Release of Free F2-isoprostanes from Esterified Phospholipids Is Catalyzed by Intracellular and Plasma Platelet-activating Factor Acetylhydrolases*

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F2-isoprostanes are produced in vivo by nonenzymatic peroxidation of arachidonic acid esterified in phospholipids. Increased urinary and plasma F2-isoprostane levels are associated with a number of human diseases. These metabolites are regarded as excellent markers of oxidant stress in vivo. Isoprostanes are initially generated in situ, i.e. when the arachidonate precursor is esterified in phospholipids, and they are subsequently released in free form. Although the mechanism(s) responsible for the release of free isoprostanes after in situ generation in membrane phospholipids is, for the most part, unknown, this process is likely mediated by phospholipase A2 activity(ies). Here we reported that human plasma contains an enzymatic activity that catalyzes this reaction. The activity associates with high density and low density lipoprotein and comigrates with platelet-activating factor (PAF) acetylhydrolase on KBr density gradients. Plasma samples from subjects deficient in PAF acetylhydrolase do not release F2-isoprostanes from esterified precursors. The intracellular PAF acetylhydrolase II, which shares homology to the plasma enzyme, also catalyzes this reaction. We found that both the intracellular and plasma PAF acetylhydrolases have high affinity for esterified F2-isoprostanes. However, the rate of esterified F2-isoprostane hydrolysis is much slower compared with the rate of hydrolysis of other substrates utilized by these enzymes. Studies using PAF acetylhydrolase transgenic mice indicated that these animals have a higher capacity to release F2-isoprostanes compared with nontransgenic littermates. Our results suggested that PAF acetylhydrolases play key roles in the hydrolysis of F2-isoprostanes esterified on phospholipids in vivo.

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‡1 PO/VPC, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine; PG, prostaglandin; IP, isoprostane; LCAT, lecithin:cholesterol acyltransferase; PON-1, paraoxonase-1; HDL, high density lipoprotein; LDL, low density lipoprotein; BAL, bronchoalveolar lavage; GC-MS, gas chromatography-mass spectrometry; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; tTS, tetracycline-controlled transcriptional silencer; tET, tetracycline.

EXPERIMENTAL PROCEDURES

Plasma PAF Acetylhydrolase—Human recombinant PAF acetylhydrolase (Pafase©) was a kind gift from ICOS Corp., Bothell, WA. The
specific activity of this preparation was 172 μmol/min/mg using PAF as the substrate, a value that compares favorably to those reported by Tew et al. (14) (170 μmol/min/mg) and by Kruse et al. (15) (180 μmol/min/mg). The affinity of the preparation for PAF (K_m = 5.2 μM) was also comparable with previously reported values of 13.7 μM (16), 7 μM (15), and 12 μM (14).

**PAF Acetylhydrolase II Expression and Purification**—The cDNA encoding human PAF acetylhydrolase II was amplified from a commercially available plasmid (American Type Culture Collection) using primers tagged with XbaI and EcoRI sites. The products were digested and then cloned into pcDNA 3.1/Zeo-“ (Invitrogen) for expression in mammalian cells. The sequence of the insert was verified and confirmed to be correct by automated sequence analysis. The cDNA (1 μg) was transfected into individual 35-mm wells coated with COS7 cells, using Lipofectamine, following the instructions provided by the manufacturer (Invitrogen). After 24–48 h, we harvested the cells using lysis buffer (Promega) and employed these extracts in enzymatic analyses that tested the suitability of PAF and esterified F_2-IPs as substrates. The specific activity of these extracts was 46.7 nmol/min/mg using PAF as the substrate, a value that compares favorably to that reported previously by Hattori et al. (17) (59 nmol/min/mg). For purification, a FLAG epitope (MDYKDDDDD) was added to the amino terminus, using PCR and Pfu polymerase (Invitrogen). Extracts from transfected COS7 cells were subjected to purification using FLAG affinity chromatography, following the instructions provided by the manufacturer (Sigma). The purified preparation had a specific activity of 4.2 μmol/min/mg using PAF as the substrate, a value similar to that reported for PAF acetylhydrolase II purified from bovine liver (17) (7.2 μmol/min/mg). The isolated protein was >99% pure as judged by electrophoresis on SDS-PAGE (supplemental Fig. S1).

**Synthesis of POVPc**—This compound was synthesized exactly as described by Ibane et al. (18) using a mixture of 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids) and the 2-14C-labeled compound (PerkinElmer Life Sciences) as the starting materials. We purified [14C]POVPc by thin layer chromatography (18) and then assessed the concentration of the final product by lipid phosphate analysis using the method of Ames and Dubin (19).

**Release of Acetate from PAF and 5-Oxovaleraldehyde from POVPc**—Enzyme activity levels were determined as described previously (20) using either [acetetyl-3H]PAF or [14C]POVPc, with minor modifications. These changes were necessary to standardize assays in which the use of esterified F_2-IP substrates required the presence of detergents for solubilization. We supplemented CHAPS (final concentration 0.8 mM) to assays using the plasma form of PAF acetylhydrolase. For assays of intracellular PAF acetylhydrolase II, we supplemented CHAPS (2 mM) and dithiothreitol (2 mM).

**Isolation of Phospholipid-esterified F_2-IPs and Measurement of Esterified and Free F_2-IPs**—Phospholipid-esterified F_2-IPs were extracted from livers of CCl_4-treated rats and purified by normal phase high pressure liquid chromatography as described previously (9). Free and esterified F_2-IPs were measured by a stable isotope dilution GC-MS assay, as described (21). The percent of F_2-IPs hydrolyzed from the phospholipids by PAF acetylhydrolases was determined by comparing the ratio of the amount of free F_2-IPs to the total amount of free plus esterified F_2-IPs.

**Release of F_2-IPs from Esterified Phospholipids**—The amount of substrate necessary for each set of assays was dried under a stream of nitrogen, resuspended in phosphate-buffered saline containing 0.8–2 mM CHAPS (see below), and then sonicated for 10 min at room temperature. Unless indicated otherwise, the assays contained esterified F_2-IPs (150 nM) for plasma PAF acetylhydrolase and 300 nM for PAF acetylhydrolase II, CHAPS (0.8 mM for plasma PAF acetylhydrolase and 2 mM for PAF acetylhydrolase II), 2 mM dithiothreitol (for PAF acetylhydrolase II only), the enzyme source, and phosphate-buffered saline in a total volume of 50 μL. The addition of CHAPS at the indicated concentrations was necessary for optimal hydrolysis; omission of detergent from the assays resulted in poor rates of F_2-IP quantitation. Initial studies were conducted to optimize the assay conditions so that product release increased as a function of time and enzyme concentration in a linear fashion. Unless otherwise stated, the incubations were conducted for 30 min at 37 °C in a water incubator and then snap-frozen at −80 °C until F_2-IP quantitation. The levels of free and total IPs were assessed in each sample. For K_m determinations, substrate concentrations were calculated from the total amount of F_2-IP in the assays, a value that was experimentally determined. These values were usually within 10–20% of the theoretical concentrations. The results were also corrected for a small amount of free F_2-IP present in the substrate.

**Construction of Inducible Transgenic Mice for Expression of PAF Acetylhydrolase in Airway Epithelium**—The human PAF acetylhydrolase cDNA was a gift of Dr. L. Tjoelker (ICOS Corp., Bothell, WA). A BstXI fragment was digested from a pcDNA-CMV vector. The overhanging ends of the fragment were filled in, and the resulting fragment was ligated into the EcoRV site within the multicloning region of a modified pBluescript II SK expression vector containing the (tet-O)-CMV promoter that consists of seven copies of the tet operator DNA binding sequence linked to a minimal cytomegalovirus promoter (obtained from Dr. J. Whitsett, University of Cincinnati). The expression vector also contained human growth hormone polyadenylation sequences to ensure transcript termination. A 2.3-kb (tet-O)-PAF acetylhydrolase microinjection fragment was released from the vector with an Ascl digestion. To prevent basal leakiness of transgene expression, a construct expressing tetracycline-controlled transcriptional silencer (tTS) under the control of the Clara cell-specific CC10 promoter (CC10-tTS) was obtained from Dr. Jack Elias (Yale University, with permission from Andrew Farner, Clontech). We purified both (tet-O), PAF acetylhydrolase and CC10-tTS constructs using a GELaseTM-agarose gel-digesting preparation kit following the manufacturer’s instructions (Epicenter, Madison, WI). These constructs were co-injected at the Vanderbilt Transgenic/ES Cell Shared Resource to generate transgenic lines of mice that have cointegrated both the (tet-O)-CMV-PAF acetylhydrolase and CC10-tTS transgenes. The mice were generated on an FVB background.

Transgenic mice were genotyped using DNA from tail biopsies. Founder animals were genotyped by Southern blot, and then further generations were genotyped by PCR analysis for increased efficiency. A 1318-bp BamHI/SphI fragment from the PAF acetylhydrolase construct and a 700-bp XbaI/BamHI fragment from the CC10-tTS construct were used as probes for Southern analysis. Primers used for PCR of the (tet-O)-CMV-PAF acetylhydrolase transgene were as follows: 5’-primer, 5’-GGA GCC CTA TAT AAC CAG AGC-3’; 3’-primer, 5’-TCC AAA GGG TGT CAA GGC GAT-3’. The product size was 548 bp. Primers used for identification of the CC10-tTS transgene were as follows: upstream, 5’-GAG TTT GCA GCA GGT TCT CC-3’; downstream, 5’-GAG CAC AGC CAC ATC TTC AA-3’. The product size was 472 bp. PCR protocols for both (tet-O)-CMV-PAF acetylhydrolase and CC10-tTS were as follows: 1 cycle at 94 °C for 2 min; 30 cycles at 94 °C for 1 min, 56 °C for 30 s, and 72 °C for 1 min; and 1 cycle at 72 °C for 10 min.

Mice transgenic for CC10-tTS/(tet-O)-CMV-PAF acetylhydrolase were mated with CC10-rtTA homozygous mice (obtained from Dr. J. Whitsett, University of Cincinnati) to generate triple transgenic mice.
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To induce transgene expression, mice were provided with 1–2 mg/ml doxycycline (Sigma) in 2% sucrose \textit{ad libitum} in drinking water. The drinking bottle was wrapped with foil to prevent light-induced doxycycline degradation. Doxycycline water was replaced twice each week.

\textbf{Ovalbumin Sensitization}—We sensitized mice to ovalbumin by intraperitoneal injection of ovalbumin/alum. Fourteen days later, we began an 8-day series of inhaled 1% ovalbumin challenges. Doxycycline (2 mg/ml) was supplemented to the drinking water for 7 days prior to treatment with aerosolized ovalbumin. A day after the last challenge, mice were anesthetized and underwent bronchoalveolar lavage (BAL) for measurement of PAF-hydrolyzing capacity and \( F_2 \)-IP by GC-MS, as described above. All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee and in accordance with their rules and regulations.

\textbf{Statistics}—The data are reported as the means ± S.D. Unless otherwise indicated, the experiments were conducted twice using similar conditions.

\textbf{RESULTS}

An Enzymatic Activity in Human Plasma Releases Free \( F_2 \)-IPs from Phospholipid-esterified Precursors—We initially explored whether the plasma fraction of blood contains an enzymatic activity that releases free \( F_2 \)-IPs from esterified precursors. We incubated human plasma samples (0.1 \( \mu \)l) or buffer with phospholipid-esterified \( F_2 \)-IPs (2 ng) for 30 min at 37 °C in a total volume of 50 \( \mu \)l. We found that 10.2 ± 3.0% of the substrate was hydrolyzed to free \( F_2 \)-IPs. Under the same assay conditions (30 min at 37 °C), PAF (4 \( \mu \)mol) and the oxidized phospholipid POVPc (0.9 \( \mu \)mol) were hydrolyzed by 9.5 ± 0.1 and 34 ± 1.6%, respectively. Thus, human plasma contains an enzymatic activity that releases \( F_2 \)-IPs from esterified phospholipids.

The Enzymatic Activity That Hydrolyzes Esterified \( F_2 \)-IPs in Plasma Comigrates with PAF Acetylhydrolase on Density Gradients—Human plasma contains a number of esterolytic activities, including lecinthin: cholesterol acyltransferase (LCAT), paraoxonase-1 (PON-1), and PAF acetylhydrolase. The first two enzymes associate with high density lipoprotein (HDL), whereas PAF acetylhydrolase is found in both HDL and low density lipoprotein (LDL) (11, 22). We subjected human plasma to ultracentrifugation on a KBr density gradient and then assayed individually for their ability to hydrolyze PAF, POVPc, and phospholipid-esterified \( F_2 \)-IPs. We found that the activities comigrated on density gradients and that the ratio between the HDL- and LDL-associated activities was the same using the three individual substrates (Fig. 1).

\textbf{Plasma from PAF Acetylhydrolase-deficient Subjects Fails to Release \( F_2 \)-IPs from the Esterified Parent Compounds}—The results from our experiments suggest that PAF acetylhydrolase is the main phospholipase in plasma with the ability to release \( F_2 \)-IPs from esterified precursors. LCAT exhibits ~1% of the hydrolytic activity of PAF acetylhydrolase using PAF as the substrate (23). If this enzymatic activity had significantly contributed to \( F_2 \)-IP release, we would have observed altered ratios between the HDL- and LDL-associated activities using individual substrates. Thus, LCAT either does not contribute to \( F_2 \)-IP release, it does so to a much lower extent than PAF acetylhydrolase, or it requires additional components for optimal activity. PON-1 has been reported previously to utilize PAF (24), oxidized phospholipids (25), and esterified IPs (25) as substrates. However, Maratha et al. (26) demonstrated that minute contamination of PON-1 preparations with PAF acetylhydrolase account for the phospholipase activity previously ascribed to PON-1, a finding later confirmed by Connely et al. (27). Thus, our data are consistent with the notion that PAF acetylhydrolase is the main phospholipase in plasma that catalyzes the release of \( F_2 \)-IPs from esterified sources and that neither LCAT nor PON-1 contribute, or do so to a much lower extent, to the HDL-associated \( F_2 \)-IP-releasing activity.

To directly test the hypothesis that PAF acetylhydrolase is the only phospholipase in plasma with the ability to release \( F_2 \)-IPs from esterified precursors, we obtained plasma from Japanese subjects that were either partially or completely deficient in plasma PAF acetylhydrolase activity because of a point mutation (V279F) that introduces a critical change near the active site of this enzyme (28). We found that plasma from PAF acetylhydrolase-deficient subjects did not hydrolyze PAF or POVPc, as expected (28), and that \( F_2 \)-IPs were not released from esterified phosphatidylcholine (Fig. 2). In addition, plasma from heterozygous subjects had 50% of the hydrolytic capacity displayed by plasma from wild-type controls (Fig. 2). These results complement those reported above and demonstrate that PAF acetylhydrolase is the main plasma activity with the ability to release \( F_2 \)-IPs from esterified precursors.

\textbf{Intracellular PAF Acetylhydrolase Utilizes Esterified \( F_2 \)-IPs as Substrates}—The occurrence of esterified \( F_2 \)-IPs in cellular as well as extracellular tissues prompted us to investigate phospholipase activities that could release \( F_2 \)-IPs from cellular precursors in the intracellular compartment. We reasoned that such activity(ies) are likely to share a number of structural features with the plasma form of PAF acetylhydrolase because they both should recognize similar substrates. The intracellular PAF acetylhydrolase II has similar substrate specificity and is 41% homologous to the plasma isoform (17). We expressed the cDNA encoding FLAG-tagged PAF acetylhydrolase II in COS7 cells, and we then tested the ability of extracts from transfected cells to release acetate
and F₂-IPs. We found that extracts from cells transfected with the PAF acetylhydrolase II cDNA released F₂-IPs from esterified precursors at a faster rate than cells transfected with empty vector (75.1 ± 2.2 and 24.3 ± 0.9 pmol/min/mg protein, respectively). The PAF acetylhydrolase–transfected cells hydrolyzed PAF efficiently (46.7 ± 1.2 nmol/min/mg protein). We detected virtually no enzymatic activity in the vector-transfected cell extracts. PAF acetylhydrolase II is the first reported intracellular phospholipase with the ability to release F₂-IPs from esterified precursors. However, these data do not rule out the possibility that other intracellular phospholipase activities, such as cytosolic phospholipase A₂, can also catalyze this reaction.

F₂-IP Levels in Transgenic Mice That Overexpress the Plasma Form of PAF Acetylhydrolase—Our next goal was to establish if the findings made using purified systems are recapitulated in vivo. We generated transgenic mice that overexpressed the plasma form of PAF acetylhydrolase under the control of the Clara cell–specific CC10 promoter. We sensitized the mice to ovalbumin and then challenged the animals with aerosolized ovalbumin, as described under “Experimental Procedures.” We found that BAL fluid from transgenic animals hydrolyzed PAF to a much larger extent compared with wild-type congenic controls, as expected (Fig. 3A). In addition, the level of F₂-IPs in the nontransgenic BAL fluid was 0.053 ± 0.008 pg/ml (mean ± S.E., n = 8) versus 0.098 ± 0.010 in the transgenic mice (p < 0.003 by unpaired t test with Welch’s correction; Fig. 3B). These results are consistent with our in vitro biochemical data, and they demonstrate that PAF acetylhydrolase increases F₂-IP release from esterified precursors in vivo.

Kinetics of F₂-IP-PC Hydrolysis by Plasma and Intracellular PAF Acetylhydrolases—To rigorously compare the relative efficiencies with which PAF, POVPC, and esterified F₂-IPs are metabolized, we studied the kinetics of hydrolysis of each substrate using purified PAF acetylhydrolases in mixed micellar assays. We investigated the efficiency of hydrolysis of PAF, POVPC, and esterified F₂-IPs at various substrate concentrations (Figs. 4 and 5). We found that the plasma form of PAF acetylhydrolase recognized esterified F₂-IPs with much higher affinity relative to PAF and POVPC (Kᵣ values of 4.5 nM and 5.2 and 19.3 μM for F₂-IP-PC, PAF, and POVPC, respectively; Fig. 4). Conversely, the rate of product release was much slower with F₂-IP-PC versus PAF and POVPC (46.3 nmol/min/mg versus 172.4 and 116.3 μmol/min/mg, respectively). The much higher rate of PAF and POVPC versus F₂-IP-PC hydrolysis was observed using enzyme sources at various stages of purification (not shown). However, the ratio of activities obtained using individual substrates increased after purification. In normal plasma, this ratio was 550 (Fig. 2), but in lipoproteins it increased to ~1,000 (Fig. 1) and to over 2,000 using the purified recombinant protein (Fig. 4). The basis for this phenomenon is unknown. The possibility that purification results in the loss of additional activities that can hydrolyze F₂-IP-PC is unlikely because we observed no F₂-IP-PC hydrolysis in plasma from PAF acetylhydrolase-deficient subjects (Fig. 2). We speculate that lipid or lipoprotein components that are lost during purification favor F₂-IP release, potentially by modifying the detergent micelle environment of the assay, without affecting the ability of the enzyme to hydrolyze PAF. Our data do not rule out the possibility that other phospholipases, such as secreted forms of phospholipase A₂, that exist in several isoforms (29), may be responsible for the release of F₂-IP in biological fluids other than plasma.

The kinetics of PAF and F₂-IP release were also investigated using purified, recombinant PAF acetylhydrolase II. We found that the kinetic parameters using the three substrates had similar relationships compared with those exhibited by the plasma form of PAF acetylhydrolase (Fig. 5). The affinity for esterified F₂-IPs (Kᵣ = 57 nM) was much higher than that observed using PAF and POVPC as substrates (Kᵣ = 13.5 and 43.1 μM, respectively). Furthermore, the rate of F₂-IP release (1.4 nmol/min/mg) was much slower compared with that of acetate (4.2 μmol/min/mg) and oxovaleraldehyde (5.0 μmol/min/mg). In addition, we observed that the ratio of activities obtained using individual substrates increased after purification (not shown). This suggests that a second intracellular activity may also catalyze F₂-IP release from esterified precursors or that a component necessary for optimal release of F₂-IPs was
lost during isolation of the enzyme. This observation is similar to that made with the plasma isoform, and because both enzymes displayed this phenomenon, we speculate that the relatively higher ratio of PAF to F₂-IP-PC hydrolysis observed after purification is related to a stimulatory effect of cellular or lipoprotein lipid components on F₂-IP-PC, but not PAF or POVPc, hydrolysis in micelles.

FIGURE 4. Kinetics of F₂-IP-PC, PAF, and POVPc hydrolysis using purified recombinant plasma PAF acetylhydrolase. Purified recombinant plasma PAF acetylhydrolase (80 pg) was incubated with F₂-IP-PC (0–100 nM; A), with PAF (0–20 μM; B), or with POVPc (0–20 μM; C) for 30 min at 37 °C. Substrate hydrolysis was assessed as described under “Experimental Procedures.” Lineweaver-Burk plots are shown as insets and were generated from the data shown in A–C to generate Kₘ and Vₘₐₓ values for individual substrates. OVA indicates oxovaleraldehyde.
A comparison of the kinetic parameters obtained using intra- and extracellular PAF acetylhydrolases revealed that the intracellular isoform has decreased substrate affinity and rate of hydrolysis using the three substrates compared with the extracellular plasma form. However, the relative efficiencies with which PAF, POVPC, and esterified F₂-IPs are recognized and hydrolyzed by each enzyme are maintained. Thus, the observations made with the secreted form of PAF acetylhydrolase were qualitatively recapitulated by the intracellular PAF acetylhydrolase II; the affinity of each enzyme for F₂-IP-PC substrates was ~200–1000-fold higher than that for PAF and POVPC, but the rates of hydrolysis were several orders of magnitude slower. These observations suggest the existence of shared mechanisms for regulation of extracel-
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lular and intracellular F$_2$-IP release by members of the PAF acetylhydrolase family.

DISCUSSION

These studies have implications from two different perspectives related to the role of F$_2$-IPs in physiology and pathophysiology. First, the physicochemical and functional properties of cell membranes, which are altered by oxidant stress and formation of esterified F$_2$-IPs (9), can potentially be restored after release of the oxidized fatty acids by PAF acetylhydrolase, thus inhibiting cellular death because of disruption of the integrity of the membrane (30). From this analysis it can be speculated that PAF acetylhydrolase participates in cellular homeostasis because of its ability to initiate metabolic steps aimed at repairing damaged cellular membranes. The second issue to consider is related to the biological activities associated with released F$_2$-IPs. In this regard, PAF acetylhydrolases are positioned to regulate the amount of F$_2$-IPs that are hydrolyzed from phospholipids and thus the biological effects that ensue from the release of free F$_2$-IPs.

These observations also have relevance to the role of PAF acetylhydrolase in human syndromes, including cardiovascular disease, for example. Several groups reported that this enzyme has anti-inflammatory and anti-atherogenic properties because of its ability to down-regulate signals mediated by PAF and structurally related phospholipids (reviewed in Ref. 31). Conversely, others propose that this enzyme is pro-atherogenic because it generates oxidized fatty acids and lyso phosphatidylcholines that can mediate cellular activation and proliferation (14, 32–34).

Paradoxically, both deficiency of plasma PAF acetylhydrolase and elevated levels of protein and enzymatic activity are associated with increased risk for vascular diseases and stroke. If PAF acetylhydrolase contributes significantly to F$_2$-IP release from esterified precursors in vivo, this would support the conclusion that this enzyme is pro-atherogenic. F$_2$-IPs are increased in LDL when it is oxidized in vitro (35–38), and elevated F$_2$-IP levels have been detected in the urine of asymptomatic patients with hypercholesterolemia (39), in atherosclerotic plaques (40), and in murine models of atherosclerosis (41). However, the data shown here indicate that individuals who are completely deficient in PAF acetylhydrolase activity have impaired abilities to release F$_2$-IPs from esterified phospholipids and presumably have lower circulating and/or urinary levels of IPs. Yet these subjects are at higher risk of developing atherosclerosis compared with those with normal PAF acetylhydrolase levels (42, 43).

These seemingly contradictory findings can be explained in various ways. First, although PAF acetylhydrolase utilizes substrates generated from increased oxidant stress (oxidized phospholipids and esterified F$_2$-IPs), the enzyme itself is sensitive to oxidative inactivation (44, 45). The fact that very low rates of hydrolysis were observed when phospholipid-esterified F$_2$-IPs were used as substrates for PAF acetylhydrolases suggests that these substrates (or the released F$_2$-IP products) are potent inhibitors of the enzyme. Studies aimed at characterizing the expression levels of PAF acetylhydrolase in human atherosclerotic plaques utilized immunological approaches from which it is not possible to predict to what extent enzymatic activity was preserved in this strongly oxidizing environment (46). Although some studies reported elevated enzymatic activity levels in rabbit atherosclerotic lesions compared with normal aortic tissue (1.5 versus 0.33 nmol/min/mg), these values were extremely low compared with the levels of activity present in mammalian plasma samples, for example. It is quite possible that these relatively low levels of PAF acetylhydrolase activity in atherosclerotic plaques reflect the presence of potent inhibitors of the enzyme, including oxidants and esterified/free F$_2$-IPs. Recent studies in which active PAF acetylhydrolase was overexpressed in balloon-injured carotid arteries had remarkable anti-inflammatory, antithrombotic, and antiproliferative effects (47). Similarly, local adenovirus-mediated transfer of PAF acetylhydrolase resulted in a significant reduction in neointima formation in balloon-denuded rabbit aortas (48). Finally, transfer of PAF acetylhydrolase to atherosclerosis-prone ApoE$^{-/-}$ mice decreased the extent of atherosclerotic lesion formation (49). These studies strongly suggest that the presence of active PAF acetylhydrolase in settings of vascular inflammation is beneficial and that the increased levels of expression observed in human atherosclerotic plaques using immunological approaches reflect, to a large extent, enzymatically inactive protein.

A second possibility is related to the relative biological actions mediated by free versus esterified F$_2$-IPs in vascular events. A number of free F$_2$-IPs have been found to exert a variety of pro-atherogenic actions, particularly in the vasculature (6, 7, 50). However, a recent study reported that cyclopentenone IPs inhibit inflammatory responses in macrophages, and suggested that these molecules may serve as negative feedback regulators of inflammation (51). Moreover, most studies have focused on the biological effects of free IPs, it has been shown recently that epoxy-IP and epoxycyclopentenone-IP esterified in phospholipids exert effects important in the regulation of atherogenesis, such as induction of endothelial synthesis of interleukin-8 and monocyte chemotactic protein-1 (52). The observations that at least one IP esterified in phosphatidylcholine can exert biological effects that would promote atherogenesis and that specific free IPs have anti-inflammatory properties are consistent with the findings in PAF acetylhydrolase-deficient subjects. These individuals presumably have decreased capacities to release free IPs from esterified precursors and have increased risk of developing coronary artery disease.

A consideration that should be addressed in more detail in future studies is related to the mechanism(s) that mediate elevated expression of PAF acetylhydrolase in vascular disorders. Our previous work (53) demonstrated that substrates such as PAF stimulate transcription of PAF acetylhydrolase in what likely reflects a positive feedback mechanism to down-regulate substrate accumulation. We also demonstrated that inflammatory stimuli modulate expression of PAF acetylhydrolase at the transcriptional level (54). It is possible that additional effectors modulate expression of the PAF acetylhydrolase gene, including oxidants and/or derivatives that result from oxidative attack of cellular components. Thus, elevated PAF acetylhydrolase levels may reflect a physiological response to injury whose role is the attenuation of signals elicited by stimuli that include biologically active pro-inflammatory substrates.

In summary, we have investigated the mechanisms that lead to the release of free IPs from esterified precursors, and we found that both plasma and intracellular PAF acetylhydrolase II have the ability to release F$_2$-IPs with high affinity but at a rate much slower compared with other substrates of these enzymes. These findings combined with our in vivo observations using PAF acetylhydrolase transgenic mice suggest that PAF acetylhydrolases play key roles in the hydrolysis of F$_2$-IPs esterified on phospholipids in vivo.

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