

Intracellular PAF catabolism by PAF acetylhydrolase counteracts continual PAF synthesis[§]

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Abstract Stimulated inflammatory cells synthesize platelet-activating factor (PAF), but lysates of these cells show little enhancement in PAF synthase activity. We show that human neutrophils contain intracellular plasma PAF acetylhydrolase (PLA2G7), an enzyme normally secreted by monocytes. The esterase inhibitors methyl arachidonoylfluorophosphonate (MAFP), its linoleoyl homolog, and Pefabloc inhibit plasma PAF acetylhydrolase. All of these inhibitors induced PAF accumulation by quiescent neutrophils and monocytes that was equivalent to agonist stimulation. Agonist stimulation after esterase inhibition did not further increase PAF accumulation. PAF acetylhydrolase activity in intact neutrophils was reduced, but not abolished, by agonist stimulation. Erythrocytes, which do not participate in the acute inflammatory response, inexplicably express the type I PAF acetylhydrolase, whose only known substrate is PAF. Inhibition of this enzyme by MAFP caused PAF accumulation by erythrocytes, which was hemolytic in the absence of PAF acetylhydrolase activity.¶ We propose that PAF is continuously synthesized by a nonselective acyltransferase activity(ies) found even in noninflammatory cells as a component of membrane remodeling, which is then selectively and continually degraded by intracellular PAF acetylhydrolase activity to modulate PAF production.—Chen, J., L. Yang, J. M. Foulks, A. S. Weyrich, G. K. Marathe, and T. M. McIntyre. **Intracellular PAF catabolism by PAF acetylhydrolase counteracts continual PAF synthesis.** *J. Lipid Res.* 2007. 48: 2365–2376.

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Platelet-activating factor (PAF) activates all cells of the innate immune system at picomolar concentrations through a single receptor (1, 2). PAF is not normally present as a circulating lipid mediator; rather, it is produced by stimulated inflammatory cells, and a few select other cell

types, as a critical component of the coordinated acute inflammatory response.

Human neutrophils (3–6) and monocytes (7) produce PAF in response to cell-specific agonists and generate far larger quantities when pharmacologically stimulated by Ca²⁺ ionophores. Two mechanisms for PAF synthesis have been described, but it is the remodeling route that acetylates alkyl (or acyl) 2-lyso glycerophosphocholine using acetyl-CoA as the donor (8) that primarily accounts for PAF synthesis in inflammatory cells (1). Neutrophil (9, 10) and monocyte (11, 12) lysates catalyze this acetyl transacylation reaction, but long-chain acyl-CoAs competitively inhibit this acetylation (13–15). This raises the possibility that the PAF synthetic activity may not be specific for acetyl-CoA as a donor and that long-chain acyl-CoA-dependent phospholipid remodeling and PAF synthesis are related. A recent description of lyso-PAFAT/LPCAT2 (ATL1) as a PAF synthase confirms that this enzyme in fact displays a strong preference for arachidonoyl-CoA over acetyl-CoA (16). Moreover, total acetyltransferase activity does not increase, as HL60 cells differentiate from cells unable to produce PAF to cells that do so in response to appropriate stimulation (17). These observations raise mechanistic and regulatory questions about the way that PAF accumulates in stimulated cells.

PAF is selectively degraded by two groups of Ca²⁺-independent phospholipases, type VII (plasma and type II isoforms) and type VIII (type Ib; α 1 dimer, α 2 dimer, or α 1/ α 2 heterodimer) (18, 19) enzymes. The plasma isoform (PLA2G7) is secreted by monocytes (20) and apparently by Kupffer cells in vivo (21) and circulates in association with low and high density lipoproteins (22). The homologous

Abbreviations: LPS, lipopolysaccharide; MAFP, methyl arachidonoylfluorophosphonate; MLnFP, methyl linolenoylfluorophosphonate; PAF, platelet-activating factor; PMN, neutrophils; SIN-1, 3-morpholininosydnonimine; TNF- α , tumor necrosis factor- α .

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type II (PAFAHII) enzyme is intracellular (23). The group VIII enzymes, unrelated to the group VII enzymes, are intracellular and are distinguished by their complete specificity for PAF as a substrate (24). In contrast, the class VII enzymes also use oxidatively damaged phospholipids (25, 26) and protect cells from oxidative insult (27). None of these enzymes attack intact, long-chain phospholipids.

PAF acetylhydrolase activity modulates PAF accumulation. One example is the lack of PAF accumulation by stimulated macrophages (7, 28), which are the physiologic source of plasma PAF acetylhydrolase, and the transformation to cells that make and accumulate PAF after chemical inhibition of this activity with PMSF (7). Similarly, PMSF augments platelet (29) and methyl arachidonoylfluorophosphonate (MAFP) augments endothelial cell (30) agonist-stimulated PAF production.

Here, we show that PAF accumulates in unstimulated neutrophils when PAF hydrolysis is blocked. The accumulation of constitutively produced PAF is as rapid and to the same extent as in cells stimulated with lipopolysaccharide (LPS) or tumor necrosis factor- α (TNF- α). Continual PAF cycling even occurs in erythrocytes that are not components of the innate immune system and provides a rationale for the presence of an enzyme in erythrocytes that can only hydrolyze PAF. These data suggest that constitutive PAF synthesis mirrors constitutive phospholipid remodeling and that intracellular PAF acetylhydrolase activity is a critical component of stimulated PAF production.

EXPERIMENTAL PROCEDURES

Chemicals and reagents

Chemicals and reagents were purchased from the following sources: sterile filtered HBSS, BioWhittaker, Inc. (Walkersville, MD); sterile tissue culture plates, Falcon Labware (Lincoln Park, NJ); human serum albumin, Baxter Healthcare (Glendale, CA); endotoxin-free PBS, Mediatech, Inc. (Herndon, VA); LPS (*Escherichia coli* O111:B4), List Biological Laboratories (Campbell, CA); TNF- α , R&D Systems (Minneapolis, MN); *Rhizopus arrhizus* phospholipase A₁, Sigma (St. Louis, MO); aminopropyl and C₁₈ SPE columns, J. T. Baker, Inc. (Phillipsburg, PA); FURA-2AM ester, Molecular Probes (Eugene, OR); recombinant human plasma PAF acetylhydrolase and its monoclonal antibody, ICOS Corp. (Bothell, WA); anti- β -actin and anti-human hemoglobin, ICN (Costa Mesa, CA); A23187, Calbiochem (San Diego, CA); and PAF and BN52021, Biomol Research Laboratories (Plymouth Meeting, PA). 1-Hexadecyl-*sn*-glycero-3-phosphocholine (lyso-PAF) was from Avanti Polar Lipids (Alabaster, AL). [³H]acetyl-CoA (3.7 Ci/mmol) was from Perkin-Elmer (Boston, MA). Antibody to type I PAF acetylhydrolase was from Abcam (Cambridge, MA), and anti-type II PAF acetylhydrolase was from Proteintech Group, Inc. (Chicago, IL). Polyclonal plasma PAF acetylhydrolase antibody and its immunogenic peptide, MAFP, methyl linolenoyl-fluorophosphonate (MLnFP), 3-morpholinodnonimine (SIN-1), and nitrotyrosine monoclonal antibody were all from Cayman Chemicals (Ann Arbor, MI). Pefabloc was from Pentapharm AG (Basel, Switzerland). Protease inhibitor cocktails were Roche Complete Mini (Roche, Mannheim, Germany), Protease Arrest (Geno Technology, Inc., St. Louis, MO), and Halt Protease (Pierce, Rockford, IL).

Cell isolation, activation, and lipid extraction

Neutrophils (31) and monocytes (7) were isolated from healthy human donors by dextran sedimentation and Ficoll centrifugation under a protocol approved by the Cleveland Clinic institutional review board as described previously. Neutrophils (PMN)s were incubated with or without agonist in HBSS containing 0.5% human serum albumin (HBSS/A) in microcentrifuge tubes for the stated times. Monocytes were isolated from the Ficoll centrifugation as described previously. Erythrocytes were isolated as described (32).

Cellular lipids were extracted in methanol and transferred to Teflon-capped tissue culture glass tubes and mixed with chloroform by the method of Bligh and Dyer (33). The resulting total lipid extract was dried under N₂ and reconstituted with HBSS/A. The reconstituted lipid extracts were sonicated and vortexed just before use. In some experiments, the lipid extracts were treated with 1 μ g/ml PAF acetylhydrolase for 3 h at 37°C before being tested for neutrophil agonists using FURA-2-labeled PMN. In some experiments, the competitive PAF receptor antagonist BN52021 (20 μ M) was preincubated with the FURA-2-labeled cells for 30 min before the start of the assay.

Intracellular Ca²⁺ measurements

PAF-like activity was detected by monitoring rapid changes in intracellular Ca²⁺ levels as described (34). Briefly, neutrophils (5.5 \times 10⁶ cells/ml) were incubated in the dark with the Ca²⁺-sensitive fluorescent dye FURA-2AM (1 μ M) at 37°C for 45 min. After loading the cells with dye, neutrophils were recovered by centrifugation and washed three times in HBSS/A before being resuspended in HBSS/A (2.75 \times 10⁶ cells/ml). Changes in intracellular Ca²⁺ were detected by dual excitation at 340 and 380 nm, with emission monitored at 510 nm (35). Data are presented as 340/380 nm ratios on the ordinate and a 5 min period as the abscissa.

Mass spectrometry

Separation of PAF-like lipids by HPLC was as described previously (34). Briefly, PMNs (10⁸ cells/5 ml) were stimulated with PBS, A23187 (10 μ M), LPS (1 μ g/ml), TNF- α (10³ U/ml), or MAFP (10–100 μ M) for 60 min before total lipids were extracted as described (33). The total lipid extracts were condensed by Rotavapor (Buchi Labortechnik, Flawil, Switzerland), dried under a stream of N₂ gas, and then reconstituted in CHCl₃. Phospholipids were separated from neutral lipids and fatty acids by aminopropyl extraction columns (36) and then fractionated by reverse-phase HPLC as described previously (34). Fractions eluting from the reverse-phase column were assayed after drying and resuspension of an aliquot in HBSS/A by analyzing their effect on FURA2-loaded PMNs as detailed above. Fractions containing PAF-like activity were then pooled, dried under a flow of N₂ gas, reconstituted in HBSS/A, and then treated with 50 units of phospholipase A₁ from *R. arrhizus* in HBSS/A at 37°C overnight to remove diacyl lipids (37). Polar alkyl phospholipids were resolved from free fatty acids with aminopropyl extraction columns, and the recovered PAF-like lipids were reconstituted in methanol for analysis by ESI-MS/MS (34, 38) compared with [²H]PAF.

PAF acetylhydrolase activity

PAF-hydrolyzing activity of intact PMNs was determined using 4 μ M [³H-acetyl]PAF as substrate. Typically, 10⁷ intact neutrophils were used per assay, and the released radioactive acetate was measured using a C₁₈ cartridge as described (39). Erythrocyte PAF acetylhydrolase activity was determined as described previously (32).

PAF acetylhydrolase Western blot

Neutrophils were lysed in the presence of three protease inhibitor cocktail buffers (Roche, Genotech, and Pierce) because preliminary work had shown that cyclooxygenase-2 was reliably detected in these protease-laden cells when all three mixes were added before lysis. Cell lysates were separated by SDS-PAGE, and the resolved proteins were transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA) and then probed with anti-PLA2G7 (plasma PAF acetylhydrolase) polyclonal antibody (1:1,000), chicken anti-type I (PAFAH1b2) polyclonal antibody (1:500), or affinity-purified antibody to type II PAF acetylhydrolase (1:400). Peroxidase-conjugated secondary antibodies were used at 1:10,000, dilution, and blots were visualized by enhanced chemiluminescence. In some aliquots, the primary antibody was preincubated with cognate immunogenic peptide to establish antibody specificity.

Acetyl-CoA:lyso-PAF acetyltransferase activity

Neutrophils (10^8 in 5 ml of HBSS/A at 37°C) were stimulated with LPS, TNF- α , or A23187 for 10 min before adding chilled HEPES buffer (10 mM, pH 7.4). The cells were sedimented (100 g, 5 min, 4°C) and resuspended in 5 ml of ice-cold HEPES buffer (10 mM, pH 7.4) containing protease inhibitor cocktails from Pierce, Roche, and Genobiotec. Lysates were prepared by subjecting neutrophils to nitrogen cavitation using a Parr cell disruption bomb (Moline, IL) at 500 p.s.i. for 5 min. The reaction mixture contained 20 μ M lyso-PAF, 100 μ M acetyl-CoA, 1 μ Ci of [3 H]acetyl-CoA, and PMN lysate corresponding to 10 million cells. The mixture was incubated at 37°C for 10 min before the reaction was terminated by adding methanol and chloroform. The lipids were extracted (33) and resolved by TLC using chloroform-methanol-water (65:35:6, v/v) as the mobile phase, and the region corresponding to a PAF standard was scraped and radioactivity was determined by liquid scintillation counting.

Hemolysis

Freshly isolated human red blood cells (10^{10}) were washed four times with PBS to remove contaminating serum and resuspended in 10 ml of RPMI medium containing 0.5% glucose. Aliquots containing 10^7 cells were treated with the stated concentration of PAF and 100 μ M MAFF, or buffer, for 24 h. Hemoglobin release was measured in the centrifuged supernatants at 541 nm. A portion of the supernatant was resolved by SDS-PAGE and immobilized on Immobilon before hemoglobin was detected using a monoclonal antibody (1:1,000) by the same procedures described above.

PLA2G7 immunocytochemistry

Neutrophils (3×10^8) were stimulated with 10 μ M A23187, 10^3 U/ml TNF- α , or vehicle for 15 min at 37°C. An aliquot (5×10^7 cells) was removed and fixed with 2% paraformaldehyde (1:1) for 20 min at room temperature before the cells were centrifuged onto Vectabond-treated coverslips with 200 μ l of HBSS as a carrier. Cells were permeabilized with 0.1% Triton X-100 for 5 min at room temperature, blocked with 10% goat serum (Sigma) in HBSS for 1 h at room temperature, and incubated overnight at 4°C with murine monoclonal anti-plasma PAF acetylhydrolase (PLA2G7) at a final concentration of 2 μ g/ml. Samples were then incubated with biotin-conjugated goat anti-mouse IgG (Invitrogen; B2763) at 2 μ g/ml for 1 h at room temperature, followed by streptavidin-Alexa₄₈₈ (Invitrogen; S32354) staining for 1 h. Cell nuclei were stained with TO-PRO-3 (Invitrogen; T3605) and visualized on an FV300 Olympus IX81 microscope using a 60 \times oil objective and a 2.5 \times zoom.

Stimulated PAF accumulation does not correlate with PAF synthase activity

Quiescent human neutrophils do not contain PAF, but extracts of cells stimulated by TNF- α , LPS, or the Ca²⁺ ionophore A23187 all contain sufficient PAF to activate other neutrophils (Fig. 1A). The amount of PAF generated by A23187-stimulated neutrophils was sufficient to induce a sustained increase in intracellular Ca²⁺ in the test cells, whereas the PAF produced by LPS or TNF- α -stimulated cells was significantly less because it caused only transient stimulation when added to naïve cells. These results are consistent with the 30-fold difference in PAF accumulation between LPS- and A23187-stimulated cells we documented previously by mass spectrometry (40).

Analysis of acetyl-CoA:lyso-PAF acetyltransferase activity in lysates of these activated cells (Fig. 1B) revealed that the PAF synthase activity in A23187-stimulated neutrophil lysates was just 2-fold greater than that of quiescent cells. Moreover, stimulation of neutrophils with LPS or TNF- α resulted in the same 2-fold increase in activity found in cells treated with A23187, so the pattern of agonist effectiveness in intact cells was not accurately reflected by the in vitro assay of their lysates.

Human neutrophils express and retain plasma PAF acetylhydrolase

Human neutrophils express the intracellular type I PAF acetylhydrolase as an α_2/α_2 homodimer (41), which we confirmed by Western blot (Fig. 2A). However, inhibitor profiles suggest that this enzyme does not account for the bulk of leukocyte PAF acetylhydrolase activity (42), so we searched for the other intracellular enzyme, type II PAF acetylhydrolase (PAFAH2), but failed to detect immunoreactive material in neutrophil lysates (data not shown). Instead, we found (Fig. 2B) that neutrophils abundantly express immunoreactive plasma PAF acetylhydrolase (PLA2G7; lipoprotein-associated phospholipase A₂). Although the neutrophil enzyme migrated as a significantly smaller protein than the recombinant enzyme, the immunoreactive band was abolished when the antibody was blocked with the cognate peptide immunogen. We found that the recovery of recombinant plasma PAF acetylhydrolase added to neutrophil lysates was reduced with the appearance of degradation products (Fig. 2C). We also found that the amount of endogenous plasma PAF acetylhydrolase was significantly less in cells that had been stimulated by A23187 than in control cells (Fig. 2C).

Immunocytochemistry shows that the neutrophil enzyme is intracellular and that it has a distinct punctate distribution (Fig. 2D). This distribution is clearly distinguished from the nuclear membrane that is the site of PAF production (43, 44). The plasma PAF acetylhydrolase is secreted by HepG2 cells (45) and monocyte-derived macrophages (20). Stimulation of neutrophils with either LPS or TNF- α reduced, but did not eliminate, intracellular plasma PAF acetylhydrolase immunostaining that remained in a punctate pattern.

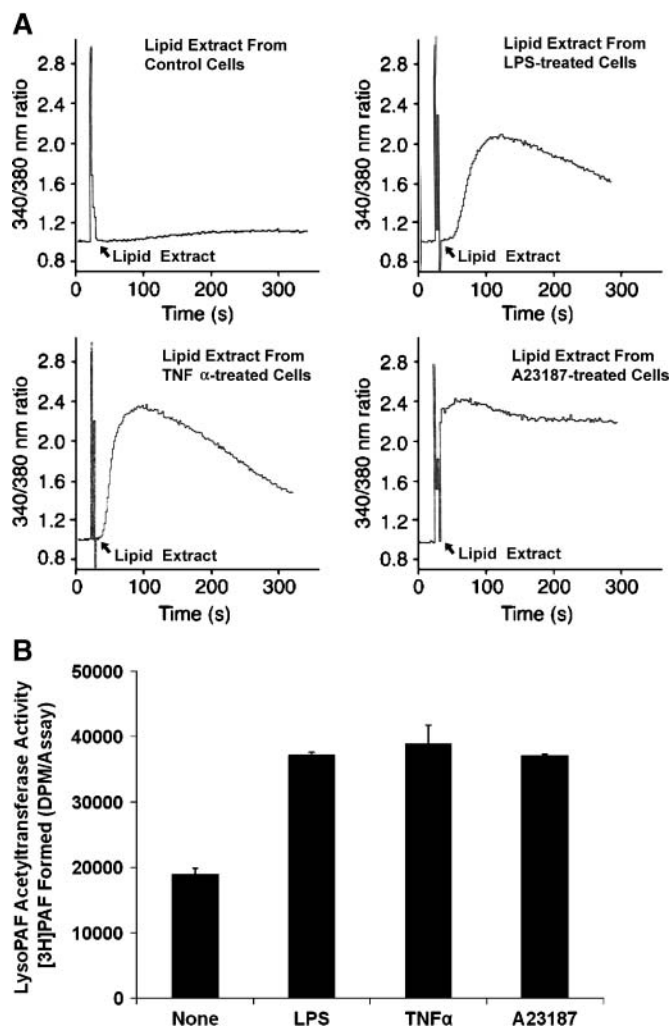


Fig. 1. Platelet-activating factor (PAF) accumulation and acetyl-CoA:lyso-PAF acetyltransferase activity in stimulated human neutrophils. **A:** PAF accumulation. Freshly isolated human neutrophils (10^7) were stimulated with $10 \mu\text{M}$ A23187, 10^3 U/ml tumor necrosis factor- α (TNF- α), or 100 ng/ml lipopolysaccharide (LPS) at 37°C for 1 h in 1 ml of HBSS containing 0.5% human serum albumin (HBSS/A). Neutrophil lipids were extracted and dried before being reconstituted in $100 \mu\text{l}$ of HBSS/A by sonication. This extract was added to fresh FURA2-labeled human neutrophils (2.75×10^6 in 1 ml), and changes in intracellular Ca^{2+} were monitored as an excitation ratio of 380 nm to 340 nm with emission monitored at 510 nm, as described in Methods. **B:** In vitro assessment of acetyl-CoA:lyso-PAF acetyltransferase activity. Human neutrophils were stimulated as in A for 10 min at 37°C in a total volume of 5 ml. Lysates were prepared by nitrogen cavitation in the presence of a mix of protease inhibitors before acetyl-CoA:lyso-PAF acetyltransferase (PAF synthase) activity was determined, as stated in Methods, using lysates corresponding to 10^7 cells, lyso-PAF ($20 \mu\text{M}$), and [^3H]acetyl-CoA ($100 \mu\text{M}$). The newly made [^3H]PAF was resolved by TLC and quantified by liquid scintillation counting. The experiments in each panel were repeated on at least 10 occasions. Error bars indicate \pm SEM.

PLA2G7 is the primary PAF acetylhydrolase of neutrophils

Proteolysis of the endogenous and added recombinant PAF acetylhydrolase in neutrophil extracts precluded an in vitro approach to define the activities responsible for

cellular PAF hydrolysis. However, we found that PAF hydrolysis by intact neutrophils was linear with time and number of leukocytes in the assay (Fig. 3A, inset). This activity was significantly (Fig. 3A), although not completely, inhibited by Pefabloc, MAFP, and NO^- derived from SIN-1, all irreversible inhibitors of the plasma PAF acetylhydrolase (30, 46, 47). The linolenoyl homolog of methyl arachidonyl-fluorophosphonate also inhibited neutrophil PAF acetylhydrolase activity, although MAFP proved to be the most robust inhibitor of this type. Pefabloc inhibited plasma PAF acetylhydrolase (Fig. 3B) but not the type I enzyme (see supplementary Fig. I).

To test the hypothesis that reduced PAF degradation complements stimulated PAF synthesis, we measured [^3H]PAF degradation by control and stimulated cells. The results (Fig. 3B) showed that stimulated cells had a reduced level of PAF hydrolysis, although the reduction was modest, and that A23187 was the most effective agent in effecting this change.

Esterase inhibitors induce PAF accumulation in unstimulated neutrophils

We tested the effect of inhibiting PAF hydrolysis on cellular PAF accumulation by treating quiescent neutrophils with varied concentrations of MAFP, MLnFP, and Pefabloc. We then isolated their polar phospholipids and defined the amount of PAF present by treating fresh, quiescent neutrophils with the extract and following changes in intracellular Ca^{2+} levels in the target cells. Treating neutrophils with each of the serine esterase inhibitors resulted in the accumulation of neutrophil agonists in a time-dependent (see below) and concentration-dependent (Fig. 4) manner. The accumulated stimulatory lipid was PAF, because for each inhibitor treatment of the recovered lipid with recombinant plasma, PAF acetylhydrolase destroyed its bioactivity, whereas treatment with phospholipase A_1 , which has no effect on the *sn*-1 ether bond of PAF, was without effect. The competitive PAF receptor antagonist BN52021 (Fig. 4) and other PAF receptor antagonists (data not shown) abolished the effect of the accumulated lipid on the test neutrophils. Addition of an excess of synthetic PAF overcame BN52021 inhibition, showing that the inhibition was competitive and the cells remained responsive. We confirmed these data by quantifying PAF accumulation by HPLC MS/MS to find 1.8, 17.4, 21.8, 32.8, and 53.2 pg of PAF per 10^6 cells after treatment by buffer or 10, 25, 50, or $100 \mu\text{M}$ MAFP, respectively. This is consistent with our previous determination of 58.8 pg of PAF in LPS-stimulated cells (40).

Esterase inhibition is as effective as agonist stimulation for neutrophil PAF production

We compared the extent of PAF accumulation after MAFP inhibition of PAF degradation with the amount that accumulated in cells stimulated by TNF- α or LPS. The data (Fig. 5A) show that the amount of PAF accumulated by neutrophils treated with just MAFP was equivalent to the amounts produced after agonist stimulation. The data also

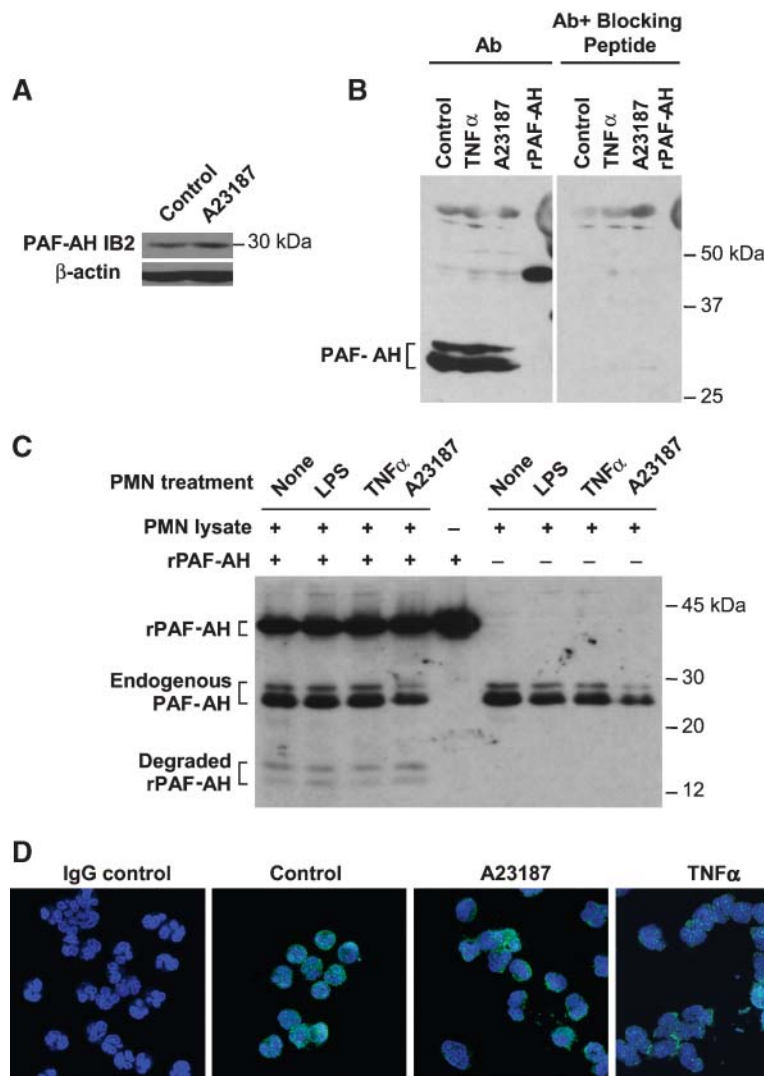


Fig. 2. Neutrophils express type I and plasma PAF acetylhydrolases. **A:** Type I PAF acetylhydrolase (PAF-AH) Western blot. Neutrophil lysates (2×10^8 cells) were prepared by nitrogen cavitation from resting and A23187-stimulated cells in the presence of protease inhibitors. Lysates (equivalent to 2×10^6 cells) were subjected to SDS-PAGE and immunoblotted for type I PAF acetylhydrolase and β -actin ($n = 5$). **B:** Plasma PAF acetylhydrolase Western blot. Lysates were prepared as in **A** and probed with PAF acetylhydrolase polyclonal antibody (Ab; $n = 9$). In a parallel experiment, the primary plasma PAF acetylhydrolase antibody was preincubated with 200 μ g/blot of the cognate immunogenic peptide. **C:** Endogenous and exogenous recombinant plasma PAF acetylhydrolases are degraded in neutrophil lysates. Neutrophils were treated with agonists or buffer as in **A**; in some aliquots, 250 ng of recombinant plasma PAF acetylhydrolase was added for 2 h at 37°C before the proteins were resolved and immunoblotted for plasma PAF acetylhydrolase as in **B** ($n = 2$). **D:** Plasma PAF acetylhydrolase immunocytochemistry. Neutrophils were stimulated as above and then fixed and permeabilized as described in Methods. The plasma PAF acetylhydrolase was detected using a monoclonal antibody, a biotinylated secondary antibody, and streptavidin-Alexa₄₈₈. Cell nuclei were counterstained blue with TO-PRO-3 ($n = 2$).

show that agonist stimulation when PAF degradation was blocked did not materially augment PAF accumulation.

MAFP also inhibits Ca^{2+} -dependent and Ca^{2+} -independent phospholipases A₂ (48) that supply the lysolipid backbone necessary for stimulated PAF synthesis (49), so their lyso-PAF product might limit PAF accumulation after MAFP treatment. We found that MAFP induced a time-dependent increase in cell-associated PAF and that the addition of lyso-PAF to these MAFP-treated neutrophils had no effect during the first 10 min of stimulation (Fig. 5B).

However, at later times, lyso-PAF itself promoted a modest increase in PAF accumulation and augmented the amount of PAF that accumulated after MAFP treatment. This indicates that the endogenous pool of lyso-PAF was initially sufficient, but eventually it began to limit PAF production.

Monocytes accumulate PAF after MAFP treatment

Human macrophages, compared with the monocytes from which they are derived, accumulate little PAF, as a

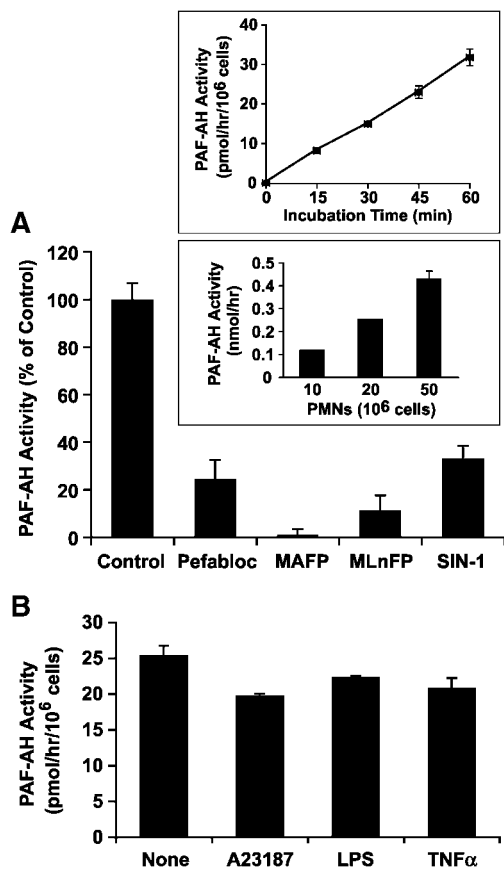


Fig. 3. Neutrophil PAF hydrolysis is sensitive to plasma PAF-acetylhydrolase (PAF-AH) inhibitors and cellular activation. **A:** Inhibitor profile. Duplicate samples of human neutrophils (10^7 /ml) were pretreated or not with 100 μ M Pefabloc, methyl arachidonoyl-fluorophosphonate (MAFP), methyl linolenoylfluorophosphonate (MLnFP), or 3-morpholinosydnonimine (SIN-1) for 30 min at 37°C ($n = 6$). The incubation was then continued for another 30 min in the presence of 4 μ M [3 H-acetyl]PAF. Released [3 H]acetate was separated from unhydrolyzed PAF over a C18 cartridge before quantification by liquid scintillation counting. Insets: PAF hydrolysis by intact neutrophils as a function of time or number of cells. **B:** PAF hydrolytic activity of stimulated neutrophils. Duplicate samples of neutrophils were stimulated as in Fig. 1 with the stated agonists for 1 h at 37°C before the hydrolysis of [3 H]PAF by intact cells ($n = 5$) was determined, as described in Methods. Error bars indicate \pm SEM.

consequence of a 260-fold increase in intracellular plasma PAF acetylhydrolase during differentiation (50). We determined whether the PAF acetylhydrolase activity present in undifferentiated monocytes regulates PAF accumulation as it does in neutrophils by treating these cells with MAFP. Indeed, quiescent monocytes, like neutrophils, accumulated PAF when treated with MAFP (see supplementary Fig. II), so PAF accumulation subsequent to disruption of its continual hydrolysis is not a response specific to neutrophils.

Type I PAF acetylhydrolase also degrades constitutively produced PAF

Neutrophils contain both the plasma and the type I PAF acetylhydrolases. To determine whether the type I enzyme

participates in constitutive PAF turnover, we sought a cell type that expressed just this isoform. We purified PAF acetylhydrolase from human erythrocytes (see supplementary Table I), because porcine erythrocytes express only the type I PAF acetylhydrolase (41). We found that the purified human protein, like the porcine type I enzyme, migrated with an apparent mass of 29–30 kDa. We confirmed the identity of this band as the human type I enzyme by mass spectrometry of the excised band. Reactive nitrogen species (41) generated by SIN-1 irreversibly inhibit circulating plasma PAF acetylhydrolase (47), and we used this observation to show that the protein migrating at 30 kDa was responsible for PAF hydrolysis by erythrocytes. The NO[•] donor SIN-1 caused a progressive inhibition of hydrolytic activity of the erythrocyte preparation that corresponded with nitration of the \sim 30 kDa band (Fig. 6A). We tested the effect of MAFP and Pefabloc on the type I enzyme to find that this PAF acetylhydrolase isoform was inhibited by MAFP but not by Pefabloc (see supplementary Fig. I).

Erythrocytes do not accumulate, secrete, or respond to PAF, but they do actively incorporate long-chain fatty acyl residues into their membrane phospholipids (51, 52). If PAF accumulation occurs through continual acyltransferase action followed by constitutive hydrolysis by type I PAF acetylhydrolase, then these cells might also accumulate PAF when their PAF hydrolytic activity is lost. We treated erythrocytes with MAFP to find (Fig. 6B) that this inhibitor caused a time-dependent increase in PAF bioactivity in these noninflammatory cells.

The complete specificity of the porcine enzyme (24) and the human enzyme we purified (data not shown) for PAF compared with oxidatively fragmented phospholipid suggests that constitutive PAF formation is a normal erythrocyte process and that the type I PAF acetylhydrolase is present to suppress inadvertent PAF accumulation. A short-chain phospholipid oxidation product hemolyzes red cells (53), suggesting that PAF, with its short chain, might be similarly disruptive. We incubated isolated human erythrocytes with varied concentrations of exogenous PAF and measured hemoglobin release by absorbance (Fig. 6C) and Western blotting (data not shown) to find that PAF had no effect on the integrity of cells with active PAF acetylhydrolase. In contrast, erythrocytes pretreated with MAFP were lysed by PAF in a concentration-dependent manner.

DISCUSSION

We find that the inhibition of PAF acetylhydrolase in several cell types is, by itself, sufficient to promote PAF accumulation in the absence of agonist stimulation. For neutrophils, this was equivalent to the levels of PAF generated in response to TNF- α or LPS stimulation. PAF is a transient agonist with a critical role in the first steps of the inflammatory response (1). Its actions at subnanomolar levels mandate tight control of its presence, and a significant component of this control is the expression by cells

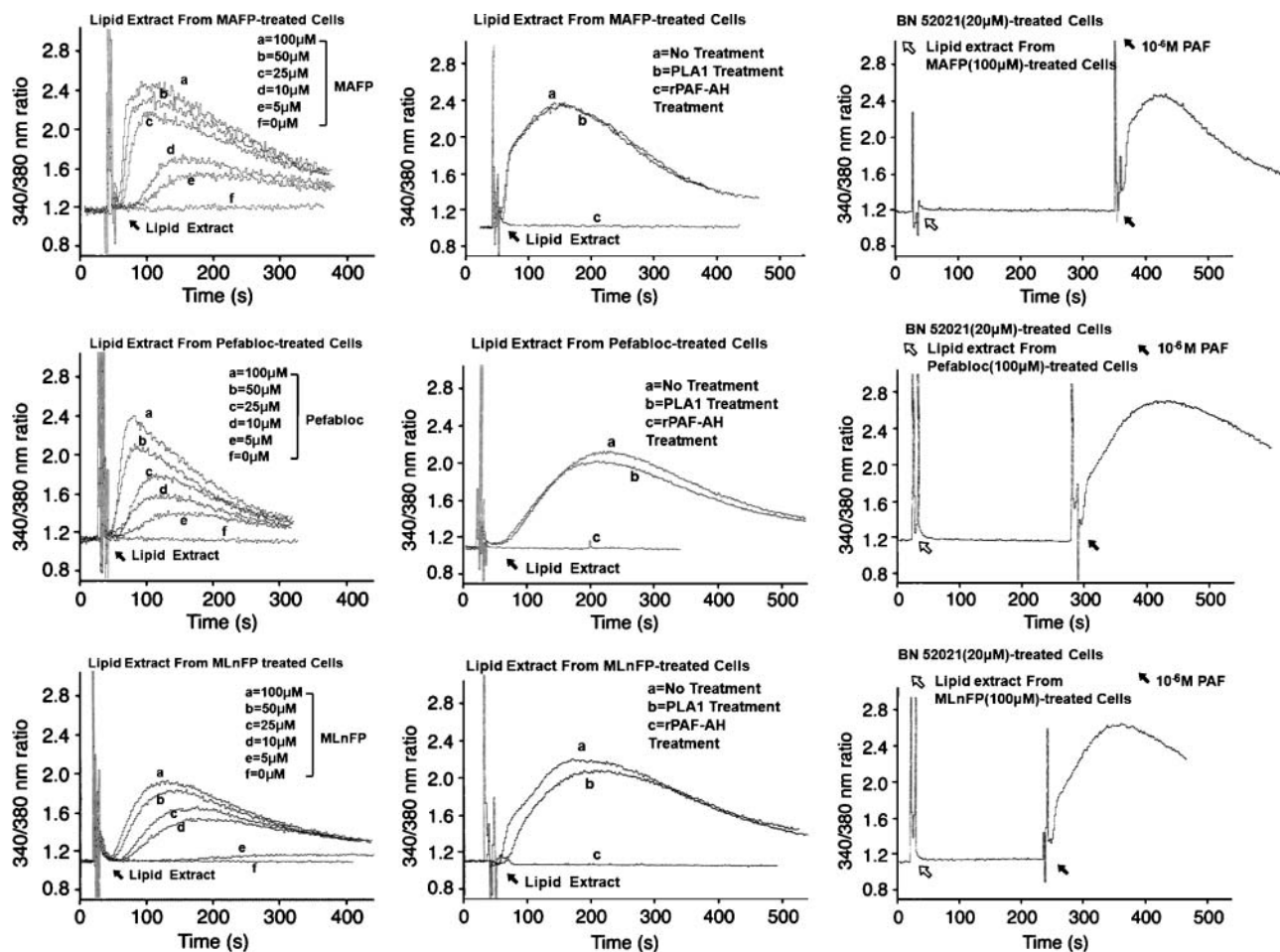


Fig. 4. Serine esterase inhibitors induce PAF accumulation by unstimulated neutrophils. Left: Neutrophils (10^7) were treated with the stated concentrations of the serine protease inhibitors MAFP, MLnFP, or Pefabloc for 1 h at 37°C . Cellular lipids were extracted and reconstituted in HBSS/A, and their effects on naïve FURA2-labeled neutrophils was measured as in Fig. 1. Middle: Effects of recombinant plasma PAF acetylhydrolase (PAF-AH) or phospholipase A_1 (PLA $_1$) on the neutrophil-stimulating activity extracted from esterase inhibitor-treated neutrophils before the addition of the extract to FURA2-labeled test neutrophils. Right: The role of the PAF receptor in the response of the target neutrophils to the lipid extracts was determined by pretreating the FURA2-labeled neutrophils with $20\ \mu\text{M}$ BN52021. The lipid extract was added at the time shown by the first, open arrow, and the incubation was continued in the presence of the PAF receptor antagonist BN52021. BN52021 is a competitive PAF receptor antagonist, and the addition (second, closed arrow) of excess PAF ($1\ \mu\text{M}$) reverses the inhibition caused by BN52021. This entire experiment was replicated three times.

and in plasma of various members of the constitutively active PAF acetylhydrolase family (18).

The role of PAF acetylhydrolase activity in controlling PAF accumulation is apparent in the accumulation of PAF by stimulated peripheral blood monocytes and the absence of PAF accumulation after they differentiate to monocyte-derived macrophages and begin to abundantly produce plasma PAF acetylhydrolase (50). Inhibition of this newly induced PAF acetylhydrolase allows PAF to once again accumulate in stimulated macrophages (7). However, inhibition of lesser amounts of plasma PAF acetylhydrolase present in undifferentiated monocytes allows PAF to accumulate in unstimulated cells, so the amount of PAF acetylhydrolase activity in monocytes is sufficient to suppress their basal production of PAF but not the larger amount produced by stimulated macrophages. Once PAF is formed in these cells, the acetyl enzyme intermediate in

the PAF acetylhydrolase reaction can distribute the activated acetyl residue among lysolipid acceptors (28, 54).

PAF production by stimulated platelets (29) or stimulated endothelial cells (30) is enhanced by PMSF or MAFP, respectively. However, we find that even unstimulated cells produce PAF when PAF degradation is blocked. The *n*-alkyl fluorophosphonates MAFP and MLnFP are potent irreversible inhibitors of plasma (55) (see above) and type I PAF acetylhydrolases, but they also inhibit group IV Ca^{2+} -dependent and group VI Ca^{2+} -independent phospholipases A_2 (48). The latter two enzymes supply lyso-PAF for stimulated PAF synthesis (49, 56) and phospholipid remodeling (57). Despite the inhibition of both phospholipase A_2 and PAF catabolic enzymes, the overall effect of MAFP was to promote spontaneous PAF accumulation so that sufficient lyso-PAF is present, or can be generated by other enzymes, for the initial period of synthesis. At later

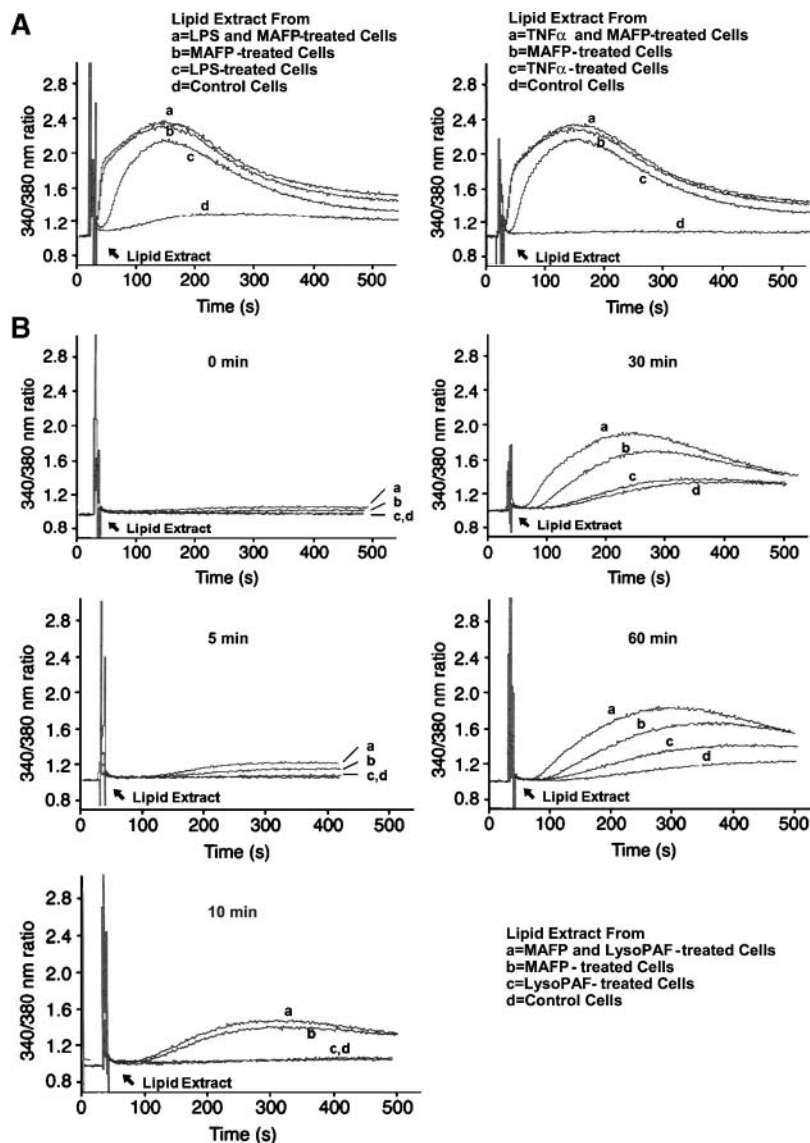


Fig. 5. MAFP-enabled PAF accumulation equals that resulting from agonist stimulation, is time-dependent, and is partially substrate-limited. **A:** Comparison of PAF accumulation by soluble neutrophil agonists with MAFP treatment. Human neutrophils (10^7 /ml) in HBSS/A were treated for 1 h at 37°C with buffer, 100 ng/ml LPS (left), or 10^3 U/ml TNF- α (right). Some aliquots were pretreated with 100 μM MAFP for 30 min before incubation with these agents. The lipid extract from these cells was tested for neutrophil agonist activity as in Fig. 1. **B:** Effect of exogenous lyso-PAF on MAFP-induced PAF accumulation over time. Neutrophils were treated with MAFP or buffer for the stated times in the presence or absence of lyso-PAF (0.1 μM) before their lipids were extracted and analyzed as in Fig. 1. $n = 3$ for both panels.

times, or when cytosolic phospholipase A_2 activity is abolished (49), lyso-PAF limits total PAF production.

The synthetic activity that generates PAF apparently does not specifically use acetyl-CoA as an acyl donor; therefore, it is not simply a PAF synthase. Both short-chain (13, 58) and long-chain (13, 14, 59, 60) fatty acyl-CoA are well established as competitors for acetyl-CoA in *in vitro* and *in vivo* (15, 61, 62) assays of PAF synthesis. Indeed, a recent molecular description of a PAF synthase also shows that the resulting gene product uses arachidonoyl-CoA in preference to acetyl-CoA (16). Acylation using long-chain fatty acyl-CoAs means that the activity participates in cellular phospholipid remodeling, a continual process in all cells. PAF accumulation subsequent to chemical inhibition of its constitutive turnover shows that PAF too is subject to continual cycling by cells in their basal state.

The contention that the acetyltransferase that forms PAF is not a specific PAF synthase is strengthened by the observation that even erythrocytes generate PAF. Erythrocytes do not respond to PAF (63) because they do not ex-

press the PAF receptor, and erythrocytes do not respond to inflammatory stimuli that control acute inflammation. Accordingly, erythrocytes have no overt link to the rapid inflammatory response. Erythrocytes, however, do remodel and recycle their phospholipid fatty acyl chains (64, 65) in a process that accounts for a daily turnover of one-third of their phospholipid pool (66). PAF synthesis accompanies membrane remodeling in these cells, as we found that PAF accumulated in erythrocytes after inhibition of their type I PAF acetylhydrolase. These results show that cellular PAF synthesis is constitutive and that continual PAF cycling occurs even in cells that are not a component of the innate immune system. Although PAF cycling may be inefficient, these data show that cell death would be a consequence of not removing this lytic lipid.

We found that human erythrocytes, like porcine cells (67), contain the type I PAF acetylhydrolase, but its presence in erythrocytes (32) creates an enigma, given that its only known substrate is PAF. All three forms of PAF acetylhydrolase are sufficiently selective that they do not

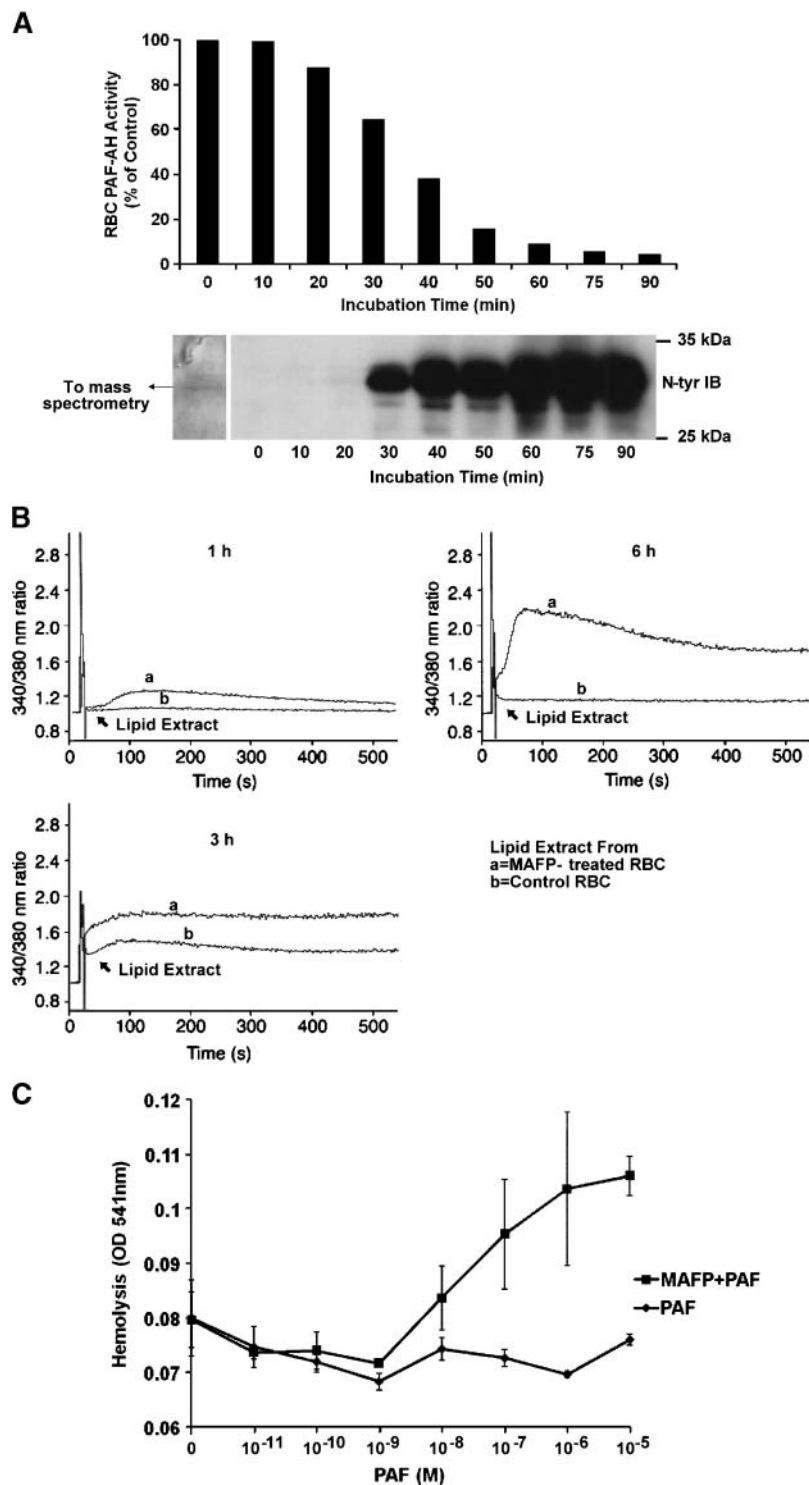


Fig. 6. Erythrocytes continuously produce PAF that is catabolized by intracellular type I PAF acetylhydrolase (PAF-AH) to suppress hemolysis. **A:** Human erythrocytes contain type I PAF acetylhydrolase. PAF acetylhydrolase was purified from human erythrocytes (see supplementary data) before the final preparation was incubated with 2.5 mM SIN-1 for the stated times. Aliquots of the treated and untreated control material were assayed for [³H]PAF hydrolysis (top), whereas a second aliquot was resolved by SDS-PAGE and then blotted for nitrotyrosine content (bottom). There was concordance between the loss of activity and nitrotyrosine derivatization of a 30 kDa band. The ~30 kDa band was stained with silver (bottom, far left lane), excised from the gel, and identified using tandem mass spectrometry with standard in-gel trypsin digestion followed by LC-MS/MS, as described in Methods and the supplementary data. **B:** Erythrocytes treated with MAFP accumulate bioactive PAF. Human erythrocytes were treated with 100 μ M MAFP for the stated times before their lipids were extracted, subjected to *R. arrhizus* phospholipase A₁ treatment to clear non-ether-linked phospholipids, and then assayed for neutrophil-stimulatory activity using FURA2-labeled target cells (34). **C:** PAF is hemolytic for erythrocytes lacking PAF acetylhydrolase activity. Human erythrocytes were treated with the stated concentrations of PAF and 100 μ M MAFP, or not, for 24 h. The absorbance of the released heme was measured at 541 nm. A portion of the medium was Western blotted with a monoclonal antibody for hemoglobin, which confirmed (not shown) that hemoglobin was released from MAFP-treated cells as a function of PAF concentration. The experiments reported in each panel were replicated on three separate occasions. Error bars indicate \pm SEM.

hydrolyze long-chain cellular phospholipids (18, 19), but the substrate specificity for the type I enzyme is precise; it hydrolyzes PAF, but not slightly longer chains produced during phospholipid oxidation (19, 24). PAF acetylhydrolases are particularly suited for constitutive PAF hydrolysis not only through their distinctive substrate specificity but also because, unlike most cellular phospholipase A₂ activity, they are Ca²⁺-independent and do not require increased intracellular free Ca²⁺ to initiate hydrolysis.

This type I enzyme is developmentally regulated in brain (68, 69) and may serve to modulate neuronal cell migration (70), but PAF plays no known role in erythrocytes. PAF slowly (half-life of 17 h) transits the erythrocyte membrane (71) to become available to the internal enzyme, but human cells lacking functional plasma PAF acetylhydrolase show that it is the lipoprotein-associated plasma PAF acetylhydrolase that hydrolyzes PAF in the plasma (72–74). The erythrocyte enzyme does not normally act on extra-

cellular PAF (41, 72) but can encounter circulating PAF after hemolysis (74).

Our data suggest that the role of the red blood cell enzyme is related to the collateral formation of PAF during phospholipid remodeling. The fact that erythrocytes generate PAF has been hidden by their expression of type I PAF acetylhydrolase. PAF had no effect on erythrocyte integrity when their PAF acetylhydrolase was active but caused a concentration-dependent lysis when the enzyme was inactivated. We propose that erythrocytes express the isoform of PAF acetylhydrolase that is specific for PAF to prevent the consequences of erythrocyte-generated PAF accumulation.

Neutrophils rapidly accumulate exogenous PAF and hydrolyze the *sn*-2 acetyl residue (75, 76). The relevant enzyme(s) remained undefined, although the intracellular type I and type II PAF acetylhydrolases were candidates. We confirmed by Western blotting that neutrophils express the type I enzyme (41), but experiments with Pefabloc, which did not inhibit the type I enzyme, indicate that this isoform was a smaller contributor to overall PAF catabolism. However, the type II enzyme was not sufficiently abundant to be detected by Western blotting. Instead, we found that neutrophils expressed and retained plasma PAF acetylhydrolase, which accounted for the majority of PAF catabolism by these cells. This is consistent with the much higher specific activity of the plasma isoform (39) compared with that of the type I enzyme. This finding is consistent with a report (77), from before the molecular identification of any PAF acetylhydrolase, that HL60 cells differentiated toward a granulocytic phenotype secrete an activity with some, but not all, properties consistent with the plasma PAF acetylhydrolase.

The distribution of the plasma PAF acetylhydrolase in neutrophils was punctate and was well dispersed from the nuclear site of PAF synthesis (43, 44). The plasma PAF acetylhydrolase is synthesized with an N-terminal signal sequence (78) that directs its secretion from monocytes (20) and transformed HepG2 cells, but not primary hepatocytes (45). The enzyme is either secreted in association with, or rapidly binds to, dense particles released from HepG2 cells (45) and then rapidly redistributes to LDL and HDL particles, as found in the circulation (22). Specific residues, such as tyrosine 205, tryptophan 115, and leucine 116, are required for the association of the plasma PAF acetylhydrolase with LDL (79), whereas the molecular basis for HDL binding is uncharacterized. Human platelets contain a membrane-associated plasma PAF acetylhydrolase that is released by activated cells as components of shed microparticles (80). The processes that determine whether the enzyme is to be secreted or retained in intracellular compartments are uncharacterized.

Our results suggest that phospholipid reacylation and PAF synthesis are mechanistically intertwined and that cells, whether engaged or not in inflammatory signaling, continuously produce PAF, supporting the early deduction (81) that lyso-PAF is either acetylated to PAF or reacylated to phospholipid in stimulated cells. We find that a similar process occurs in unstimulated cells and that a

critical role for the widespread expression of PAF acetylhydrolases is to control this PAF expression. ■

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