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Human platelets stimulated by thrombin produce platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) when the degrading enzyme acetyl hydrolase is blocked

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It has been shown [Touqui, Jacquemin & Vargaftig (1983) *Thromb. Haemostasis* **50**, 163; Touqui, Jacquemin & Vargaftig (1983) *Biochem. Biophys. Res. Commun.* **110**, 890–893; Alam, Smith & Melvin (1983) *Lipids* **18**, 534–538; Pieroni & Hanahan (1983) *Arch. Biochem. Biophys.* **224**, 485–493] that rabbit platelets inactivate exogenous PAF (platelet-activating factor, PAF-acether) by a deacetylation–reacylation mechanism. The deacetylation step is catalysed by an acetyl hydrolase sensitive to the serine-hydrolase inhibitor PMSF (phenylmethanesulphonyl fluoride) [Touqui, Jacquemin, Dumarey & Vargaftig (1985) *Biochim. Biophys. Acta* **833**, 111–118]. We report here that human platelets can produce PAF on thrombin stimulation. This production is marginal and transient, reaching a maximum at 10 min and decreasing thereafter. In contrast, 10–12 times more PAF is produced when platelets are treated with PMSF and stimulated with thrombin. Under these conditions, the maximum formation is observed at 30 min and no decline occurs for up to 60 min after stimulation. In addition, these platelets (treated with PMSF and stimulated with thrombin) incorporate exogenous labelled acetate in the 2-position of PAF, probably by an acetyltransferase-dependent mechanism. Production of PAF by human platelets during physiological stimulation can be demonstrated when PAF degradation is suppressed by the acetyl-hydrolase inhibitor PMSF.

Platelet-activating factor (PAF; Benveniste *et al.*, 1977; Demopoulos *et al.*, 1979) is a phospholipid mediator of anaphylaxis and inflammation that induces platelet aggregation and secretion (Chignard *et al.*, 1979; Cazenave *et al.*, 1979). It is formed by various cell types (Benveniste *et al.*, 1979; Vargaftig *et al.*, 1979), including platelets, during stimulation by agents such as thrombin, collagen and the ionophore A23187 (Chignard *et al.*, 1980), by a Ca^{2+}-dependent deacylation–reacetylation reaction involving a membrane-bound precursor, alkylacyl-GPC (1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine) (Touqui *et al.*, 1985). The initial step in PAF biosynthesis is the activation of a phospholipase A_2 (Benveniste *et al.*, 1982; Albert & Snyder, 1983; Touqui *et al.*, 1985), leading to the biologically inactive intermediate lyso-PAF (alkyl-lysoglycerophosphocholine). Subsequent acetylation of lyso-PAF by an acetyltransferase generates PAF. The activity of the latter enzyme appears to be the rate-limiting step in the PAF biosynthesis (Wykle *et al.*, 1980; Chap *et al.*, 1981; Alonso *et al.*, 1982; Ninio *et al.*, 1982; Albert & Snyder, 1983).

Exogenous PAF can be converted by platelets into its precursor alkylacyl-GPC through the combined activities of an acetyl hydrolase and an acyltransferase (Touqui *et al.*, 1983*a*,*b*, 1985; Alam *et al.*, 1983; Pieroni & Hanahan, 1983). In contrast with its biosynthesis, the conversion of PAF into alkylacyl-GPC is independent of platelet activation and of extracellular Ca^{2+} (Touqui *et al.*, 1985).

Abbreviations used: PAF, platelet-activating factor (PAF-acether, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); alkylacyl-GPC, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine; lyso-PAF, alkyl-lysoglycerophosphocholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TTBSA, Tris Tyrode's buffer (Mustard *et al.*, 1972) containing 0.25% bovine serum albumin; DMSO, dimethyl sulphoxide; CP/CPK, creatine phosphate (phosphocreatine)/creatine phosphokinase.

The production of PAF by human platelets stimulated with thrombin has not been demonstrated conclusively. In the one successful demonstration (Chignard *et al.*, 1983), only marginal amounts of PAF were detected. We hypothesized that rapid inactivation and conversion of PAF into the corresponding alkylacyl-GPC may explain the inability to demonstrate the formation of PAF by human platelets during physiological stimulation.

In the present study we show that human platelets preincubated with the acetyl-hydrolase inhibitor PMSF (Touqui *et al.*, 1985) can produce large amounts of PAF after stimulation by thrombin. Under these conditions, the formation of PAF could be detected with thrombin concentrations as low as 0.1 unit/ml. Moreover, PAF formed by platelets stimulated in the presence of [^{3}H]acetate incorporated the latter, indicating the involvement of an acetyl transferase with PAF biosynthesis.

Experimental

Materials

Fat-free bovine serum albumin, PMSF, PC, PE, phosphocreatine, creatine phosphokinase, ADP and arachidonic acid were from Sigma (St. Louis, MO, USA). Bovine thrombin was from Hoffmann–LaRoche (Basel, Switzerland). [^{3}H]PAF [1′,2′-^{3}H]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, 120Ci/mmol) and ACS II were from Amersham International. Sodium [^{3}H]acetate (2.0Ci/mmol) was from New England Nuclear Corp. Synthetic PAF and lyso-PAF were prepared in the laboratory of Professor J. J. Godfroid (Université Paris VII, Paris, France). The phospholipase A_2 subunit of crotoxin from rattlesnake (*Crotalus durissus terrificus*) venom was purified in our laboratory by Dr. F. Radvanyi and Dr. C. Bon as described by Hendon & Fraenkel-Conrat (1971). Plastic silica-gel plates for t.l.c. were from Merck. The column used for h.p.l.c. was a Lichrosorb Si 60 from Spectra-Physics, Paris, France. All solvents used were of h.p.l.c. grade from Fisons, Loughborough, Leicestershire, U.K.

Methods

Preparation and stimulation of platelets. Whole blood (9vol.) from normal adult volunteers, who took no medication for at least 10 days before venipuncture, was collected and anticoagulated with 0.13M-trisodium citrate (1vol.). Platelet-rich plasma was produced by centrifugation (20min at 200*g*) and platelets were washed by the method of Mustard *et al.* (1972), giving a final platelet suspension of 5×10^8/ml in TTBSA. Portions of washed platelets (5ml) were incubated with PMSF (2mM) or DMSO (vehicle) (0.25%) in controls for 15min. Platelets were then centrifuged (15min at 1400*g*) and resuspended in fresh TTBSA to remove PMSF. Thereafter, 1ml portions were stirred and challenged with thrombin (2.5 units/ml final concn.) for the periods indicated in Fig. 1 (below) or with various concentrations of thrombin (0.1, 0.25, 0.5, 1 or 2.5 units/ml final concn.) for 30min. At the appropriate times, reactions were terminated by the addition of 3ml of chloroform/methanol (1:2, v/v) at 4°C.

Extraction and bioassay of PAF. Lipids were extracted by the method of Bligh & Dyer (1959) and stored at −20°C. The organic phase was removed and evaporated to dryness under O_2-free N_2. Lipid extracts were redissolved in 250μl of water containing bovine serum albumin (0.25%) and kept at 4°C. PAF was detected by using washed rabbit platelets as a bioassay (Chignard *et al.*, 1980). In the presence of aspirin [2-(acetyloxy)-benzoic acid; 0.1mM] and CP/CPK (0.7mM/13.9 units/ml) these platelets failed to aggregate in response to arachidonic acid (0.5mM) or to ADP (100μM). The amounts of PAF produced by human platelets were determined by comparing the extent of aggregation induced by the unknown with that induced by known amounts of synthetic PAF.

H.p.l.c. analysis of lipid extracts. Lipid extracts were pooled, redissolved in 20μl of chloroform/methanol (2:1, v/v) and resolved by h.p.l.c. on a

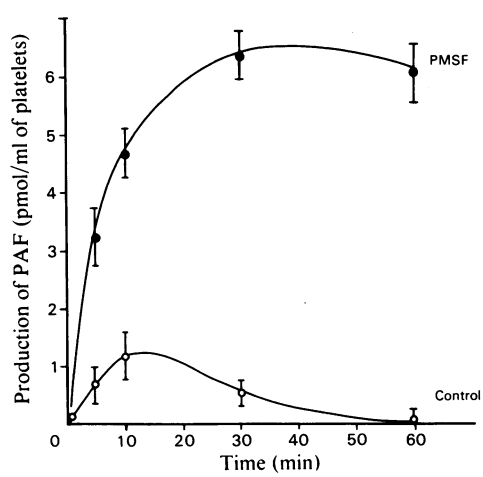

Fig. 1. *Enhanced production of PAF by PMSF-treated human platelets on thrombin stimulation*

Platelets were incubated for 15min at 37°C with PMSF (2mM final concn., ●) or its solvent DMSO (control, ○) and washed. Portions (1ml) were collected and stimulated with thrombin (2.5 units/ml) while being stirred. The reactions were stopped, at the intervals indicated, by transferring the suspensions into 3ml of chloroform/methanol (1:2, v/v). PAF production was measured as indicated under 'Methods'. Values are means±S.D. (three experiments).

Lichrosorb Si 60 column under isocratic conditions. The mobile phase consisted of dichloromethane/methanol/water (12:10:1, by vol.) at a flow rate of 0.5ml/min. The retention time of PAF was determined by co-eluting extracts with synthetic [^{3}H]PAF. Eluates were evaporated to dryness and redissolved in 250μl of bovine serum albumin solution (0.25%). Liquid-scintillation counting and the bioassay were employed to determine the radioactivity and biological activity present in each eluate.

Incorporation of [^{3}H]acetate into PAF. Portions of platelets suspensions (1 ml), treated with PMSF or DMSO (in controls), were incubated with [^{3}H]acetate (50μCi/ml; 2.0Ci/mmol), stirred and challenged with thrombin (2.5 units/ml) for 30min. The reactions were terminated and lipids extracted as described above. To remove free [^{3}H]acetate the organic phase was washed twice with 2vol. of methanol/water (1:1, v/v) before evaporating it to dryness. The extracts were redissolved in 50μl of chloroform/methanol and the lipids separated by t.l.c. with chloroform/methanol/NH_3 (70:35:7, by vol.) as the solvent system. Chromatograms were divided into 17 bands and the radioactivity associated with each band was determined by liquid-scintillation counting. The radioactive fractions which co-migrated with authentic PAF were then analysed by h.p.l.c. To determine whether [^{3}H]acetate was incorporated into the *sn*-2 position of PAF, the following procedure was adopted. Samples (250μl) of PAF, purified by h.p.l.c., were incubated for 60min with snake-venom phospholipase A_2 (10μg/ml final concn.) (see under 'Materials'). In controls, either synthetic [^{3}H]PAF or [^{3}H]acetic acid were substituted for the extracted lipids. After the addition of 3vol of chloroform/methanol to terminate the reactions, [^{3}H]acetic acid was recovered almost entirely (98%) from the aqueous phase, whereas [^{3}H]lyso-PAF (90%) was recovered from the organic phase.

Metabolism of PAF by platelets and their cytosolic fractions

PMSF-treated or control platelets were washed and resuspended in nominally Ca^{2+}-free TTBSA. Cytosolic fractions were prepared by homogenizing 1ml (5×10^8 cells) by the method described by Barber & Jamieson (1970). Cytosolic and particulate fractions were separated by centrifugation (60min at 105000*g*, 4°C). Either 1ml of intact platelets, total homogenates or their equivalent cytosolic fractions were incubated with synthetic [^{3}H]PAF at 37°C. The reactions were terminated with chloroform/methanol as described above, at the time points indicated in Table 2, and lipid extracts were analysed as described by Touqui *et al.* (1985).

Results

Control platelets stimulated with thrombin synthesized marginal amounts of PAF only. In contrast, this formation was markedly increased when platelets were pretreated with PMSF, washed and then stimulated with thrombin (Fig. 1). Washing platelets to remove PMSF from the medium was required to obtain platelets sensitive to thrombin, since omission of the washing procedure, i.e. stimulation in the presence of PMSF, resulted in no aggregation (results not shown). DMSO alone, at the 0.25% final concentration used as the vehicle for PMSF, had only a marginal effect on the production of PAF (Table 1). The PMSF-treated platelets, when compared with the control (DMSO-treated) platelets, formed about 12 times more PAF within 30min stimulation with thrombin (Table 1). The amounts of PAF formed by the PMSF-treated thrombin-stimulated platelets remained practically unchanged for up to 60min. In contrast, PAF was only detected transiently in control thrombin-stimulated plate-

Table 1. *Effect of the pre-exposure of platelets to PMSF on PAF production*

Platelets treated with PMSF, with its solvent DMSO, or with saline (0.9% NaCl), were washed and resuspended in fresh TTBSA. Then 1 ml portions were challenged, while being stirred, with thrombin (2.5 units/ml final concn.) or with saline and the reactions were stopped at the indicated times by the addition of 3ml of chloroform/methanol (1:2, v/v). The lipids were extracted and PAF production was measured as described under 'Methods'. Values are means ± S.D. (three expts.). Abbreviation used: N.D., not detected.

Drug or vehicle	Agonist	PAF production (pmol/ml of platelets) after: 10min	30min
Saline	Saline	N.D.	N.D.
	Thrombin	1.11 ± 0.13	0.19 ± 0.19
PMSF	Saline	N.D.	N.D.
	Thrombin	4.74 ± 0.96	6.57 ± 0.47
Solvent (DMSO)	Thrombin	1.37 ± 0.78	0.51 ± 0.11

lets (Fig. 1). The increased formation of PAF did not result from an intrinsic effect of PMSF, since when the latter was used in the absence of thrombin, no such synthesis was seen (Table 1).

The identity of PAF formed by human platelets was checked by its h.p.l.c. and pharmacological properties. The fractions collected from h.p.l.c. that were active in inducing aggregation had a retention time similar to that of synthetic [^{3}H]PAF (22min). The aggregating activity was insensitive to aspirin and CP/CPK, which inhibit respectively arachidonate- and ADP-induced aggregation (Chignard *et al.*, 1979). It was lost when the extracts were pre-incubated with snake-venom phospholipase A_2 (results not shown).

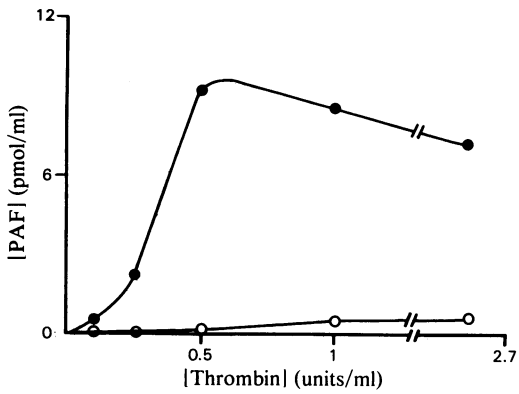

Fig. 2. *Dose-dependent production of PAF by thrombin-stimulated platelets pre-exposed to PMSF*
Platelets treated with PMSF (●) were stimulated at 37°C with thrombin at a concentration of 0.1, 0.25, 0.5, 1 or 2.5 units/ml. After 30min the reactions were stopped and the amounts of PAF formed by platelets were determined as indicated under 'Methods' (results are representative of two experiments).

The formation of PAF by thrombin-stimulated human platelets was dose-dependent (Fig. 2). It was detected down to a concentration of 0.1 unit/ml thrombin applied to the PMSF-treated platelets and was maximal at 0.5 unit/ml. In contrast, control platelets required more than 1 unit of thrombin/ml to produce detectable amounts of PAF (0.45pmol/ml of platelets). When thrombin was replaced by ionophore A23187, a similar increase in the PAF production by PMSF-treated platelets was observed (results not shown).

These results suggested that the increased production of PAF results from the inhibition, by PMSF, of PAF deacetylation, leading to its accumulation. To test this possibility, intact platelets, their homogenate or the cytosolic fraction, were incubated with radioactive PAF. As shown in Table 2, the intact platelets failed to metabolize PAF and the cytosolic fraction converted it into lyso-PAF. Finally, the platelet homogenate transformed PAF into alkylacyl-GPC. Formation of lyso-PAF or of alkylacyl-GPC by the cytosolic fraction and the homogenate respectively were inhibited when the platelets were

Table 2. *Inhibition by PMSF of the PAF conversion by platelet homogenates and their cytosolic fractions*
A 5ml portion of platelets was incubated as detailed in the text with 2mM-PMSF or with its solvent, DMSO. After one centrifugation (15min at 1400*g*), platelets were homogenized as described by Barber & Jamieson (1970). Then, [^{3}H]PAF (10^4c.p.m./ml final concn.; 120Ci/mmol) was incubated with 1ml of intact platelets, 1ml of total homogenates or 1ml of their cytosolic fractions (105000*g*, 60min supernatant). The reactions were stopped 10 or 30min later by the addition of 3ml of chloroform/methanol (1:2, v/v) and the lipid extracts were subjected to t.l.c., with chloroform/methanol/NH_3 (70:35:7, by vol.) as solvent system, and analysed as described by Touqui *et al.* (1985). The values represent the percentage of each indicated component as compared to the total plate radioactivity (two separate expts.). Abbreviation used: N.D., not detected.

	Percentage conversion of [^{3}H]PAF into:			
	[^{3}H]Lyso-PAF		[^{3}H]Alkylacyl-GPC	
Time ...	10min	30min	10min	30min
Intact platelets				
Control	N.D.	N.D.	5	10
PMSF	N.D.	N.D.	2	2
Total homogenates				
Control	5	5	20	60
PMSF	2	2	5	10
Cytosolic fractions				
Control	40	60	5	10
PMSF	10	10	2	5

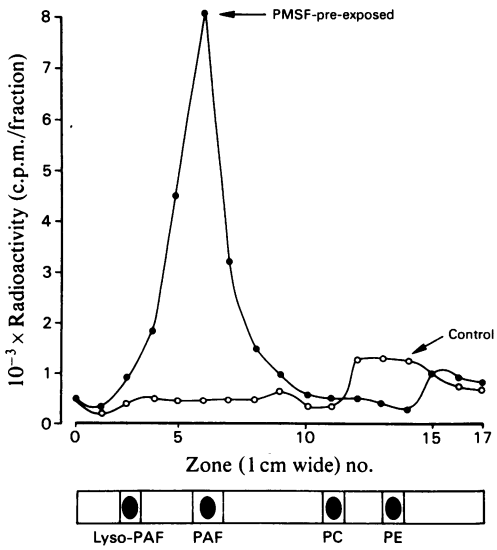

Fig. 3. *Incorporation of radioactive acetate into PAF by platelets pre-exposed to PMSF and stimulated with thrombin*

A 1 ml portion of PMSF (●)- or DMSO (○)-treated platelets was challenged for 30 min at 37°C with thrombin (2.5 units/ml) in the presence of [^{3}H]acetic acid (50 μCi/ml final concn.; 2.0 Ci/mmol). The reactions were stopped by the addition of 3 ml of chloroform/methanol (1:2, v/v) and the chloroform phases containing lipids were washed twice with methanol/water (1:1, v/v) to remove excess free acetate. Portions of the lipid extracts were subjected to t.l.c. on silica-gel plates (Merck), with chloroform/methanol/NH_3 (70:35:7, by vol.) as a solvent, and radioactivity was measured by conventional scintillation counting. The pooled fractions were purified by h.p.l.c. and incubated for 60 min with rattlesnake (*Crotalus durissus terrificus*) venom phospholipase A_2 (10 μg/ml final concn.). After phase partition by the method of Bligh & Dyer (1959), over 80% of the radioactivity was found in the aqueous upper layer, indicating that the acetate group was cleaved from the *sn*-2 position of PAF.

incubated with PMSF and washed before cell fractionation (Table 2).

Finally, pre-exposure of platelets to PMSF, followed by their stimulation with thrombin in the presence of radioactive acetate, resulted in a marked incorporation of the label into PAF. No significant incorporation of radioactive acetate was observed in PMSF-untreated platelets after 30 min stimulation with thrombin (Fig. 3). The incubation of the acetate-labelled PAF derived from platelets with purified phospholipase A_2 showed that acetate was linked in the 2-position of PAF, since this enzyme is known to cleave phospholipids specifically in the *sn*-2 position.

Discussion

We (Touqui *et al.*, 1985) and others (Pieroni & Hanahan, 1983; Alam *et al.*, 1983) have shown that the metabolism of PAF by platelets is initiated by its deacetylation, catalysed by a specific acetyl hydrolase as shown by Blank *et al.* (1981*a*,*b*) in other cells, and leading to the biologically inactive intermediate lyso-PAF. This deacetylation may represent a detoxification of the highly active PAF.

In the present studies we demonstrate that human platelets, when stimulated with the physiological agonist thrombin, form PAF and that this formation is greatly increased (10–12 times) by PMSF. Indeed, in the presence of PMSF, which alone does not enhance the formation of this lipid, human platelets can synthetize PAF at a thrombin concentration as low as 0.1 unit/ml. We have shown previously that PMSF inhibits the deacetylation of PAF, an event mediated by a cytosolic acetyl hydrolase (Kramer *et al.*, 1984). Hence the enhanced formation of PAF, in the presence of PMSF, would appear to be due to inhibition of the enzyme involved in its degradation. Under our conditions, intact human platelets, in contrast with rabbit platelets (Touqui *et al.*, 1983*a*), were not able to convert exogenous PAF (Table 2). This is probably due to the low rate of PAF uptake by human platelets in comparison with that by rabbit platelets (Hwang *et al.* 1983; Inārrea *et al.*, 1984).

In previously reported studies (Wykle *et al.*, 1980; Chap *et al.*, 1981; Alonso *et al.*, 1982; Ninio *et al.*, 1982; Albert & Snyder, 1983) the evaluation of the acetyltransferase activities and/or PAF synthesis were certainly underestimated because of the presence of an acetyl hydrolase, which inactivates PAF.

Our studies also show that human platelets incorporate radioactive acetate into PAF by a stimulus-dependent mechanism. This incorporation is markedly increased when platelets are first exposed to the acetyl-hydrolase inhibitor PMSF. These findings support the concept that an acetylation process is involved in the biosynthesis of PAF by human platelets and that acetylation may be better observed when deacetylation of PAF is blocked by PMSF.

Overall, the present study demonstrates that an acetyl-hydrolase activity plays an important role in regulating the concentration of PAF produced by these cells. PMSF, by inhibiting the degradation of this lipid, may provide a useful technique for detecting small or transient formations of PAF.

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