC during incubation and no evidence of formation of myristoyl lysoPC.

The Effect of Pefabloc on the Formation of Lysophosphatidylcholine

Oxidation of PLPC or PAPC in the presence of 0.2 mM pefabloc did not affect the formation of lysoPC

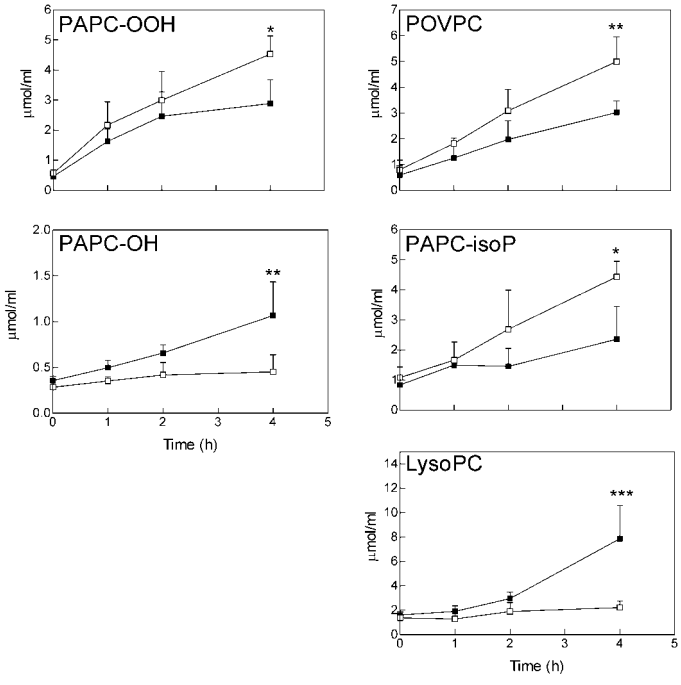


FIG. 2. Accumulation of PC monohydroperoxides (PAPC-OOH), PC monohydroxides (PAPC-OH), PC core aldehyde (POVPC), PC isoprostanes (PAPC-isoP), and palmitoyl lysophosphatidylcholine (lysoPC) during oxidation of PAPC in the presence (closed squares) or absence (open squares) of PON-1. Details of PAPC oxidation and LC/ESI/MS operating conditions were as described under Experimental Procedures. Values represent means ± SD of three separate experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

(Fig. 4, lower panels). Pefabloc did not affect the extent of PC oxidation or the hydrolysis of PC core aldehydes or PC isoprostanes, whether generated by SIN-1 oxidation or exogenously added (data not shown).

DISCUSSION

The present experiments extend our previous observations with two important new findings: (i) PON-1 retains its phospholipase activity toward PAPC oxidation products for at least 4 h in the presence of the strong oxidant SIN-1; and (ii) PON-1 shows phospholipase activity toward PC isoprostanes. Previous studies by others have shown that PON-1 destroys the bioactivity of oxidized PC (2, 6, 8). Oxidized phospholipids constitute the major portion of bioactivity in each of class oxidized lipoprotein (28). These products have been specifically shown to be responsible for the promotion of monocyte binding to endothelial cells by oxidized lipoproteins (29). Watson *et al.* (2) have shown that oxidized phospholipids, isolated from minimally modified LDL (MM-LDL), as well as products of oxidized PAPC, can mimic the biological activity of MM-LDL (30). In addition, POVPC and its corresponding carboxylate, 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC) and epoxyisoprostane *sn*-glycero-3-phosphocholine have been identified as inflammatory compounds, capable of inducing monocyte binding to endothelial cells (31). The products of PLPC oxidation have relatively less proinflammatory activity (32). The significance of the formation of short chain fatty acyl PC core aldehydes, such as PONPC, was recently reinforced by the observation that oxidized LDL, measured using an antibody that reacts with PONPC and proteins modified by PONPC, is elevated in patients with acute myocardial infarction (33).

The action of PON-1 during peroxidation of PC by SIN-1 appears to be biphasic, in that it decreases hydroperoxides and increases hydroxides at early time points, and decreases accumulation of POVPC, PONPC, and PC isoprostanes and increases formation of lysoPC at later time points. The modest level of conversion of hydroperoxides to hydroxides under our experimental conditions, compared to other products, suggests that this is a minor activity of PON-1. It may be a consequence of the oxidation of the single free cysteine residue of PON-1 and thus not a true catalytic peroxidase activity. Evidence that HDL is involved in the conversion of PC-OOH to PC-OH has been obtained under non-oxidizing conditions (34, 35). Further studies are required to determine whether PON-1 can function as a peroxidase in a reducing environment, in contrast to the simultaneous exposure to lipid hydroperoxides and oxidant used in this study.

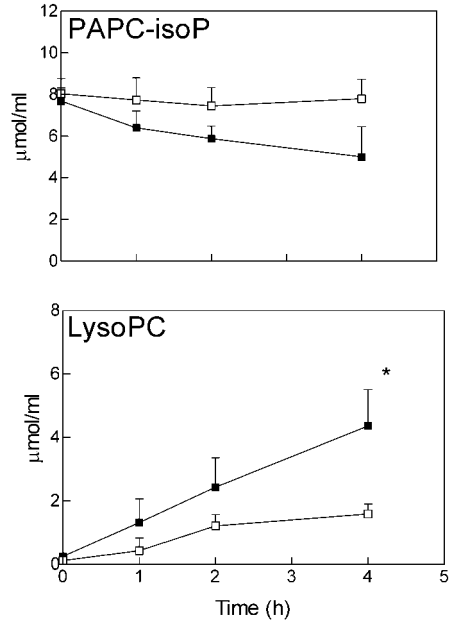


FIG. 3. Hydrolysis of PC isoprostane proteoliposomes and formation of palmitoyl lysophosphatidylcholine in the presence (closed squares) and absence (open squares) of PON-1. Preparation of proteoliposomes and details of LC/ES/MS operating conditions were as described under Experimental Procedures. Values represent means ± SD of three separate experiments. $*P < 0.001$.

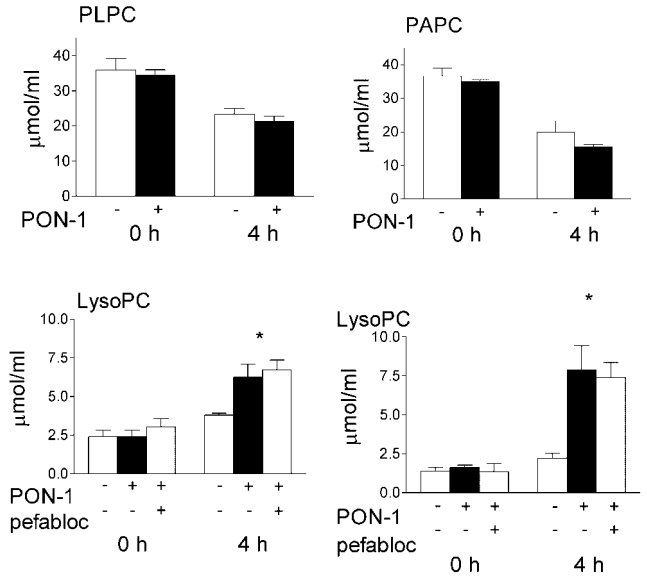


FIG. 4. Concentration of PLPC or PAPC following oxidation with SIN-1 in the presence (closed bars) or absence (open bars) of PON-1 (upper panels). The concentration of palmitoyl lysophosphatidylcholine during oxidation of PLPC (PLPC-lysoPC) or PAPC (PAPC-lysoPC) in the presence (solid bars) or absence (open bars) of PON-1 or PON-1 plus pefabloc (shaded bars) (lower panels). Details of LC/ESI/MS operating conditions were as described under Experimental Procedures. Values represent means ± SD of three separate experiments.

Under our experimental conditions, PON-1 must compete with apoA-I for lipid hydroperoxides. We have previously shown that apoA-I efficiently converts PC fatty acid hydroperoxides to PC core aldehydes (15). We propose a scheme whereby apoA-I and PON-1 form a coupled system that, in the presence of an oxidant, efficiently converts PC fatty acid hydroperoxides to lysoPC (Scheme 1).

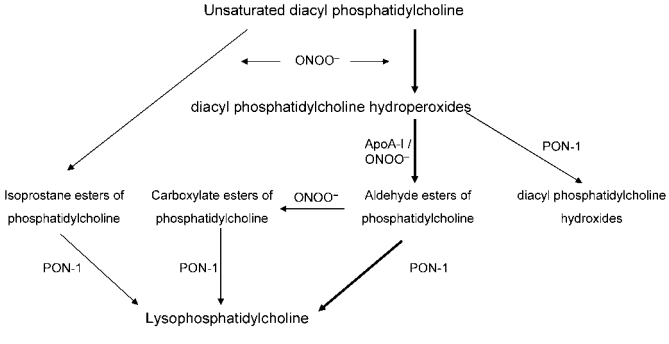
Previously, Stafforini *et al.* (36) demonstrated that PAF-acetyl hydrolase readily attacks oxidatively fragmented phosphatidylcholines in oxidized LDL. The present preparations of apoA-I and PON-1 were specifically shown to be free of PAF-acetyl hydrolase and lecithin cholesterol acyltransferase and thus the enzyme activity can be attributed only to PON-1. The hydrolysis of PC oxidation products in the presence of pefabloc, which has been shown to inhibit PAF- acetyl hydrolase (10, 16), suggests that hydrolysis of oxidized phospholipids by PON-1 occurs by a serine-independent mechanism. Consistent with this report, Rodrigo *et al.* (37) demonstrated that PON-1 can hydrolyze PAF, in the presence of SB-222657, a potent serine esterase inhibitor.

Isoprostanes are prostaglandin-like compounds that are known to affect vascular and platelet function (38, 39). The biological importance of PC isoprostanes, that are produced as regioisomers by direct oxidation of arachidonic acid (40) *in vivo,* has yet to be determined. Roberts and Morrow (40) reported that the majority of plasma isoprostanes exist esterified to phospholipid and are subsequently slowly released by phospholipase(s), although the nature of phospholipase(s) had not been determined previously. Our experiments show that PON-1 is one of these phospholipases. However, this does not exclude the possibility that other enzymes could also be involved.

The concentration of PON-1 used in this study is about half of that found in normal serum, as estimated in terms of arylesterase activity. This activity is able to effectively inhibit accumulation of oxo-phospholipids in the presence of peroxynitrite. Although PON-1 arylesterase activity was inhibited by PC hydroperoxides, as observed in the current and previous studies (41, 42), this inhibition apparently has little effect on the PON-1 hydrolytic activity toward PC core aldehydes and PC isoprostanes. This suggests that the active sites involved in these two activities are distinct (43, 44). Previously PON-1 has been shown to hydrolyze H_2O_2 (42). PON-1 did not decrease the concentration of peroxynitrite in our experiments (data not shown).

Incubation of phospholipid with PON-1 in the presence of apoA-I, in our experiments, is likely a critical determinant of the results. Oda *et al.* (45) recently demonstrated that PON-1 arylesterase activity is modulated by apoA-I, only in the presence of lipids. In addition, mutation of specific amino acids residues in the N-terminal region of apoA-I, significantly affected PON-1 specific activity.

These results demonstrate that PON-1, in the presence of apoA-I, alters the oxidation products of PLPC and PAPC, without protecting these PCs from oxidation by SIN-1. The major activity of PON-1 is to hydrolyze PC core aldehydes, generated by SIN-1 oxidation in the presence of apoA-I, to lysoPC, by a phospholipase A_2-like activity. In addition, PON-1 modestly reduced the concentration of PC monohydroperoxides by conversion to PC monohydroxides. Finally, PON-1 hydrolyses PC isoprostanes to lysoPC (Scheme 1).



SCHEME 1. Proposed roles for apoA-I and PON-1 in the oxidative conversion of unsaturated fatty acyl glycerophosphocholines to major end products. The predominant steps are indicated with bold arrows.

The interrelationships between PON-1, lecithin cholesterol acyltransferase, and PAF-acetyl hydrolase, in addition to the effect of PON-1 genetic polymorphisms on the accumulation of oxo-phospholipids, are important questions to be addressed in future studies.

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