Human Platelets Secrete the Plasma Type of Platelet-Activating Factor Acetylhydrolase Primarily Associated With Microparticles

John V. Mitsios, Maria P. Vini, Dominique Stengel, Ewa Ninio, Alexandros D. Tselepis

Objective—Platelet-activating factor acetylhydrolase (PAF-AH) expresses a Ca$^{2+}$-independent phospholipase A$_2$ activity and hydrolyzes platelet-activating factor as well as oxidized phospholipids. Two major types of PAF-AH have been described: the plasma type, which is associated with lipoproteins, and the intracellular type II PAF-AH.

Methods and Results—We investigated the type(s) of PAF-AH expressed in human platelets as well as the mechanism and the enzyme type secreted from platelets during activation. The majority of the enzyme activity (75.1±14.3% of total) is found in the cytosol, whereas 24.9±7.3% is associated with the membranes. Immunofluorescence microscopy studies and Western blotting analysis showed that platelets contain the plasma type as well as the intracellular type II PAF-AH. Furthermore, platelets contain high levels of the mRNA of plasma PAF-AH, whereas only a small quantity of the type II PAF-AH mRNA was detected. On activation, platelets secrete the plasma type of PAF-AH mainly associated with platelet-derived microparticles (PMPs). The enzyme activity was also detected on circulating PMPs in plasma from normolipidemic healthy subjects.

Conclusion—This is the first indication that in addition to lipoproteins, PAF-AH in human plasma is carried by PMPs, suggesting that the PMP-associated PAF-AH may play a role in the dissemination of biological activities mediated by these particles. (Arterioscler Thromb Vasc Biol. 2006;26:1907-1913.)

Key Words: atherosclerosis ■ inflammation ■ PAF ■ PAF-acetylhydrolase ■ PMPs

Platelet-activating factor (PAF) is a biologically active phospholipid involved in diverse pathologies such as inflammation and atherosclerosis. PAF can activate various cell types including platelets. In the presence of PAF, platelets aggregate and degranulate, releasing biologically potent agents. Furthermore, platelets activated by other stimuli, such as thrombin, synthesize and secrete PAF. A prominent role in the regulation of PAF activity in vivo plays PAF acetylhydrolase (PAF-AH), an enzyme that expresses a Ca$^{2+}$-independent phospholipase A$_2$ activity and degrades PAF into the inactive metabolite lyso-PAF. Two major types of PAF-AH have been described, namely the extracellular (plasma) and the intracellular (cytosolic) type. The plasma enzyme is a single polypeptide that originates mostly from cells of the hematopoietic lineage, primarily from monocytes/macrophages. In human plasma, PAF-AH is predominantly associated with low-density lipoprotein (LDL), whereas a small proportion also binds to high-density lipoprotein (HDL). Thus, PAF-AH is also denoted as lipoprotein-associated phospholipase A$_2$. Apart from PAF, plasma PAF-AH can effectively hydrolyze biologically potent oxidized phospholipids containing short-chain peroxidized sn-2 residues. Several types of the intracellular form of PAF-AH have been described. The intracellular type II PAF-AH consists of a single 40-kDa polypeptide chain that exhibits a similar substrate specificity and a 42% amino acid identity with that of the plasma enzyme. A multimeric PAF-AH (IB), which is composed of $\alpha$, $\beta$, and $\gamma$ subunits, was also described in the brain.

It has been shown previously that type II PAF-AH is the major enzyme type present in human platelets. On activation, platelets secrete PAF-AH activity; however, the mechanism and the type of PAF-AH secreted remain to be established. Platelet activation either by shear stress or by the combination of thrombin plus collagen leads to the secretion of PAF, which is mainly associated with microparticles. Indeed, platelet activation by shear stress or various agonists results in the shedding of submicroscopic membrane vesicles (platelet-derived microparticles [PMPs]), which are enriched in bioactive lipids. PMPs are also enriched in bioactive platelet-derived proteins and express several platelet receptors such as the integrin receptor $\alpha_{IIbeta}$. Because of the above characteristics, PMPs express a wide range of biological actions.
In the present study, we show for the first time that platelets contain both the plasma type (mRNA and protein) as well as the intracellular type II PAF-AH protein only. On activation, platelets secrete the plasma type of PAF-AH, which is primarily associated with PMPs.

Materials and Methods
For detailed Materials and Methods, please see the online data supplement, available at http://atvb.ahajournals.org.

Results
PAF-AH Secretion From Activated Platelets
Thrombin induced a dose- and time-dependent secretion of the enzyme, with a maximum of $716 \pm 144 \text{ pmol/10}^9 \text{ cells per hour}$ observed at 30 minutes of platelet activation with a thrombin concentration of 0.2 IU/mL (supplemental Figure I, available online at http://atvb.ahajournals.org). Importantly, no enzyme activity was detected in the supernatants from resting platelets, whereas no cell lysis was observed during platelet activation because lactate dehydrogenase was not released during aggregation. In contrast to thrombin, ADP failed to induce PAF-AH secretion at any concentration used, although it induced a dose-dependent platelet aggregation (maximum aggregation $41 \pm 7\%$ at a concentration of $5 \mu\text{mol/L}$; $n=5$).

Characterization of the Platelet PAF-AH
Thrombin-activated platelets secreted $22 \pm 5\%$ of the total PAF-AH activity measured in the lysate of resting platelets ($3254 \pm 654 \text{ pmol/10}^9 \text{ cells per hour}$). The total enzyme activity in the platelet lysate after activation (3048 $\pm 598 \text{ pmol/10}^9 \text{ cells per hour}$) was similar to that measured before activation. On lysate ultracentrifugation, the majority of the enzyme activity (2444 $\pm 306 \text{ pmol/10}^9 \text{ cells per hour}$), representing $75.1 \pm 14.3\%$ of total, remained in the supernatant (cytosolic fraction), whereas $24.9 \pm 7.3\%$ was associated with the membrane-rich pellet. PAF-hydrolyzing activities in the cytosolic fraction, on the membrane-rich pellet, and secreted into the medium were characterized as PAF-AH because they were Ca$^{2+}$-independent and were inhibited either by 1 mmol/L of the serine esterase inhibitor Pefabloc$^21$ or by 30 $\mu\text{mol/L}$ CV-3988, a specific PAF receptor antagonist$^22$ that also inhibits PAF-AH. Furthermore, PAF-AH activity was specific for short acyl chains at the sn-2 position because it was not inhibited in the competition studies by the sn-2 long-chain phosphatidylcholine. Importantly, the secreted and the membrane-associated enzyme activities were resistant to treatment with 1 mmol/L of the sulfhydryl agent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). In contrast, the cytosolic enzyme activity was significantly reduced by DTNB treatment, suggesting the presence of a free cysteine residue essential for catalysis, thus resembling type II PAF-AH, (supplemental Table II).

The platelet-associated PAF-AH was also characterized by immunofluorescence microscopy. Platelets are positively stained with the monoclonal antibodies against the plasma type and the intracellular type II of PAF-AH, although the staining for the plasma type of PAF-AH is weaker compared with that of type II PAF-AH (Figure 1A and 1B). Additionally,
these results revealed that the plasma type of PAF-AH might not be glycosylated because it does not exhibit a broadband characteristic for the N-glycosylated PAF-AH associated with LDL (Figure 1A).25

We next searched for the mRNA of both types of PAF-AH in platelets; platelets contain high levels of mRNA corresponding to the plasma type of PAF-AH, with the cycle threshold (CT) value in quantitative polymerase chain reaction (QPCR) of 24.3 when using 20 ng of cDNA (Figure 1C and 1D). The mRNA of the type II PAF-AH was undetectable in classical polymerase chain reaction (PCR; data not shown), whereas an extremely low quantity with a CT value of 30.4 was detected by using a more sensitive method of QPCR (Figure 1D). Finally, among the brain type of PAF-AH (IB) subunits, only the $\gamma$ subunit was slightly detectable in 2 separate platelet preparations (Figure 1D) because the CT values for the $\alpha$ and $\beta$ subunits were $>31$. Of importance, the specific leukocyte transcripts CD11c/$\beta_2$ integrin and CD14 were not found in the RNA preparations of these platelets (Figure 1D), thus excluding the possibility of contamination by other circulating cells. The mRNA extracted from human cultured monocyte/macrophages, which express all types of PAF-AH and both the CD11c/$\beta_2$ integrin and CD14, served as a positive control for all PCR procedures (CT ranging from 24 to 26).

**The PAF-AH Secreted From Platelets Is Associated With PMPs**

Based on previous studies indicating that PAF secreted by platelets is associated with PMPs,13,14 we asked whether such PMPs could also be the carriers of PAF-AH secreted from thrombin-activated platelets. Indeed, thrombin (0.2 IU/mL) induced a time-dependent shedding of PMPs, denoted by an increase in the population in the lower left quadrant of the forward side scatter (FSC) versus side scatter (SSC) dot plot (Figure 2), which paralleled the secretion of PAF-AH as it is shown in supplemental Figure IB.

To investigate whether platelet aggregation is a prerequisite step for PAF-AH secretion, we activated platelets in the presence of Arg-Gly-Asp-Ser (RGDS), which inhibits platelet aggregation by preventing the binding of fibrinogen to the activated form of the integrin receptor $\alpha_{\text{IIb}}\beta_3$.26 RGDS (0.2 mmol/L) completely inhibited thrombin-induced platelet aggregation (Figure 3A); however, it failed to significantly inhibit either PAF-AH secretion (Figure 3B) or PMP production: 79.2% total expression of both annexin V and CD61 in the absence of RGDS versus 65.5% total expression of both annexin V and CD61 in the presence of RGDS (Figure 3C).

To further investigate whether PAF-AH secretion is independent of platelet aggregation, we activated platelets in the presence of Ca$^{2+}$-ionophore A23187 under nonstirring conditions that induces platelet vesiculation as opposed to platelet aggregation,27 and therefore, this treatment is considered one of the best inducers of PMP production.18 Ca$^{2+}$-ionophore A23187 (10 $\mu$mol/L) induced a time-dependent shedding of PMPs in parallel to the secretion of PAF-AH, similarly to that obtained with thrombin stimulation. Importantly, RGDS neither influenced PAF-AH secretion nor PMP production (Figure 4A and 4B).

To show whether the secretion of PAF-AH induced by Ca$^{2+}$-ionophore A23187 is associated with the production of PMPs; we used calpeptin, a specific inhibitor of calpain and PMP formation.27,28 Calpeptin (300 $\mu$mol/L) significantly inhibited Ca$^{2+}$-ionophore A23187-induced PAF-AH secretion (Figure 4C), along with PMP production; in the presence of calpeptin, 15.0% of total events expressing CD61 were found in the lower left quadrant of the FSC versus SSC dot plot, which corresponds to the PMP population, versus 61.1% of total events found in the absence of calpeptin (Figure 4D).
This suggests that vesiculation of the platelet membrane is a prerequisite to PAF-AH secretion.

Characterization of the PAF-AH Associated With PMPs

Subsequently, we characterized the PAF-AH associated with the PMPs produced on platelet activation with Ca\(^{2+}\)-ionophore A23187. PMPs were isolated by ultracentrifugation of the supernatant of activated platelets and characterized by flow cytometry. The PMPs population was found to be \(\geq 98\%\) positive for CD61. PMPs were characterized further by their staining with annexin V and anti-CD41a (supplemental Figure IIIA). Importantly, all PAF-AH activity secreted in the supernatant was recovered in isolated PMPs. PMPs were enriched in PAF-AH, exhibiting a higher specific activity compared with the platelet lysate (10.1±2.4 nmol/mg protein per hour versus 4.9±0.3 nmol/mg protein per hour, respectively; \(P=0.01; n=7\)). The PMP-associated PAF-AH activity was inhibited by 30 \(\mu\)mol/L CV-3988, but it was not affected by 1 mmol/L DTNB, suggesting that this enzyme is of the plasma type (supplemental Figure IIIB). Additionally, the PMP-associated PAF-AH was immunoprecipitated with a rabbit polyclonal antiserum raised against the recombinant plasma PAF-AH (supplemental Figure IIIC).

Association of PAF-AH With Circulating PMPs

The above results provide strong evidence that PMPs produced by activated platelets in vitro contain the plasma type of PAF-AH. However, it remains to be established whether circulating PMPs are also carriers of PAF-AH in plasma. To explore this possibility, we prepared plasma from peripheral blood of 10 normolipidemic apparently healthy volunteers and investigated whether a proportion of circulating plasma PAF-AH is associated with PMPs. Circulating microparticles were detected in plasma by flow cytometry as a population of particles that exhibited a typical FSC versus SSC profile; \(10\%\) of the total population was positive for annexin V. Among the gated annexin V–positive particles, 89±3% express both CD41a and CD31 whereas 11±2% express only CD41a. There were no detectable amounts of CD45 or CD14 in the annexin V–positive population. Because CD41a is a specific marker of PMPs, whereas CD31 (platelet-endothelial cell adhesion molecule-1) characterizes both the PMPs as well as the endothelial cell-derived microparticles, we suggest that all of the annexin V–positive particles found in plasma are PMPs, a finding that is in accordance with previously published results.29 In contrast, in the annexin V–negative population, 5±1% were positive for CD31 (endothelial cell-derived microparticles). No detectable amounts of CD41a, CD45, or CD14 were observed in this population. This finding is in accordance with previously published observations50 showing that most of the plasma endothelial cell-derived microparticles are negative for annexin V. To assess the possibility of nonspecific binding, fluorescein isothiocyanate– and phycoerythrin-labeled isotype-matched mouse monoclonal IgG antibodies were tested. Unspecific binding was negligible. Representative dot plots showing the flow cytometric profile of the gated annexin V–positive particles are shown in supplemental Figure IV.

We next studied whether PMPs are carriers of PAF-AH in plasma by using an ELISA method. PAF-AH activity was detected only in the wells that were coated with anti-CD61 (Figure 5), showing that exclusively platelet-derived \(\alpha_{\text{IIb}\beta_{3}}\)-containing microparticles that were captured by ELISA contained PAF-AH activity. Similar results were obtained when isolated PMPs were used as a positive control for PAF-AH.
These data provide for the first time evidence that circulating PMPs are carriers of PAF-AH in human plasma.

**Discussion**

The present study shows that human platelets contain 2 types of PAF-AH: the plasma type as well as the intracellular type II. Importantly, 75% of total enzyme activity is found in the cytosolic fraction and resembles type II PAF-AH, a finding that is in accordance with previously published data, whereas 25% of enzyme activity is associated with the cell membranes and shares similar characteristics with that of the plasma type PAF-AH. Platelets are capable of absorbing several proteins from plasma, such as fibrinogen or albumin on endocytosis and are able to store them in their granules. In addition, it has been shown that tissue factor–bearing microvesicles (of monocyte origin) are able to fuse with activated platelets and transfer their protein as well as their lipid content to the platelet surface. Thus, it might be envisioned that platelets absorb the plasma type of PAF-AH either from circulating lipoproteins or from microvesicles derived from monocytes/macrophages, the main cellular source of the plasma type of PAF-AH. However, the above possibilities are unlikely because both the plasma and the monocytes/macrophage-secreted PAF-AH are N-glycosylated, whereas we show here that platelet PAF-AH lacks glycosylation. Furthermore, we showed that platelets contain high levels of mRNA corresponding to the plasma type of PAF-AH, suggesting that these cells may synthesize de novo plasma PAF-AH. Previous reports have provided evidence that platelets can synthesize proteins such as interleukin-1β in an activation-dependent manner. In our studies, the total enzyme activity determined in the platelet lysate before activation was similar to that measured after activation with thrombin. This suggests that translation of the mRNA for the plasma type of PAF-AH by platelets is independent of their activation state.

A substantial proportion of the platelet PAF-AH is secreted during thrombin-induced aggregation, a finding that is in accordance with our previous findings. Importantly, PAF-AH secretion occurred in parallel to the shedding of PMPs observed during platelet aggregation, although the latter is not required either for PAF-AH secretion or for PMP production, as documented in the experiments with the peptide RGDS.
The presence of PAF-AH in PMPs may be pathophysio-
logically important because PMPs express several protein
receptors and ligands and contain biologically active lipids
including PAF, which allows their interaction with various
cells, especially PAF, because it plays an important role
in cell-to-cell interactions, as observed in models of acute and
chronic inflammation. Thus, it has been suggested that
most signaling by PAF may occur between closely juxta-
posed cells (endothelial cells, neutrophils, monocytes), and
that PAF can be recognized by its receptor on target cells
while associated with the plasma membrane of the signaling
cell. Consequently, the PMP-associated PAF-AH may be
important in regulating the activity of PMP-associated PAF in
the juxtacrine signaling. It has been shown recently that
PMPs are elevated in the circulation of patients with acute
coronary syndromes. In this context, we demonstrated that
the balance between PAF production and secretion as well
as PAF-AH secretion from platelets of patients undergo-
ing coronary angioplasty is significantly altered before
angioplasty as well as 48 hours afterward. This suggests that
alterations in the balance between PAF and PAF-AH secreted
by activated platelets may be of importance in coronary
atherothrombosis and in the inflammatory response elicited
during intracoronary injury induced by angioplasty. Recent
data from large population studies consistently report a
positive association between the risk for atherosclerotic
events and the mass or activity of total plasma PAF-AH (denoted as lipoprotein-associated phospholipase A2), which
mainly reflects the LDL-associated enzyme. In contrast,
the HDL-associated PAF-AH may exhibit antiatherogenic
properties. Therefore, the role of PMPs in disseminating
PAF-AH on inflammation and atherosclerosis remains to be
elucidated. In addition, the usefulness for quantitation of
PMP-associated PAF-AH in plasma as a prognostic or diag-
nostic tool in atherosclerotic diseases should be investigated
in large clinical studies.

In conclusion, the present study shows for the first time
that human platelets contain 2 types of PAF-AH: type II
PAF-AH, primarily found in the cytosol, and plasma PAF-
AH, which was associated with membranes. Only the mRNA
coding for plasma PAF-AH was detected in platelets. On
activation with thrombin or Ca2+-ionophore A23187, platelets
secreted exclusively plasma PAF-AH that was mainly asso-
ciated with PMPs. Collectively, we provide strong evidence
that in addition to plasma lipoproteins, the platelet-borne
PMPs are also efficient carriers of PAF-AH in circulation.

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Disclosures
None.


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Fatty acid-free bovine serum albumin (FFA-BSA), L-a-phosphatidylcholine dipalmytoyl (PC), 5,5'-dithiobis (2-nitrobenzoic acid), (DTNB), RGDS, Ca$^{2+}$-ionophore A23187, apyrase, prostaglandin E$_1$ (PGE$_1$), CV-3988, sepharose-protein A, adenosine diphosphate (ADP), Tris-base, ethylenediaminetetraacetic acid (EDTA), N-2-hydroxyethylpiperazine-N$'$_2-ethanesulfonic acid (HEPES) and 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine (C16:0 PAF) were purchased from Sigma (St. Louis, MO). Titrplex III was form Merck, calpeptin and fibrinogen from Calbiochem (San Diego, CA) and thrombin from Chronolog corp. (Havertown, PA). 1-O-hexadecyl-2-[3H-acetyl]-sn-glycero-3-phosphocholine solution in ethanol (10 Ci/mmol) was purchased from New England Nuclear, Boston, MA. An 1 mmol/L [3H-acetyl]PAF solution in 2.5 mg/mL FFA-BSA solution in saline (FFA-BSA/saline) was prepared as previously described and used as a substrate in the PAF-AH assays. Human plasma recombinant PAF-AH and rabbit polyclonal antisera raised against this PAF-AH were from Cayman (Ann Arbor, MI). Pefabloc SC [4-(2-aminoethyl)-benzenesulfonyl fluoride; Pefabloc] and bicinechonic acid (BCA) assay reagent were from Pierce (Rockford, IL). DEAE-Sepharose CL-6B, and hydroxylapatite were from Pharmacia LKB Biotechnology (Uppsala, Sweden), BioGel A-1.5m was from Bio-Rad (Hercules, CA), whereas liquid scintillation fluid (OptiPhase HiSafe 3) and the Western Lighting™chemiluminescence kit was supplied by PerkinElmer Life Sciences (Boston, Mass., USA). Dr. C. Macphee, SmithKline Beecham, generously provided monoclonal antibodies against the plasma type and the intracellular type II of PAF-AH, conjugated with fluroscein isothiocyanate (FITC)-IgG anti-mouse secondary antibody was purchased from the Dianova Company (Hamburg, Germany). Monoclonal antibodies anti-CD41a labeled with FITC, anti-CD61 labeled with peredinin chlorophyll protein (PerCP) and anti-CD62p labeled with phycoerythrin (PE), were purchased from Becton Dickinson (San Jose, CA, USA). Annexin V-FITC, anti-CD14-FITC, anti-CD45-PE, anti-CD31-PE, and purified anti-CD61 were purchased from PharMingen (Becton Dickinson, San Jose, CA, USA). Restriction enzymes, plasmids, and molecular markers for DNA size (Promega, Charbonnières, France) were used according to the manufacturer's specifications. "RNA plus", phenol were from Bioprobe System (Montreuil-sous-Bois, France). Oligonucleotides were from Sigma-
Genosys. Thermostable DNA polymerase was obtained from Appligene. Superscript reverse transcriptase was from Gibco-BRL (Bethesda, USA). Absolute QPCR SYBR Green was from Abgene (UK). LDL was prepared from freshly isolated plasma by sequential ultracentrifugation as previously described.2

Preparation and stimulation of washed platelets

Washed platelets were prepared from peripheral blood of 12-hour fasting normolipidemic volunteers, as previously described.3 Briefly, the platelet-rich plasma (PRP) was initially prepared.4 Apyrase (1 U/mL) and PGE₁ (0.1 mg/mL), were added to PRP and then centrifuged at 650xg for 10 min. The contaminating erythrocytes were subjected to lysis by resuspending and incubating the precipitated platelet pellet in a solution containing 156 mmol/L NH₄Cl, 97 µmol K₂EDTA, and 10 mmol/L KHCO₃, pH 7.4 for 15 min at 4°C. Platelets were subsequently washed twice in a Tyrode’s buffer pH 7.0, containing 70 mmol/L citric acid, 110 mmol/L sodium citrate, 1 U/mL apyrase, and 0.1 mg/mL PGE₁. Platelets were then re-suspended to a concentration of 1×10⁹/mL in a Tyrode’s buffer, pH 7.4 without citrates, apyrase and PGE₁ but containing 2 mmol/L CaCl₂. Platelets were stimulated in the presence of either thrombin at concentrations ranging from 0.05 to 0.5 IU/mL or ADP at concentrations 2.5 to 10 µmol/L. Platelet activation was performed at 37°C for various time intervals up to 1 h under continuous stirring. It should be noted that in experiments where ADP was used as an agonist; washed platelets were activated in the presence of fibrinogen (200 µg/mL) as previously described.5 In selected experiments platelet aggregation was recorded in a platelet aggregometer (model 560, Chronolog corp. Havertown, PA, USA). In other experiments platelets were activated by the Ca²⁺-ionophore A23187 (10 µmol/L) for various time intervals up to 1 h at 37°C under non-stirring conditions. In some experiments, platelets were incubated with 30 µmol/L calpeptin dissolved in dimethyl sulfoxide (DMSO) for 30 min or with 0.2 mmol/L RGDS for 1 min at 37°C prior to platelet activation. The DMSO concentration did not exceed 1% (v/v) in the final volume of the platelet suspension and did not affect platelet function. Platelet activation was terminated by the addition of 0.01% (v/v) EDTA and cooling in an ice bath. Samples were then centrifuged at 1,500×g for 10 min at 4°C to sediment aggregated platelets and all supernatants were collected and stored at 4°C for further analyses which were performed within 2 days from preparation. In selected experiments, untreated platelet suspension was centrifuged at 1,500×g for 10 min at 4°C to sediment resting platelets. Cells
were then suspended into a HEPES buffer (4.2 mmol/L HEPES, 137 mmol/L NaCl, 2.6 mmol/L KCl, 2 mmol/L Titriplex III), pH 7.4 supplemented with 0.01% EDTA and disrupted by sonication in a tube surrounded by ice for three periods of 10 sec at 10 sec intervals using a Vibra cell sonicator (Sonic and Materials, Danbury, CT. USA). A portion of the cell lysate containing less than 3% of unbroken platelets was stored at 4°C for the measurement of PAF-AH activity whereas the remaining lysate was centrifuged at 100,000xg for 1 h at 4°C. The supernatant containing the platelet cytosolic fraction was collected and the pellet enriched in platelet organelles and membranes was suspended in a HEPES buffer, pH 7.4 supplemented with 0.01% EDTA by brief sonication. Both fractions were stored at 4°C for the measurement of PAF-AH activity.

**Preparation of platelet-derived microparticles**

PMPs were prepared as described earlier with some modifications.6,7 Washed human platelets were activated with 0.2 IU/mL of thrombin under continuous stirring or with 10 µmol/L of Ca²⁺-ionophore A23187 under non-stirring conditions for 30 min at 37°C. Platelet stimulation was terminated by the addition of 0.01% (v/v) EDTA and cooling in an ice bath. Samples were centrifuged at 1,500×g for 15 min to sediment the remnant platelets. Each supernatant was then overlaid onto 20% sucrose gradient and centrifuged at 3,000×g for 10 min at room temperature.8 The PMPs-rich supernatant was then centrifuged at 100,000×g for 2 h at 4°C. The sedimented PMPs were then re-suspended in 10 mmol/L PBS, pH 7.4. PMPs were quantitated by determination of the total protein concentration measured by the BCA method.

**Analysis of PMPs by flow cytometry**

PMPs were analyzed by flow cytometry using a Becton Dickinson FACScalibur (Becton Dickinson, San Jose, CA, USA). The FACScalibur was used in the standard configuration with a 15 mW, 488 nm air-cooled argon laser, and the standard band-pass filter for FITC fluorescence (530/30 nm). PMPs were stained with anti-CD41a-FITC (against the platelet αIIbβ3 receptor), annexin-V-FITC and anti-CD61-PerCP (recognizes the β3 subunit of the platelet αIIbβ3 receptor). In all flow cytometric analyses, platelets were identified with anti-CD61, which labelled all platelets. Platelets were gated according to staining for the platelet specific antigen, CD61.
The platelet and/or PMPs population evaluated was found to be ≥98% positive for CD61. To differentiate between platelets and PMPs, the lower limit of the platelet gate was set at the left hand border of the forward scatter profile of resting platelets. Analysis was performed on an Apple computer using the Cellquest program (Becton Dickinson).

**PAF-acetylhydrolase assay**

PAF-AH activity was measured by the trichloroacetic acid (TCA) precipitation procedure with some modifications. For the routine assay, 90 µL of platelet supernatants or lysates, or the platelet cytosolic or membrane fraction were used as the source of the enzyme. Ninety µl of PMPs suspension in 10 mmol/L PBS containing 0.01% EDTA were also used as the source of the enzyme. The reaction was initiated by adding 10 µL of the 1 mmol/L [3H-acetyl]PAF solution in FFA-BSA/saline (100 µmol/L final concentration in the reaction mixture) and the PAF-AH assay was performed for 60 min at 37°C. The detection limit of the method is 60 pmol of [3H-acetyl]PAF degraded per hour per 10⁹ platelets. The inter- and intra-assay coefficient of variation of the assay was 7.3% and 5.7%, respectively. In some experiments the effect of 100 or 500 µmol/L PC, 0.5 mmol/L Pefabloc or 30 µmol/L CV-3988 was studied as previously described. Finally the effect of DTNB on PAF-AH activity was studied by incubating each source of PAF-AH with 1 mmol/L DTNB for 30 min at 37°C prior to the addition of [3H-acetyl]PAF.

**PAF-AH semipurification**

PAF-AH in pooled platelet lysates was semipurified by using batch and column DEAE-Sepharose CI-6B steps, a gel-filtration step on a BioGel A-1.5m column, and a hydroxylapatite column step, essentially as previously described. In all purification steps, 10 mmol/L CHAPS was used as a detergent.
Gel electrophoresis and Western blot analysis

Semipurified PAF-AH was analyzed by gradient SDS-PAGE on 5–19% gels. After electrophoresis, samples were transferred to a nitrocellulose membrane by electrotransfer. Immunoblotting was performed using monoclonal antibodies against the intracellular type II and the plasma type of PAF-AH diluted 1:500 (v/v). Protein bands were visualized with a chemiluminescence kit.10

Immunofluorescence Microscopy

The immunofluorescence of platelets was done as previously described with some modifications.11,12 Glass coverslips were coated with fibrinogen (100 µg/mL) and incubated for 2 h at 37°C. The coverslips were subsequently blocked with 1% FFA-BSA in PBS (pH 7.4), for 1.5 h in a humidified staining tray. Washed platelets prepared as described above, were diluted 2.0×10^7/mL and allowed to adhere to the fibrinogen-coated slides for 60 min. Slides were then washed with 10 mmol/L PBS and then fixed with ice-cold methanol (7 min). Slides were then blocked with 1% FFA-BSA for 20 min. Primary antibodies (1:5 v/v dilution for monoclonal anti-CD41a-FITC and 1:50 (v/v) dilution for monoclonal antibodies against both the plasma type and the intracellular type II PAF-AH were incubated for 60 min, in a humidified staining tray. After extensive washing 1:50 (v/v) dilution of the secondary conjugated antibody was incubated for 30 min in a humidified staining tray. Slides were washed and mounted in a fluorescent mounting medium and then the coverslips were inverted onto microscope slides and examined in a Leica TCS-SP scanning confocal microscope (Leica Microsystems AG, Wetzlar, Germany) equipped with an argon/krypton laser and Leica TCS software. Digital recordings were obtained using a Leica TCS NT camera (Leica Microsystems AG). All experiments were carried out at room temperature.

RNA isolation, and first strand cDNA synthesis

PRP was prepared as described above and the pellet containing platelets was placed immediately in an ice bath. Total RNA was isolated with RNA Plus (200 µL per 5×10^8 cells) and its concentration determined by
spectrophotometry at 260 nm. In some experiments, the platelet mRNA was subjected to treatment with DNAse to degrade any genomic DNA that could be present in the platelet mRNA preparations. First strand cDNA synthesis was performed with 5 µg of total RNA in the presence of oligo dT (2 µg) and 500 µU of SuperScript reverse transcriptase.\(^\text{13}\)

**PCR and QPCR**

Detection and quantification of PAF-AH mRNA was performed by RT-PCR in the presence of three specific oligonucleotides, PAFAH1 (ATACAAGTACTGATGGCTGCTGCAAG), PAFAH2 (TCTTGGAACACACTGGCTTATGGGC), PAFAH4 (GACCTGGCATCTCATGG) with PAFAH6R (GGACTGAACCCCTGATTGTAA) and compared to the amplification of the PAF receptor (L1/C1), or β actin as described.\(^\text{13}\) PCR (35 cycles) were performed under conditions previously described.\(^\text{13}\)

Real-time quantitative PCR (QPCR) was performed in MX4000 Stratagene using SYBR green detection kit. In all assays, cDNA was amplified using a standardized program (15 min Taq polymerase activation step; 40 cycles of 30s at 95°C and 1 min at 60°C; a 1 min step at 95°C and dissociation step: 36 cycles of 30s between 60°C-95°C). All Q-PCR determinations were performed in a final volume of 20 µL using primers at final concentrations of 0.33 µmol/L. Two preparations of platelet mRNA were tested, each point were performed in duplicate. Control experiment was performed in parallel using mRNA extracted from macrophages. Two housekeeping genes were used: β actin and RPL13A. Additionally the amplification of 2 leukocyte-specific transcripts: CD11c/ β2 integrin and CD14 was performed to insure the purity of platelet’s preparations. All primers were designed using primer Express 2 and are listed in Table I.

**Immunoprecipitation**

Immunoprecipitation experiments were performed using sepharose-protein A coupled to rabbit polyclonal antiserum raised against the recombinant plasma type of PAF-AH. Isolated PMPs in 10 mmol/L PBS containing 10 mmol/L CHAPS were used as the source of the enzyme. Incubations were performed for 4 h.
The PAF-AH activity was determined in the supernatants after centrifugation at 12,000×g, 15°C for 1 min. Rabbit preimmune serum was used in control experiments.

**Characterization of microparticles in plasma**

Plasma-containing microparticles was prepared as described by Osumi et al.\textsuperscript{14} with some slight modifications. Blood samples from apparently healthy normolipidemic volunteers were drawn into tubes containing acid-citrate dextrose as an anticoagulant (Vacutainer, Becton Dickinson) and centrifuged at 150×g for 10 min to obtain PRP. The resultant PRP was then mixed with 0.1% EDTA/saline, at a ratio of 3:2 (v/v) and then centrifuged at 1,500×g for 20 min to obtain the plasma-containing microparticles. Flow cytometry was then performed, to verify the presence of microparticles and to characterize their type in the sample. Initially, microparticles in plasma were labeled with annexin-V-FITC, which recognizes the population of microparticles that express anionic phospholipids. Furthermore, in an attempt to determine the cellular origin of the microparticles monoclonal antibodies that recognize specific antigens characteristic of various cell-types were used. Samples were stained with anti-CD41a-FITC specific for platelets, anti-CD14-FITC specific for monocytes, anti-CD31-PE specific for platelets and endothelial cells and anti-CD45-PE a general marker for leukocytes.

**Detection of PAF-AH activity on plasma PMPs using a captured ELISA method**

ELISA plates were coated with the monoclonal antibody anti-CD61, which specifically recognizes the β3 subunit of the platelet integrin αIIbβ3. This integrin is also located on PMPs. Thus only microparticles of platelet origin would be captured by the anti-CD61-coated wells of the microtiter plate. Fifty μL of purified anti-CD61 (100 μg/mL in PBS) were added in each well of a 96-well microtiter plate (Costar, maxisorp). In separate wells, 50 μL of either anti-CD45 (100 μg/mL in PBS) or the isotype-matched mouse monoclonal IgG (100 μg/mL in PBS), were added. Coating was performed overnight at 4°C.\textsuperscript{14} The plate was then washed three-times with 50 mmol/L Tris-saline buffer (pH 7.6) to remove any excess antibody. To block the non-reacted surfaces, 300 μL of 1% FFA-BSA in PBS (pH 7.4) were added to each well and incubated for 2.5 h at 37°C.
Wells were subsequently washed three-times with PBS and then 100 µL of either plasma-containing microparticles or PMPs (150 µg of protein/mL) prepared from washed platelets activated with Ca²⁺-ionophore A23187 as described above, were placed into the wells coated with any of the above antibodies and incubated for 2 h at 37°C. The plate was washed three-times with a HEPES buffer supplemented with 0.01% EDTA (PAF-AH assay buffer) and then PAF-AH assay was performed in each well by adding 90 µL of the PAF-AH assay buffer preheated at 37°C, and 10 µL of the 1 mmol/L [³H-acetyl]PAF solution in FFA-BSA/saline (100 µmol/L final concentration in the reaction mixture). The PAF-AH assay was performed for 60 min at 37°C. The reaction was terminated by the addition of 20 µL of cold 100 mg/mL FFA-BSA/saline solution in each well and the plate was immediately placed in an ice bath for 15 min. The total reaction mixture of each well was then transferred into separate Eppendorf tubes containing 80 µL of cold 20% TCA. The procedure for the determination of the enzyme activity was then processed as previously described by our group. The inter- and intra-assay coefficient of variation of the assay was 8.2% and 7.1%, respectively.

**Statistical analysis**

Results are expressed as mean ± SD. Mean values were compared by the Student's t test, with significance defined at a value of P<0.05.

**References**


Table I: All QPCR primers were designed using primer Express 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
<th>Bp</th>
<th>Position</th>
<th>Genbank</th>
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Table II: Enzymatic characteristics of PAF-AH associated with subcellular platelet fractions or secreted by activated platelets

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PAF-AH activity, % of total</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cytosolic fraction</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Ca(^{2+}), 1 mmol/L</td>
<td>97</td>
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<tr>
<td>Ca(^{2+}), 10 mmol/L</td>
<td>93</td>
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<tr>
<td>EDTA, 1 mmol/L</td>
<td>110</td>
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<td>EDTA, 5 mmol/L</td>
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</tr>
<tr>
<td>Pefabloc, 1 mmol/L, 30min, 37°C</td>
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</tr>
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<td>DTNB, 1 mmol/L, 30min, 37°C</td>
<td>10</td>
</tr>
<tr>
<td>PC, 0.1 mmol/L</td>
<td>98</td>
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<td>PC, 0.5 mmol/L</td>
<td>94</td>
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</tbody>
</table>

Values represent the mean from 3 different platelet preparations. PAF-AH activity was determined with the TCA precipitation procedure and control. DTNB was dissolved in DMSO whereas PC was dissolved in ethanol. The DMSO concentration in the reaction mixture was 1% (v/v) whereas the ethanol concentration was 0.5% (v/v).
Legends to the supplemental Figures

**Supplemental Figure I.** A) Dose-dependent secretion of PAF-AH from platelets stimulated with thrombin. B) Time-dependent secretion of PAF-AH from platelets activated with 0.2 IU/mL of thrombin expressed as the % total enzyme activity found in the platelet lysate. PAF-AH activity was determined by the TCA precipitation procedure using 90 µL of platelet sample as the source of the enzyme. Values represent the mean ± SD from 10 different platelet preparations. *P<0.03 compared to the PAF-AH secretion at 15 min.

**Supplemental Figure II.** Representative immunofluorescence microscopy image of PAF-AH in washed platelets: Adherent platelets to fibrinogen-coated coverslips were stained with monoclonal antibodies against A) Type II PAF-AH, B) plasma PAF-AH, C) Mouse IgG, D) CD41a-FITC. Experiments were performed in triplicate.

**Supplemental Figure III.** A) Flow cytometric profile of isolated PMPs from the supernatant of platelets activated with Ca²⁺-ionophore A23187. The shaded region represents the IgG control antibody and the open region represents the positive staining with respect to annexin-V and CD41a respectively. Data is representative from 10 different platelet preparations. B) The effect of 30 µM CV-3988 or 1 mM DTNB on the PAF-AH specific activity expressed by PMPs. Data represents the mean ± SD from three different platelet preparations. *P<0.02 compared to the control sample. C) Immunoprecipitation of PAF-AH associated with PMPs with a rabbit polyclonal antiserum raised against the plasma type of PAF-AH. Data represents the mean ± SD from three different platelet preparations.

**Supplemental Figure IV.** Representative dot plots showing the flow cytometric profile of the gated annexin-V positive particles (A). Non-specific binding was assessed using FITC- and PE-labeled isotype-matched mouse monoclonal IgG antibodies (B). The upper right quadrant in the dot plot represents positive expression for both antigens; for PMPs (C) and for microparticles of leukocyte origin (D). Values represent the % total expression in regards to the entire population being studied. Microparticles in plasma from ten normolipidemic apparently healthy volunteers were evaluated.
Supplemental Figure I

A) [Graph showing the relationship between Thrombin (IU/mL) and PAF-AH activity (pmol/10^6 cells/h).]

B) [Bar graph showing the secretion of PAF-AH% of total over time (min).]
Supplemental Figure II

A) 

B) 

C) 

D)
Supplemental Figure III

A)

B)

C)

PAF-AH specific activity (nmol/mg protein/h)

Control  DTNB  CV-3988

PAF-AH activity (nmol/h)

Control  Preimmune serum  Antiserum