Platelet-activating Factor Acetylhydrolase, and Not Paraoxonase-1, Is the Oxidized Phospholipid Hydrolase of High Density Lipoprotein Particles*

Paraoxonase-1 (PON1), an high density lipoprotein (HDL)-associated organophosphate triesterase, suppresses atherosclerosis in an unknown way. Purified PON1 protects lipoprotein particles from oxidative modification and hydrolyzes pro-atherogenic oxidized phospholipids and the inflammatory mediator platelet-activating factor (PAF). We find human PON1 acted as a phospholipase A₂ but not as a phospholipase C or D through cleavage of phosphodiester bonds as expected. PON1 requires divalent cations, but EDTA did not block the phospholipase A₂ activity of PON1. In contrast, a serine esterase inhibitor abolished phospholipase activity even though PON1 has no active-site serine residues. PAF acetylhydrolase, an oxidized phospholipid phospholipase A₂, is a serine esterase associated with specific HDL particles. Western blotting did not reveal detectable amounts of PAF acetylhydrolase in PON1 preparations, although very low amounts of PAF acetylhydrolase might still account for PON1 phospholipase A₂ activity. We revised the standard PON1 purification by first depleting HDL of PAF acetylhydrolase to find PON1 purified in this way no longer hydrolyzed oxidized phospholipids or PAF. Serum from a donor with a propensity toward PAF or pro-atherogenic oxidized phospholipids. Oxidatively fragmented phospholipids, like PAF, are substrates for PAF acetylhydrolase (28, 29), a phospholipase A₂.
that only hydrolyzes phospholipids with sn-2 residues that are short or contain an oxy function introduced during oxidative fragmentation. This enzyme is a serine esterase with the signature catalytic triad (30), and so is sensitive to serine esterase inhibitors but not divalent ion chelators. PAF acetylhydrolase circulates in association with LDL and HDL particles and can migrate between them in a pH-dependent fashion (31). PON1 is a phosphotriesterase (37) and might be expected to inactivate phospholipid mediators by hydrolysis of the head group. That is, its enzymatic mechanism would suggest a potential mechanism that could account for the vascular protective effect of PON1. The existence of two distinct enzymes in the same lipoprotein particle apparently catalyzing the same reaction has yet to be tested directly. Here, we show that trace amounts of PAF acetylhydrolase contaminate PON1 preparations and that PON1 lacking PAF acetylhydrolase displays no phospholipase activity.

MATERIALS AND METHODS

Cibacron blue 3GAG-agarose (type 3000-CL), DEAE-Sepharose 6B, concanavalin A-Sepharose 4B, deoxycholate, methyl-α-D-mannopyranoside, IGEPAL CA-630, potassium bromide, phenyl acetate, phospholipase A2 (bee venom), phospholipase C (Bacillus cereus), and phospholipase D (cabbage) were from Sigma, and Extracti-gel was from Pierce. Lipase D (cabbage) was from Sigma, and Cibacron blue 3GA-agarose (type 3000-CL), DEAE-Sepharose 6B, phenyl acetate (2), 20 mM Tris-Cl (pH 8.0), 1 mM CaCl2, and 1 mM MgCl2. Blanks without enzyme were used to correct for spontaneous hydrolysis. Activity clot was passed from the molar extinction coefficient at 270 nm (using differences in the absorbance of phenol versus phenyl acetate) of 1310 M⁻¹ cm⁻¹. One unit of arylesterase activity was defined as a micromole of phenyl acetate hydrolyzed per min.

We measured PAF acetylhydrolase activity using [3H]acetate-PAF as described by Stafforini et al. (31, 34) and also by bioassay. For this, human neutrophils were isolated by dextran sedimentation and centrifugation over Ficoll as before (35). Neutrophils (2.25 × 10⁶/ml) were labeled with Fura-2 AM as before (23), and changes in intracellular calcium concentration were measured by dual excitation at 340 and 380 nm with emission collected at 510 nm. The amount of PAF stated in the figures (typically at 10⁻¹⁰ M) was pretreated with detergent-free PON1 or recombinant PAF acetylhydrolase for the specified time at 37 °C before the entire reaction was tested in the Ca⁺⁺ mobilization assay. We find as little as 5 to 10 ng of PAF acetylhydrolase in 15 min abolishes the calcium signal generated by 0.1 nM PAF. In some experiments, purified PON1 was pretreated for 1 h at 37 °C with either EDTA (100 μM) or the serine esterase inhibitor Pefabloc (100 μM) prior to assay. In some experiments PAF or 3-O-methyl PAF were treated with 10 μg of bee venom phospholipase A2 (in 0.5% human serum albumin in Hank's balanced salt solution with 10 mM Ca⁺⁺), 10 units of phospholipase C (B. cereus), or 10 units of phospholipase D (cabbage) for 2 h at 37 °C and then tested in the Ca⁺⁺–fluor bioassay. The concentration of PAF was as described (23, 36).

RESULTS

Standard Preparations of Human PON1 Hydrolyze PAF and the Oxidized Phospholipids of Oxidized LDL—We purified PON1 as described (2, 33) and assayed its hydrolysis of PAF or the PAF-like phospholipids produced during LDL oxidation (19, 22, 23). We chose a bioassay to examine hydrolysis because inactivation of these lipid mediators is the important outcome, and because the extreme potency of these mediators makes assays other than biologic ones impractical. Here we loaded freshly isolated human PMN with the Ca⁺⁺–sensitive dye Fura-2 and followed increases in intracellular Ca⁺⁺ by increases in the fluorescent ratio of the dye. We found that PMN were maximally stimulated by 10⁻¹⁰ M synthetic PAF (Fig. 1A) and by the PAF-like phospholipids created during oxidized LDL oxidation (Fig. 1B). PAF receptor antagonists abolished both responses (not shown) (23). Pre-incubation of either PAF (Fig. 1C) or the oxidized phospholipids isolated from oxidized LDL (Fig. 1D) with purified PON1 abolished the ability of these lipids to stimulate PMN. Heat inactivation of PON-1 (Fig. 1E) destroyed the effectiveness of the preparation in degrading PAF. The positive control, recombinant PAF acetylhydrolase destroyed the biologic activity of PAF as expected (Fig. 1F). PON1 therefore displays the reported (5, 32) anti-inflammatory activity and inactivates PAF and PAF-like phospholipids generated during LDL oxidation.

Phospholipid Hydrolysis by PON1 Preparations Occurs at the sn-2 Position—PON1 is a phosphotriesterase (37) and might be expected to inactive phospholipid mediators by hydrolysis of the phosphodiester bonds of the phosphocholine head group. That is, its enzymatic mechanism would suggest that it functions as either a phospholipase C or phospholipase D. We tested this postulate in the following way. The PAF receptor is stimulated by PAF (Fig. 2A) but also by an sn-2 ether analog of PAF, 2-O-methyl PAF. This analog cannot be hydrolyzed at the sn-2 position by esterases, yet remains susceptible to the actions of phospholipases C and D. We first showed that phospholipase D (Fig. 2B) and phospholipase C (Fig. 2C) were just as effective as recombinant PAF acetylhydrolase, phospholipase A2, and PON1 (Fig. 2, D–F) in hydrolyzing and inactivating PAF. We performed a parallel experi-
ment with 2-O-methyl PAF to find that it was an active agonist (Fig. 2G) and that phospholipase D (Fig. 2H) and phospholipase C (Fig. 2I) effectively inactivated this PAF analog. We found as expected that neither recombinant PAF acetylhydrolase (Fig. 2J) nor phospholipase A2 (Fig. 2K) hydrolyzed and inactivated this phospholipid. However, we also found that purified PON1 did not inactivate this PAF analog (Fig. 2L). Additionally, we found that a second non-hydrolyzable PAF analog, 2-carbamoyl-PAF, also was impervious to treatment with PON1 (not shown). The inference from this series of experiments is that PON1 functions as an \( sn \)-2-directed esterase and does not attack phosphodiester bonds to inactivate PAF or related phospholipids.

Hydrolysis of PAF by Purified PON1 Requires an Activated Serine Residue—PON1 is an EDTA-sensitive phosphotriesterase, yet the above data showed it to function as an \( sn \)-2-directed esterase. We therefore determined whether the serine esterase inhibitor Pefabloc, which reacts with activated serine residues of enzymes such as phospholipases \( A_2 \) and PAF acetylhydrolase, would suppress PON1 phospholipid hydrolysis. We found that Pefabloc effectively suppressed hydrolysis and inactivation of PAF by PON1 (Fig. 3C). In contrast, EDTA, which completely destabilizes and inactivates PON1 (2, 37), had no effect on PAF hydrolysis by PON1 preparations (Fig. 3D). As a control for the effectiveness of EDTA, we measured the aryl esterase activity of PON1. We found that the pattern of EDTA and Pefabloc effects on this activity of PON1 was the complete reverse of that for PAF hydrolysis; EDTA destroyed PON1 aryl esterase activity, whereas Pefabloc was without effect on this reaction (Fig. 3E).

This outcome was unexpected should the anti-atherogenic effect of PON1 reside in its hydrolysis of PAF and oxidized phospholipids. We considered the possibility that PAF acetylhydrolase, which is also a constituent of certain HDL particles (31), might contaminate PON1 preparations. We tested for the presence of PAF acetylhydrolase in PON1 preparations of increasing purity by Western blotting. We found (Fig. 3F) that PON1 fractions isolated from Cibacron columns (which very effectively bind PAF acetylhydrolase (34, 38)) still contained detectable amounts of immunoreactive PAF acetylhydrolase. Fractions eluting from the DEAE column containing PON1 still showed significant amounts of PAF acetylhydrolase contamination, whereas the final concanavalin A step of the PON1 purification scheme suppressed PAF acetylhydrolase contami-
FIG. 2. Purified human PON1 displays phospholipase A₂, but not phospholipase C or D activity. A. PAF induces a Ca²⁺ flux in PMN that is destroyed by phospholipase D (PLD; B), phospholipase C (PLC; C), recombinant PAF acetylhydrolase (rPAFAH; D), phospholipase A₂ (PLA₂; E), and purified human PON1 (F). In contrast, the Ca²⁺ flux induced by 2-O-methyl-PAF (2-O-Me-PAF; G), a PAF analog with a non-hydrolyzable sn-2 methyl function, is inactivated by phospholipase D (H) and phospholipase C (I), but not by PAF acetylhydrolase (J), phospholipase A₂ (K), or PON1 (L).
What we cannot be sure of, however, is to what extent Western blotting detects PAF acetylhydrolase contamination at levels sufficient to confer phospholipase A2 activity to purified PON1.

**PAF Hydrolytic Activity, but Not PON1, Migrates from HDL to LDL in a pH-sensitive Fashion**—PAF acetylhydrolase has been reported to be difficult to separate from PON1 (2, 6), and both are components of HDL particles. PAF acetylhydrolase is also associated with LDL particles, and its distribution between these lipoprotein species is modulated by pH. We determined whether the distribution of PON1 aryl esterase activity among lipoprotein species is modulated by pH. We determined whether the distribution of PON1 aryl esterase activity among lipoprotein species was affected by pH in parallel with shifts in PAF hydrolytic activity. The data in Fig. 4 show that aryl esterase activity is exclusively found in fractions corresponding to HDL particles at either acidic (Fig. 4A) or basic pH (Fig. 4B). In contrast PAF hydrolysis is largely found at a density corresponding to HDL under acidic conditions, whereas under basic conditions the preponderance of PAF hydrolytic activity is found at the density of LDL. This shift of PAF hydrolytic activity corresponds to the distribution of immuno-reactive PAF acetylhydrolase protein (Fig 4C), as expected (31).

**Detection of PAF Acetylhydrolase by Immunoblotting Is Not Sufficiently Sensitive to Detect PAF Acetylhydrolase Contamination**—We noted from the Western blot in Fig. 4C that we could detect 100 ng of the recombinant PAF acetylhydrolase standard but could not reliably detect 10 ng of the enzyme. PAF acetylhydrolase at levels less than 10 ng would therefore be undetectable by immunoblotting and might be an invisible contaminant of PON1 prepared from HDL, a source of both activities. We determined the minimum amount of PAF acetylhydrolase that would be sufficient to inactivate PAF in the Ca²⁺/H₁₀₀₁₀ flux bioassay by testing a graded amount of recombinant enzyme on the 0.1 nM PAF we use as an agonist in these assays. We found (Fig. 5A) that 800 pg or 1 ng of PAF acetylhydrolase did not diminish the effectiveness of PAF in the bioassay but that 5 ng of the enzyme noticeably reduced the amount of available PAF. We also found that 10 ng completely suppressed the PAF-induced Ca²⁺ response of PMN. We also varied the amount of PAF acetylhydrolase in a radiometric assay of [³H]acetyl-PAF hydrolysis and confirmed that 10 ng of the recombinant enzyme is sufficient to inactivate half of the added substrate (Fig. 5B) in just 15 min. Assays of PON1 inactivation

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**Fig. 3.** The serine esterase inhibitor Pefabloc, but not EDTA, inhibits PAF hydrolysis by PON1. PAF (10⁻¹⁰ M) was treated with purified human PON1 (25–35 units) or PON1 pretreated with 100 µM of the serine esterase inhibitor Pefabloc or 100 µM EDTA for 60 min. The effect of PAF (A) or PAF treated with PON1 (B), PAF treated with Pefabloc-inhibited PON1 (C), or PAF after incubation with EDTA-treated PON1 on FURA2-loaded PMN (D) was determined as in Fig. 1. E, the effect of Pefabloc or EDTA on the aryl esterase activity of PON1 was determined as stated under "Materials and Methods." F, Western blot for PAF acetylhydrolase in PON1-containing fractions eluting from the Cibacron blue-agarose (cibacron), second DEAE-Sepharose (DEAE), or concanavalin A-Sepharose (Con-A) columns. Each lane contained 75–100 µg of protein.
of oxidized phospholipids may extend for many hours (5), so trace amounts of PAF acetylhydrolase may be problematic.

**PON1 Can Be Separated from PAF Acetylhydrolase**—We purified PON1 to apparent homogeneity (Fig. 6A) and a final aryl esterase specific activity of 238–400 units/mg. We noted that different preparations of PON1 expressed a variable ratio of PAF hydrolysis to aryl esterase activity (not shown), an observation consistent with PAF acetylhydrolase co-purifying with PON1 (2, 6). We reasoned that if we started the purification of PON1 from a source that contained less PAF acetylhydrolase, that at a minimum we would garner evidence for PAF acetylhydrolase contamination of purified PON1 or that we may succeed in completely separating PAF acetylhydrolase from PON1. We pre-incubated plasma overnight at an alkaline pH to deplete PAF acetylhydrolase content of HDL, isolated this HDL, and used this as a starting source for PON1 purification. We examined fractions obtained from neutral plasma as the starting source to find that there were detectable amounts of PAF acetylhydrolase in the first two steps of the purification (Fig. 6B), but that even the first step of PON1 purification from PAF acetylhydrolase-depleted HDL was free of this contaminating enzyme by immunoblot analysis.

However the above results show that even when PAF acetylhydrolase cannot be detected by an immunoblot, there still may be enough enzyme to inactivate significant amounts PAF. We then compared PAF hydrolysis by the PON1 preparation apparently free of PAF acetylhydrolase to material purified in the standard way from neutral plasma, which had the same specific final aryl esterase activity. We found that this strategy was effective in separating aryl esterase activity from PAF hydrolytic activity, because PON1 purified from HDL depleted of PAF acetylhydrolase by pre-incubation at an alkaline pH did not hydrolyze PAF (Fig. 6C), whereas PON1 purified using a standard protocol hydrolyzed PAF as before. We tested the PON1 preparation devoid of PAF hydrolytic activity for its ability to inactive the PAF-like lipids found in oxidized LDL. PON1 devoid of PAF acetylhydrolase, in contrast to a standard preparation of PON1, also lacked oxidized phospholipid phospholipidase activity (Fig. 6C). Therefore PON1 does not, by itself, hydrolyze either PAF or oxidized phospholipids possessing PAF-like bioactivity.

**Genetic Ablation of PAF Acetylhydrolase Shows PON1 Has No Role in PAF or Oxidized Phospholipid Metabolism**—There are genetic polymorphisms and mutations in the human PAF acetylhydrolase gene, and the more common of these produces catalytically inactive PAF acetylhydrolase that does not persist in the circulation (39). Plasma from such an individual offers the opportunity to test the contribution of PON1 to PAF catabolism. We found that plasma from an individual with wild-type PAF acetylhydrolase completely inactivated PAF, whereas plasma from an individual with a mutant form of the gene did not (Fig. 7A). This difference in PAF hydrolytic capability did not arise from the presence of an inhibitory agent as a mixing experiment showed that the plasma from the mutant donor did not affect PAF hydrolysis by normal plasma or recombinant PAF acetylhydrolase. We repeated this experiment (Fig. 7B) using oxidized phospholipids as the substrate with similar results; a point mutation in the gene encoding PAF acetylhydrolase abolishes the hydrolysis of oxidized phospholipids with PAF-like bioactivity. We then measured aryl esterase activity in plasma from wild-type and mutant PAF acetylhydrolase to find that there was no diminution in PON1 activity by this mutation (Fig. 7C). The bulk of the plasma aryl esterase activity in both types of plasma was sensitive to EDTA and therefore reflects PON1 activity. Thus even though both PON1 and PAF acetylhydrolase are components of HDL, their expression is independent of one another.

**DISCUSSION**

We discovered that highly purified preparations of human PON1 inactivate PAF and PAF-like oxidatively fragmented phospholipids by hydrolyzing the sn-2 residue to produce two inactive products, lysoPAF and a short chain free fatty acid. This reaction suppresses atherogenesis, and it protects lipoprotein particles from chemically reactive phospholipids. Genetic deletion of PON1 activity in mice creates animals that are susceptible to organophosphate intoxication and, importantly, demonstrate an increased propensity to form atherosclerotic lesions when placed on a high fat diet or an apoE knockout background (7, 40, 41). Conversely, transgenic mice with increased levels of circulating PON1 are less susceptible to developing atherosclerosis (42, 43), as are mice expressing PAF acetylhydrolase as a transgene (44). These observations, coupled with extensive literature (4–13) indicating that paraoxonase is beneficial and hydrolyzes PAF-like phospholipid oxidation products that are formed during LDL oxidation (19, 23) to inactive products, suggest that this is the way PON1 exerts its
salubrious effects. Hydrolysis of oxidatively modified and fragmented phospholipids that are PAF receptor agonists (19, 23, 45), peroxisome proliferator-activated receptor ligands and agonists (46), and structurally inappropriate and disruptive phospholipids (47–50) are of unquestioned value in protecting against atherosclerosis, but this reaction is not accomplished by PON1.

PON1 is an organophosphatase (51) that protects animals that express it against intoxication by organophosphate insecticides (7). If PON1 attacks the phosphodiester of biologically active

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**Fig. 5.** The amount of PAF acetylhydrolase sufficient to rapidly inactivate PAF is less than can be detected by Western blotting. A, PAF (10^{-10} M) was treated with the stated concentration of recombinant PAF acetylhydrolase and then assayed as a Ca²⁺-mobilizing agonist for FURA-2-loaded PMN as in Fig. 1. B, [³H]PAF hydrolysis as a function of PAF acetylhydrolase concentration. Hydrolysis of [³H]PAF in 15 min by the stated amount of PAF acetylhydrolase was determined as stated under “Materials and Methods.”
phosphatidyglycerolines then it would inactivate 1-O-hexadecyl-2-O-methyl-sn-glycerol-3-phosphocholine, a PAF analog that is not a substrate for the esterolytic activity of phospholipases A₁ or A₂. It did not. Instead we find that purified human PON1 inactivated PAF, as reported (32), where the single bond susceptible to esterolytic activity resides at the sn-2 position. This is the same type of activity displayed by the PAF acetylhydrolase that is also a component of certain HDL particles (31).

**Fig. 6.** PON1 purified from HDL depleted of PAF acetylhydrolase does not act as a phospholipase. A, Coomassie Blue-stained SDS-PAGE of fractions obtained during PON1 purification from PAF acetylhydrolase-depleted HDL. Lane 1, molecular mass markers; lane 2, Cibacron fraction; lane 3, DEAE fractions 39–45; lane 4, DEAE fractions 46–52; lane 5, DEAE fractions 28–34; lane 6, second DEAE column fractions 39–45; lane 7, PON1 control; lane 8, Concanavalin A fraction. B, Western blot for PAF acetylhydrolase in PON1 fractions isolated from neutral plasma or from HDL isolated from plasma incubated overnight at an alkaline pH to shift PAF acetylhydrolase out of HDL. C, effect of PON1 purified in two ways on PAF or oxidized phospholipids extracted from oxidized LDL. PON1 was purified from plasma at a neutral pH or from HDL depleted of PAF acetylhydrolase as above and tested for the ability to inactivate PAF as an agonist for Fura-2 loaded PMN as described in Fig. 1.
FIG. 7. Plasma from an individual with a mutation in the PAF acetylhydrolase gene contains paraoxonase activity but does not hydrolyze PAF. A, plasma from an individual with a mutation in the PAF acetylhydrolase gene does not hydrolyze PAF, and mixing experiments with recombinant PAF acetylhydrolase or normal plasma show the lack of activity does not arise from the presence of an inhibitor. B, plasma from an individual with a mutation in the PAF acetylhydrolase gene does not hydrolyze oxidized phospholipids extracted from oxidized LDL. Mixing experiments show the loss of this activity is not because of an inhibitor. C, plasma from an individual lacking PAF acetylhydrolase retains a normal level of PON1 aryl esterase activity. Aryl esterase activity was measured as described under “Materials and Methods” with or without a preincubation with 1 mM EDTA to destabilize and inhibit PON1.
PON-1 Is Not a Phospholipase

Physical resolution of PON1 and PAF acetylhydrolase has been found to be particularly challenging (2, 6, 32). We, like other investigators (32), tested our PON1 preparation for the presence of PAF acetylhydrolase by Western blotting for the enzyme with negative results. Other investigators have searched for PAF acetylhydrolase contamination by mass spectrometry (12, 13) and sequencing (32). But whether any of these methods is sufficient to detect PAF acetylhydrolase contamination at low levels, but levels still sufficient to account for the observed catalytic activity, have not been investigated. We find that we easily detect 100 ng, but not 10 ng, of PAF acetylhydrolase by Western blotting. We also find, however, that as little as 5 to 10 ng of PAF acetylhydrolase is sufficient to account for all of the phospholipase activity in our purified PON1 preparations.

Could low levels of PAF acetylhydrolase confer phospholipase activity to purified PON1? Certainly this is of concern when prolonged incubations are employed (5) when we find a few nanograms of PAF acetylhydrolase degrades PAF and oxidized phospholipids in a little as 15 min. We find that hydrolysis of PAF and PAF-like oxidized phospholipids by purified human PON1 was insensitive to EDTA, yet PON1, and especially human PON1 (2), is not stable in the absence of Ca$_{2+}$ and other divalent cations. Human PON1 is even inactivated by choosing EDTA over heparin during a blood draw (3). We find (34) that PAF acetylhydrolase is a Ca$_{2+}$-dependent lipoprotein phospholipase A$_{2}$ that is insensitive to EDTA.

Conversely, PAF acetylhydrolase with its classic serine esterase GXXG motif is inhibited by compounds like Pefabloc (52) that derivatize activated serines. Pefabloc effectively inhibited PAF and oxidized phospholipid hydrolysis by purified PON1, but PON1 does not contain a signature serine esterase motif (53). Rather, histidine, aspartate, glutamate, and a tryptophan residues are essential for paraoxonase activity (54). Therefore, histidine, aspartate, glutamate, and a tryptophan residues are essential for PAF acetylhydrolase activity to purified PON1? Certainly this is of concern when prolonged incubations are employed (5) when we find a few nanograms of PAF acetylhydrolase degrades PAF and oxidized phospholipids in a little as 15 min. We find that hydrolysis of PAF and PAF-like oxidized phospholipids by purified human PON1 was insensitive to EDTA, yet PON1, and especially human PON1 (2), is not stable in the absence of Ca$_{2+}$ and other divalent cations. Human PON1 is even inactivated by choosing EDTA over heparin during a blood draw (3). We find (34) that PAF acetylhydrolase is a Ca$_{2+}$-dependent lipoprotein phospholipase A$_{2}$ that is insensitive to EDTA.

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