Phospholipid hydroperoxide (PLOOH) degrading activity of high density lipoprotein (HDL)-derived paraoxonase-1 (PON1) was investigated, using peroxidized 1-palmitoyl-2-oleoyl phosphatidylcholine (PCOOH) as substrate and high performance thin layer chromatography for quantitative peroxide analysis. Incubation of PCOOH with PON1 resulted in decay of the latter and reciprocal buildup of oleic acid hydroperoxide (OAOOH) at rates unaffected by GSH or other reductants. A serine esterase inhibitor blocked this activity and a recombinant PON1 was devoid of it, raising the possibility that the activity represents platelet-activating factor acetylhydrolase (PAF-AH), an esterase that co-purifies with PON1 from HDL. This was verified by showing that a recombinant PAF-AH recapitulates the ability of natural PON1 to hydrolyze PCOOH and release OAOOH while having essentially no effect on parental PC. Furthermore, recombinant PAF-AH and natural PON1 were shown to have similar \( K_m \) values for PCOOH hydrolysis. Finally, we found that recombinant PAF-AH, but not PON1, catalyzes PLOOH hydrolysis in peroxidized low density lipoprotein. We conclude from this study that PON1 is neither a PLOOH peroxidase nor hydrolase and that the phospholipase \( A_2 \)-like activity previously attributed to PON1 in natural enzyme preparations was actually due to novel PLOOH hydrolytic activity of contaminating PAF-AH.

Paraoxonase-1 (PON1)\(^6\) is a calcium-dependent enzyme found exclusively in high density lipoprotein (HDL) particles, where it reportedly protects HDL against oxidative modification and inhibits lipid hydroperoxide (LOOH) accumulation in atherosclerotic lesions (1, 2). HDL is known to suppress atherogenic oxidation of low density lipoprotein (LDL), and PON1 has been implicated in this (3, 4), although these findings have been questioned (5). Both peroxidatic and hydrolytic functions have been described for PON1, either or both of which could play a role in its antioxidant activity (2). Previous studies (6, 7) showed that HDL-derived PON1 readily hydrolyzes truncated sn-2 fatty acyl groups of phospholipids from free radical-peroxidized LDL. The enzyme also inhibited monocyte chemotaxis elicited by oxidized phospholipids and lipoproteins (6). However, subsequent studies showed that this phospholipase \( A_2 \)-like activity (8, 9) did not arise from PON1 itself but rather from a trace contamination of another enzyme, platelet-activating factor acetylhydrolase (PAF-AH) (5).

Like PON1, PAF-AH is a 43-kDa HDL-associated protein, but unlike PON1, it can be found in LDL as well as HDL (10). Individuals who are homozygotic-null for this enzyme (11) exhibit an increased propensity for stroke and hemorrhage (12). PAF-AH cleaves the sn-2 acetyl group from the potent inflammatory mediator PAF but unlike most phospholipases \( A_2 \), does not act on phospholipids containing medium or long chain fatty acyl groups at this position. Phospholipids undergoing free radical-mediated (chain) peroxidation often become truncated at the sn-2 position, and these species can serve as PAF-AH substrates (13). Some oxidatively fragmented phospholipids with sufficiently short sn-2 residues can stimulate the PAF receptor, and PAF-AH inactivates these (14). The substrate specificity of PAF-AH is uniquely restricted by the length of the sn-2 residue (13, 15). However, if the \( \omega \)-end contains an oxy function (formyl or carboxyl), then even relatively long fragments become good substrates, e.g. the nine carbon fragment from linoleoyl oxidation (15) and esterified \( F_2 \)-isoprostanes (16). Thus, residue length is not the only factor involved in substrate recognition.

Knowing that hydrolysis of short sn-2 fatty acyl chains previously attributed to PON1 (9) was actually due to contaminating PAF-AH (5), we asked whether apparent PON1-catalyzed hydrolysis of oxygenated long chains, e.g. hydroperoxide interme-
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diates, might also reflect PAF-AH activity. Using 1-palmitoyl-2-hydroperoxyoleoyl-sn-glycero-3-phosphocholine (PCOOH) as a substrate, we have found that recombinant PON1 exhibits no significant peroxidatic or hydrolytic activity. However, recombinant PAF-AH readily hydrolyzed PCOOH, releasing stoichiometric amounts of oleic acid hydroperoxide (OAOOH) in the process. These and related other findings suggest that the PLOOH hydrolyzing/detoxifying role often attributed to PON1 on the basis of in vitro studies with enzyme isolated from HDL (6) actually reflects a new activity of PAF-AH, which co-purifies with PON1 (5).

EXPERIMENTAL PROCEDURES

Materials—DFO, EDTA, GSH, Igepal CA-630, pepstatin A, phenyl acetate, Tergitol NP-10, TPD, oleic acid, liver phosphatidylethanolamine, brain phosphatidylserine, and brain sphingomyelin were obtained from Sigma. Biomol (Plymouth Meeting, PA) supplied the MAAP. Non-radioactive 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (PA) were obtained from Avanti Polar Lipids (Birmingham, AL) and used without further purification. HPLC-grade solvents were from Mallinkrodt (Paris, KY). Amersham Biosciences supplied the 1-palmitoyl-2-[1-14C]oleoyl-sn-glycero-3-phosphocholine ([14C]PC, ~55 mCi/ml), which was HPLC-purified immediately before use (17). Human recombinant PAF-AH was obtained from ICOS Corp. (Bothell, WA). Low density lipoprotein (LDL) was prepared from freshly isolated human plasma as described previously (18).

Generation and Characterization of Phospholipid and Fatty Acid Hydroperoxides—Unlabeled or radiolabeled PCOOH was generated by photodynamic action, singlet oxygen-generating AlPcS2 serving as the sensitizer (19). PC or [14C]PC (2 mg/ml in water, 39% hexane, and 10% water (19)). Monitoring of effluents of irradiation (4 °C, using a 90-watt quartz-halogen lamp (19, 20). After 30 min of irradiation (~40 J/cm² fluence), samples were dried under N₂, dissolved in isopropyl alcohol, and subjected to normal-phase HPLC, using a Supelcosil column (Supelco, Bellefonte, PA) and a mobile phase consisting of water (A) and acetonitrile (B), each with the following gradient profile: (0-35 min) 0% B to 90% B; (35-45 min) 90% B to 0% B. The iodometrically determined content of a bacterial lysate with 55% (w/v) ammonium sulfate was 101 nmol/mg protein. Details of the procedure, including the use of DEAE-Sephrose 6B chromatography with gradient elution for final purification, were as described previously (5). Stock preparations of PON1 in 50 mM Tris-Cl, 1 mM CaCl₂, 0.1% Igepal CA-630 (pH 8.0) were analyzed for protein content by Bradford assay (24). Preparations were stored at 4 °C and used for experiments within 2 weeks.

Expression and Purification of Recombinant PON1—A His-tagged rabbit recombinant PON1 (rPON1-G3C9), which is highly similar to the human counterpart (25), was expressed in Escherichia coli, as described (26). Protein precipitated by treatment of a bacterial lysate with 55% (w/v) ammonium sulfate was recovered by centrifugation, dissolved in 50 mM Tris-Cl, 1 mM CaCl₂, 0.1 mM dithiothreitol, 1 mM NADPH, 1 mM pepstatin A, 0.1% Tergitol (pH 8), dialyzed overnight at 4 °C against buffer-2, and then against 50 mM Tris-Cl, 50 mM NaCl, 1 mM CaCl₂, 0.1% Tergitol (pH 8) (buffer-2) for 4 h. The dialysate was passed over a nickel-nitrilotriacetic acid column, and bound protein was recovered by washing with buffer-2 supplemented with 50 mM imidazole. Fractons containing PON1 activity were assessed for purity by SDS-PAGE, then pooled, dialyzed extensively against buffer-2, and stored at 4 °C.

PON1 and PAF-AH Activity Assays with Standard Substrates—Ca²⁺-dependent aryl esterase activity of PON1 was determined by spectrophotometric assay (27). Reaction mixtures contained 1 mM phenyl acetate and 1 mM CaCl₂ in 50
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mm Tris-Cl (pH 8.0) at 30 °C. The rate of increase of $A_{270}$ was measured after the addition of PON1 in negligible volume. Background-corrected activity was determined using an extinction coefficient of 1.31 (mM)$^{-1}$ cm$^{-1}$, 1 unit corresponding to 1 micromole of phenyl acetate hydrolyzed per min.

PAF hydrolyzing activity of PAF-AH was determined as described (28), using $^3$Hacetyl-PAF and C18 cartridges for substrate-product separation.

Enzyme Incubations with PCOOH and with Peroxidized LDL—A typical reaction mixture for testing lipolytic activity of nPON1 or rPON1 contained 1 mM CaCl$_2$, 0.1 mM DFO, 0.1 mM EDTA, 0.05% (v/v) Igepal CA-630, enzyme sample, and PCOOH (75 μM, added last) in 50 mM Tris-Cl (pH 8.0). GSH (5 mM) or cysteine (10 mM) was included when peroxidatic activity was checked for. Reaction mixtures for assessing lipolytic activity of rPAF-AH contained 0.1 mM DFO, 0.1 mM EDTA, 0.05% (v/v) Igepal CA-630, enzyme sample (~0.05 mg of protein/ml), and PCOOH (typically 75 μM) in Chelex-treated PBS (pH 7.4). For $K_m$ and $V_{max}$ determinations on PON1 or PAF-AH, [PCOOH] was varied over the 5–500 μM range and initial rates were calculated from PCOOH decay rate constants. Where indicated, $[^{14}C]$PC or $[^{14}C]$PCOOH was used instead of unlabeled PCOOH. Photoperoxidized LDL (~1 mg of protein/ml) was used as a test substrate in other experiments with rPAF-AH; Igepal CA-630 was omitted in this case. Starting volume for all reaction mixtures was typically 0.5 ml. At various time points during incubation at 37 °C, 50-μl aliquots were removed, diluted with 0.2 ml of PBS, and extracted with 0.4 ml of ice-cold chloroform/methanol (2:1, v/v). A 0.2-ml portion of the organic phase was recovered, dried under N$_2$, and analyzed by normal-phase high performance thin layer chromatography, using chloroform/methanol/water/ammonium hydroxide (60:38:3:1 by volume) as the mobile phase. After chromatography, peroxide analytes were visualized by spraying the plate with TPD as described (19), the overall procedure being referred to as HPTLC-TPD. When $[^{14}C]$PC or $[^{14}C]$PCOOH was used as a substrate, extracted lipids were chromatographed similarly and detected by means of phosphorimaging; this procedure is referred to as HPTLC-PI (29).

Statistics—The two-tailed Student's $t$ test was used for determining the significance of perceived differences between experimental values, $p \geq 0.05$ being considered statistically insignificant.

RESULTS

Reactivity of Natural PON1 Preparations with PCOOH—The specific hydrolytic activity of freshly prepared nPON1 was found to be $\sim$600 units/mg of protein using phenyl acetate as substrate and $\sim$0.2 unit/mg of protein using PAF (Table 1), in good agreement with previous determinations (5, 30). These assays were carried out in the absence of metal ion chelators, PON1 requiring Ca$^{2+}$ for both structural stability and catalysis (1, 5), whereas PAF-AH does not (28). However, when PCOOH was tested as a substrate it was necessary to prevent, or at least minimize, any non-enzymatic peroxide turnover catalyzed by redox metal ions such as iron and copper. This was especially important when a reductant like GSH was used in reaction mixtures because these metals can catalyze the one-electron reductive degradation of LOOHs to free radical species (31). The chelators DFO and EDTA were included to block any redox cycling of iron and copper, respectively (32, 33). In the case of nPON1, the chelator concentrations used (10% of [CaCl$_2$]) were sufficient to inactivate trace metal ion contaminants while not interfering with Ca$^{2+}$-dependent esterase activity (results not shown).

In initial experiments testing nPON1 reactivity with PCOOH, we used HPTLC with TPD spraying for substrate/product analysis. Hydroperoxides oxidize TPD to its purple radical cation (Wurster’s blue), which can be quantitated by densitometric scanning with appropriate measures to maximize analyte signals relative to background (19). As shown by the HPTLC-TPD profiles in Fig. 1A, a control without nPON1 showed no change in PCOOH band intensity over a 4 h incubation period, whereas the nPON1-containing system showed a progressive loss of PCOOH with reciprocal accumulation of a more mobile peroxide species. The latter co-migrated with authentic OAOOH. Thus, phospholipase A$_2$-like action on the peroxidized sn-2 oleoyl moiety of PCOOH was apparent. Lyso-PC, which migrated below PCOOH and was visualized by exposing the plate to I$_2$ vapors (not shown), accumulated concurrently with OAOOH in the PON1 system. A plot of the HPTLC-TPD data revealed that nPON1-stimulated PCOOH decay occurred with apparent first-order rate kinetics (Fig. 1B), the rate constant for the system described being $\sim$4.2 $\times$ 10$^{-3}$ min$^{-1}$. From this, we calculated a specific activity of 0.11 unit/mg of protein (Table 1). When 5 mM GSH (Fig. 1B) or 10 mM cysteine (not shown) was included in the reaction mixture, there was no significant change in the rate of PCOOH disappearance, suggesting that peroxidatic action, at least that stimulated by thiol reductants, played no significant role. Boiling a sample of nPON1 for 5 min resulted in complete loss of apparent lipolytic activity (not shown), which argues that this activity was enzymatic rather than non-enzymatic. As shown in Fig. 2A, the initial rate of PCOOH loss with increasing substrate concentration at a given nPON1 level exhibited saturation kinetics, the $K_m$ value obtained from a Lineweaver-Burk plot of the data (Fig. 2A, inset) being $\sim$95 μM.

Increasing the EDTA concentration of a nPON1 reaction mixture to 5 mM, i.e. five times the added CaCl$_2$ concentration, abolished the aryl esterase activity of nPON1 (Fig. 3A) but had no significant effect on PCOOH loss (Fig. 3B) or OAOOH buildup, implying that some enzyme(s) other than PON1 was responsible for the latter. The phospholipase PAF-AH was con-

| Enzyme | Phenyl acetate$^a$ | PAF$^b$ | PCOOH$^c$ |
|--------|-------------------|--------|----------|
| nPON1  | 579 ± 41          | 0.2 ± 0.1 | ND$^d$ |
| rPON1  | 5.0 ± 0.1 × 10$^5$ | ND$^d$ | ND$^d$ |
| nPAF-AH| ND$^d$           | 156 ± 10 | 83 ± 12 |

$^a$ One unit of activity represents 1 μmol of phenyl acetate hydrolyzed per min at 37 °C.
$^b$ One unit of activity represents 1 μmol of PAF hydrolyzed per min at 37 °C.
$^c$ One unit of activity represents 1 nmol of PCOOH hydrolyzed per min at 37 °C.
$^d$ ND, not detectable.
sidered to be a good candidate because earlier studies (5) showed that it co-purifies with conventionally prepared nPON1 and is responsible for the hydrolytic inactivation of PAF and oxidatively fragmented PAF-like phospholipids, effects that previously been attributed to PON1 (6, 9, 34). To begin testing for this, we treated nPON1 with the serine esterase inhibitor MAFP, which irreversibly inactivates A2-type phospholipases, including PAF-AH (35). MAFP was found to have no significant effect on aryl esterase activity (Fig. 3A) but strongly inhibited PCOOH loss (Fig. 3B). Taken together, the results are consistent with the idea that the lipolytic activity described was not due to PON1 itself but rather some other enzyme(s) isolated with it, PAF-AH being a logical possibility.

Recombinant PON1 and PAF-AH: Comparative Reactivity with PCOOH—To obtain more direct evidence for PAF-AH rather than PON1 involvement, we tested preparations of pure recombinant enzymes. As shown in Fig. 4A, PCOOH lipolysis by rPON1 was negligible compared with that observed for nPON1 (Fig. 1), even though the former was used at >800 times the level of aryl esterase activity. Including 5 mM GSH in the rPON1 reaction mixture had no significant effect (not shown), indicating that a reductant was not the limiting factor. Several other PLOOH families, including PEOOH, PSOOH, SMOOH, and PAOOH, were also found to be essentially unreactive with rPON1, either in the absence or presence of GSH (not shown). In contrast to rPON1, rPAF-AH readily catalyzed the hydrolysis of PCOOH, with corresponding release of OAOOH (Fig. 4B). The specific activity for this reaction was 83 units/mg of protein or 750 times greater than that for nPON1 (Table 1). Thus, rPAF-AH, but not rPON1, mimicked the lipolytic activity observed with nPON1. As in the case of nPON1, we observed saturation kinetics with respect to increasing [PCOOH] for rPAF-AH (Fig. 2B). The $K_m$ value was found to be ~86 μM (Fig. 2B, inset), which is similar to that determined for nPON1 (~95 μM). This supports our premise that the active PCOOH phospholipase in nPON1 is PAF-AH.
Lipolytic Activity of Recombinant PAF-AH on PCOOH Versus PC—It was of interest to compare PCOOH with parental PC in terms of susceptibility to PAF-AH-catalyzed hydrolysis. To study this, we used PC and PCOOH radiolabeled in the carbonyl carbon of the sn-2 oleoyl moiety. HPTLC-PI analysis allowed us to simultaneously track and compare substrate loss and fatty acid appearance for both substrates. As shown by the chromatogram in Fig. 5A, and in agreement with the Fig. 4B results, [14C]PCOOH was hydrolyzed relatively rapidly by rPAF-AH with corresponding accumulation of [14C]OAOOH. Importantly, the rate constant for substrate disappearance was essentially the same as that for OAOOH buildup, indicating that no other products were formed. Consistent results were obtained when peroxide-detecting HPTLC-TPD was used for comparative rate analysis (not shown), confirming that peroxide groups were conserved during the reaction. In striking contrast to PCOOH, non-oxidized PC was almost completely resistant to rPAF-AH-catalyzed hydrolysis (Fig. 4, A and B). This agrees with earlier evidence showing that plasma-isolated PAF-AH, unlike most phospholipases A₂, is essentially unreactive with natural phospholipid sn-2 groups exceeding 6 carbons in length (15).

Lipolytic Action of Recombinant PAF-AH on LDL PLOOH—rPAF-AH not only catalyzed the sn-2 lipolysis of Igepal-solubilized PCOOH but also that of PLOOHs in photoperoxidized LDL. As shown by the HPTLC-TPD profiles in Fig. 6A, the LDL displayed two major zones of TPD-reactive material, the lower one representing PCOOHs and the upper cholesteryl ester hydroperoxides (CEOOHs) (36). Lesser amounts of the partially resolved hydroperoxides of free cholesterol (ChOOHs) and triacylglycerol were also observed. Control incubation under redox-inhibited conditions resulted in a slow decrease in PCOOH band intensity (~10% drop after 1 h), with no detectable FAOOH buildup (Fig. 6B). In the presence of rPAF-AH, PCOOH loss was greatly accelerated, reaching ~95% after 1 h (Fig. 6B), with parallel accumulation of FAOOH. Most of the latter was probably linoleate-derived, since this is the most abundant fatty acid in LDL (37). There was no obvious loss of CEOOH during incubation with PAF-AH, suggesting that the lipolysis reaction was specific for oxidized LDL PLOOHs and emphasizing the lack of peroxidatic activity.

DISCUSSION

Phospholipid and cholesteryl ester hydroperoxides generated in or acquired by LDL particles are known to be nascent promoters of atherosclerosis (38). A PLOOH catabolizing activity in the circulation, variously reported as either reductive (2, 4, 39, 40) or hydrolytic (2, 6, 7), has been attributed to PON1. However, some of the evidence presented is questionable, based on uncertainties about purity of the PON1
preparations used and adequacy of the methods employed to track PON1 reactions with PLOOHs and other LOOHs. In this study, we examined the effects of natural and recombinant PON1 and PAF-AH on a typical peroxidized phospholipid, PCOOH, using a recently developed assay method (HPTLC-TPD (19)) that allowed us to monitor substrate consumption and product accumulation simultaneously. With this approach, we could track PCOOH loss due to either peroxidase or phospholipase activity, the latter producing well separated OAOOH. Redox-active metal ions such as iron and copper can catalyze LOOH degradation, and this could be mistaken for enzymatic activity. We found that PCOOH and OAOOH were completely stable in control incubations without enzymes, indicating that the measures employed to chelate/inactivate redox metal ions (inclusion of EDTA and DFO) were effective. Thus, the reactions observed were strictly enzymatic, with no significant background interference. We showed that nPON1 could degrade PCOOH, whereas rPON1 had no effect on it, even in the presence of GSH. Peroxidatic activity of PON1 was ruled out on this basis. Other PLOOHs, including PEOOH, PSOOH, SMOOH, and PAOOH, were also found to be non-substrates for rPON1, indicating that the lack of reactivity was not limited to PCOOH. OAOOH buildup during incubation of nPON1 with PCOOH demonstrated that a phospholipase A₂-like activity was being observed. Since rPON1 exhibited no such activity, we reasoned that it must have been due to some phospholipase(s) that contaminated the nPON1 preparations used. By employing a specific serine esterase inhibitor and comparing $K_m$ values for nPON1 and rPAF-AH, we established that nPON1 itself was not responsible for PCOOH hydrolysis but rather PAF-AH that contaminated the nPON1. Previous work (5) showed that PAF-AH typically co-purifies with PON1 and is difficult to separate from the latter by conventional chromatographic means. It appears likely, therefore, that PLOOH hydrolysis ascribed to nPON1 in earlier studies (3, 4) was actually catalyzed by accompanying PAF-AH. One might ask whether PON1 and PAF-AH co-purify simply because of limitations of existing separation methods or whether they pre-exist as an avid complex in vivo for some functional purpose. The latter possibility can be ruled out on the following grounds: (a) either enzyme in recombinant form is fully active in the absence of

**FIGURE 5.** Comparison of PCOOH and PC as substrates for rPAF-AH-catalyzed lipolysis. [14C]PC or [14C]PCOOH in PBS containing 0.05% (v/v) Igepal CA-630 and 0.1 mM each of DFO and EDTA was incubated at 37°C in the presence of rPAF-AH (50 μg protein/ml), and timed samples were extracted for lipid analysis. A, HPTLC-PI patterns of radiolabeled analytes. B, first-order plots for [14C]PCOOH disappearance (○) and [14C]OAOOH accumulation (●) or for [14C]PC disappearance (□) and [14C]OA accumulation (▲). Mean values from duplicate experiments are shown.

**FIGURE 6.** rPAF-AH-catalyzed lipolysis of PLOOH in oxidized LDL. Photoperoxidized LDL (1.0 mg of protein/ml) in PBS containing 0.1 mM each of DFO and EDTA was treated with rPAF-AH (50 μg/ml). A control without rPAF-AH was prepared alongside. At various time points during incubation at 37°C, samples were extracted and recovered lipid fractions analyzed by HPTLC-TPD. A, HPTLC-TPD profiles. Std denotes PCOOH and OAOOH standards; CEOOH denotes cholesteryl ester hydroperoxides. B, first-order plots showing PCOOH decay (○) and FAOOH accumulation (●) in the presence of rPAF-AH. Also shown is PCOOH decay in the absence of enzyme (▲). Mean values from duplicate experiments are shown.
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the other (40, 41), thus complex formation is not critical for functionality; (b) PON1-knock-out animals have undiminished PAF-AH activity (42), and PAF-AH-inactivating mutations have no effect on PON1 activity (5); (c) active PAF-AH is present in LDL as well as HDL (5, 28), whereas active PON1 is found exclusively in HDL (1, 2).

Plasma PAF-AH, a member of the group VII family of phospholipases A2, circulates in plasma as an integral component of LDL and HDL (43) and acts on substrates presented in the context of a lipid-water interface (28). Phospholipases A2 are grouped by sequence homologies into eleven families (44). Only the family IV isozyme cPLA2, along with family VII and VIII members, exhibit selectivity for properties of the sn-2 group being hydrolyzed. For cPLA2, the preferred group is an arachidonoyl moiety; whereas for family VII short or modified sn-2 residues are required. Family VII member PLA2g7 was originally purified (28) and cloned (45) on the basis of its selective hydrolysis of PAF, a phospholipid with a distinctively short (acetyl) sn-2 group. The PLA2g7 gene product, plasma PAF-AH does not specifically recognize the sn-2 acetyl group. Rather, it fails to recognize long chain fatty acyl moieties and so does not attack normal membrane or lipoprotein phospholipids. PAF-AH will, however, readily hydrolyze a variety of oxidatively fragmented phospholipids with shortened and/or derivitized sn-2 groups (15) and thereby functions as a highly selective oxidized phospholipid phospholipase. Plasma PAF-AH has additionally been found (16) to hydrolyze phospholipids containing sn-2 F2-isoprostane groups, which are free radical-generated prostaglandin-like arachidonoyl derivatives (46) whose effective length is decreased by a cyclopentene ring. We showed for the first time in this work that PAF-AH also recognizes a typical PLOOH in which the only modification is addition of a hydroperoxide group to the long chain sn-2 fatty acyl moiety. Preliminary data (not shown) have revealed that PAF-AH-catalyzed lipolysis is not limited to PCOOH but that other PLOOHs with different head groups, including PEOOH, PSOOH and PAOOH, are also substrates for the enzyme. However, in-depth studies on the comparative kinetics have not yet been carried out. Not surprisingly, SMOOH, with an amide-rather than ester-linked sn-2 group, was found to be unreactive with PAF-AH (not shown).

Numerous studies have suggested that PON1 plays an inhibitory role in the pathogenesis of atherosclerosis and coronary heart disease. Key evidence has indicated that depletion of PON1 by genetic ablation increased atherogenesis in a murine model (42, 47), whereas global (48) or targeted (49) transgenic overexpression suppressed disease progression. In addition, PON1 polymorphisms have been shown to be associated with an increased risk of coronary vascular disease (50, 51). However, this link is tenous because of the complicated relationship between genetic polymorphisms and enzyme activity levels (52), PON1 activity correlating better with disease status than with genotype (53). Accounting for the anti-atherogenic role of PON1 is further complicated by the fact that the enzyme has several different catalytic activities involving different residues (54, 55) and that its physiologic substrate(s) have not yet been defined. Thus, which of these activities underlie the salutary effects of PON1 in coronary artery disease is still not clear. The enzyme originally was purified and cloned as an esterase that detoxifies organophosphate insecticides (56), substrates clearly not related to evolutionary pressure. PON1 is known to function as an effective lactonase (57–59), and the catalytically essential residues for this reaction are distinct from those involved in ester hydrolysis (54). A recent study (55) indicated that the two histidine residues required for the lactonase activity of PON1 are also essential for protecting LDL against oxidative modification. These findings argue against any possible non-catalytic mechanism (60) and also paraoxonase action per se (55) as being relevant to anti-atherogenic role of PON. However, they do not settle the question of which substrates are physiologically relevant vis-à-vis atherogenesis or how PON1 acts on them. Possible candidates may be phospholipids with sn-2 fatty acyl residues bearing 5-hydroxy functions, which could be hydrolyzed by PON1 acting as a lactonase (55). Our results indicate that a previously proposed mechanism of LDL protection by PON1, viz. hydrolysis of free radical-generating PLOOHs, is neither tenable with regard to how PON1 acts in vivo to suppress disease progression nor relevant to the question of how PON1 polymorphisms permit atherogenesis.

The kinetics of nPON1 and PAF-AH action on isolated PLOOHs have not been studied previously. We used the similar $K_m$ values of nPON1 and rPAF-AH for PCOOH (Fig. 2) as supporting evidence that the phospholipase A2-like activity observed in nPON1 was attributable to co-purified PAF-AH. The average $K_m$ determined (~90 μM) is significantly greater than that recently reported for PAF-AH hydrolysis of PAF itself (~14 μM) or F2-isoprostane-esterified PC (~60 nM) but only twice as high as that for 5-oxovaleroyl-esterified PC (43 μM) (16). Thus, the affinity of the enzyme for PCOOH, which still bore a long chain sn-2 acyl group (but now with one -OOH substituent), was only somewhat weaker than that for the 5-carbon oxovaleryl counterpart. By striking contrast with PCOOH, PC was found to be essentially unreactive with PAF-AH (Fig. 5), as had been observed previously for the unoxidized phospholipid (15). The molecular basis for this remarkable discrimination between PCOOH and PC as a substrate is not yet clear but may relate to the greater accessibility of the sn-2 chain in its peroxidized state.

Could PAF-AH-catalyzed PLOOH hydrolysis in HDL or LDL in vivo play an antioxidant and anti-atherogenic role, as had been previously proposed for PON1 (2, 3), and if so, how might this be explained? Phospholipase A2-like action of PAF-AH on resident PLOOHs would generate lysophospholipids and FAOOHs. Being more hydrophilic than their precursors, FAOOHs would be more prone to diffuse away from the lipoprotein, thus reducing the oxidative pressure on the latter, i.e. potential for peroxide-induced free radical oxidation (29). FAOOHs are excellent substrates for the type-3 glutathione peroxidase found in plasma (61) and, thus, could be reductively inactivated by this enzyme if the GSH levels in bulk plasma were sufficient (61). Apolipoprotein AI in HDL has been reported to reduce and detoxify PLOOHs and CEOOHs at the expense of endogenous methionine residues (62), and this was implicated in the anti-atherogenic effects
of HDL. In addition to directly reducing PLOOHs, apoAI might act on PAF-AH-liberated FAOOHs. In these ways, PAF-AH could play a role in overall disposal of HDL PLOOHs, although the specifics of this role and its importance relative to other detoxification mechanisms remains to be established.

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