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Interaction between blood components and a spin-labeled analogue of PAF-acether

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The interactions between a spin-labeled analogue of PAF-acether (designated as (0,2)PAF) and different human blood components (platelets, erythrocytes, and serum) have been studied. The rate of spin probe reduction by cytosol provided information about the internalization processes when the hydrolysis rate was also available. Although erythrocyte reactivity is lower than that of platelets, erythrocytes, because of their greater numbers, removed (0,2)PAF from whole blood faster than platelets. Lastly, erythrocytes may be more efficient traps for (0,2)PAF than serum acetylhydrolase. Criteria for extending these results to genuine PAF-acether are also discussed.

Introduction

An ether-linked phospholipid, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine or PAF-acether, has been shown to produce many pronounded physiological effects [1]. Platelet aggregation [2], hypotension [3], inflammation, anaphylaxis [4] and allergic responses [5] have been elicited by very low concentrations of PAF-acether. Its mechanism of action is not known, but probably involves Ca^{2+} transport and/or phosphatidylinositol hydrolysis [6].

The metabolism of PAF-acether, however, is now well known [7]. Its biosynthesis involves acetylation of 2-lyso-PAF-acether by acetyl-CoA, catalyzed by an acetyl transferase [8]. The lyso derivative comes from 1-alkyl-2-acyl-PC and PAF-acether via phospholipase A_2 and acetyl hydrolase, respectively [7,9]. These reactions occur at different sites, either inside the cells or on the plasma membrane. Hence, the rate of internalization of PAF-acether in different cell types is relevant to the mechanism of action and to the metabolism of this product. As noted elsewhere [10], although the plasma membranes of platelets appear to contain high-affinity binding sites [11,12] it is very difficult to distinguish between the trapping of PAF-acether inside cells and specific binding to a receptor on intact cells, unless there is good evidence against a fast penetration of the product into the cell. The results given in this paper indicate the fate of a paramagnetic analogue of PAF-acether (which we call (0,2)PAF) in the presence of different cell types. This provides a means of easily measuring the rate of internalization and hydrolysis in the presence of platelets, erythrocytes, and whole blood.

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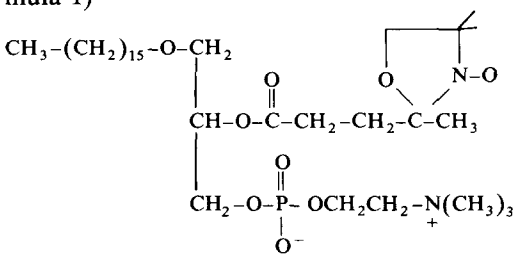
Abbreviations: PAF-acether, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; (0,2)PAF, 1-*O*-hexadecyl-2-*O*-(4-doxylpentanoyl)-*sn*-glycero-3-phosphocholine; (0,2)A, 4-doxylpentanoic acid; PC, phosphatidylcholine; CMC, critical micellar concentration; ESR, electron spin resonance; NMR, nuclear magnetic resonance; PMSF, phenylmethylsulfonyl fluoride; TLC, thin-layer chromatography.

Materials and Methods

Synthesis. The paramagnetic analogue of PAF-acether, (0,2)PAF is identical to PAF-acether, except that there is an ester bound to a 4-doxylpentanoic acid ((0,2)A) residue at position 2 (Formula 1)



Formula 1. (0,2)PAF.

Spin-labeled carboxylic acid ((0,2)A) was synthesized and purified as previously described [13]. (0,2)PAF was synthesized according to a previous method [14] from 4-doxylpentanoic anhydride and 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (2-lyso-PAF-acether, a gift from J.J. Godfroid's laboratory, which is gratefully acknowledged). It was purified by preparative TLC using $CHCl_3$/MeOH/H_2O (65 : 35 : 6, v/v) as the solvent system. The R_F of the spin-labeled analog of PAF-acether is identical to that of the genuine sample of PAF-acether (a gift from J.J. Godfroid's laboratory). The (0,2)PAF zone was immediately isolated and extracted with $CHCl_3$/MeOH (50 : 50, v/v) through a sintered glass filter. The product was stored at −20 °C without solvent. The purity of the (0,2)PAF sample was checked by TLC before each experiment. It contained one nitroxide radical, as measured by ESR (see below) for every phosphorus atom (according to the method of Bartlett [15]). ^1H- and ^{31}P-NMR spectroscopy of our sample showed that (0.2)PAF was not contaminated by the positional isomer, 1-*O*-hexadecyl-3-*O*-(4-doxylpentanoyl)-*sn*-glycero-2-phosphocholine. (0,2)PAF was added to cells in an ethanol solution giving a final ethanol concentration of 1% (v/v) which did not provoke any cell activation.

ESR spectroscopy. ESR spectra were recorded on a Brucker ER 200D apparatus interfaced with an Apple II + (48K) microcomputer. Free radical concentrations were determined by comparison with a standard after subtracting the base-line and double-integrating the signal. All experiments were done at 37 °C. Measurements of (0,2)PAF hydrolysis were performed directly by ESR spectroscopy. It is well known that since the hydrolysis product (0,2)A, is entirely dissolved in water phase, it exhibits much thinner lines than (0,2)PAF itself, both in membrane and in serum albumin. It is thus possible to quantify the amount of hydrolysis by electronic subtraction and double-integrating the ESR signals.

Biological preparations. Cells were prepared at room temperature from fresh human blood anticoagulated with a 0.15 volume of ACD (85 mM trisodium citrate/111 mM dextrose/71 mM citric acid). Blood donors had not received any medication in the 3 weeks preceding donation. Blood was centrifuged at $100 \times g$ for 15 min. The platelet-rich plasma (PRP) was carefully removed to avoid any contamination from the buffy-coat and red blood cells, and then centrifuged for 20 min at $900 \times g$. The platelet pellet was suspended in a modified tyrode-Hepes buffer (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 mM $MgCl_2$, 0.55 mM dextrose, 5 mM Hepes and a 0.15 volume of ACD at pH 6.4) and again centrifuged at $900 \times g$ for 20 min. The platelets were washed twice in the same buffer and, the concentration was adjusted to $2 \cdot 10^9$ platelets/ml.

The red blood cells were washed three times by centrifugation at $1000 \times g$ for 10 min with an isotonic solution (150 mM NaCl at pH 7) and the concentration was adjusted to 10^9 erythrocytes/ml.

The aggregation tests were performed by Dr. Benveniste's laboratory, which we gratefully acknowledge.

Results

1. Spin probes were reduced by the internal content of the cells

Nitroxide radicals react with the internal contents of cells to produce a non-paramagnetic residue, invisible on the ESR spectra, with which the internalization rate of any nitroxide-bearing molecule can be determined [16]. The spin probe interactions with the cytosol are thus expressed by a decrease in ESR signals. Before all experiments were cells, it was important to know if (0,2)A and

(0,2)PAF would react similarly with a water-soluble reactant such as sodium ascorbate. In other words, we had to check if (0,2)PAF would react with ascorbate in the membrane or in the water medium, as is the case with (0,2)A, which is a water-soluble product. Fig. 1A shows that (0,2)PAF was quickly reduced by ascorbate in a model membrane. It was not possible to use (0,2)PAF in water solution because the concentrations needed for ESR spectroscopy were higher than the CMC of this compound [14]. The reduction reaction of the spin probe did not occur with (0,2)PAF in the water phase since the reaction rate did not depend on the lipid concentration (Fig. 1A.).

No reducing power was found in the external medium of washed platelets or erythrocytes. By contrast, the internal content of disrupted cells reacted with nitroxide radicals (Fig. 1B). In addition, there was a good correspondence between the internalization rate of (0,2)A in platelets and the reduction rate of ESR signals (Fig. 1B). The internalization of the probe was thus the determining step in the overall process. As expected, the reduction was faster with intact cells than with disrupted ones, since the dilution factor was about [20].

We were unable to clarify the mechanism of spin reduction by the internal contents of cells. It has generally been supposed that the thiol groups of reduced glutathione are the major reducing entity in cells [16]. The internal concentration of glutathione in cells should be on the order of magnitude of several mM. However, Fig. 1C shows that either with or without added Fe^{2+} ions, this concentration did not suffice to reduce the doxyl radical quickly. Another approach consists of inhibiting the reducing power of glutathione by treating the cells with a reagent of the thiol groups such as *N*-ethylmaleimide and studying their reaction in the presence of free radicals. Fig. 1C shows that a preincubation of platelets ($2 \cdot 10^9$ cells/ml) with *N*-ethylmaleimide (0.2 mM) for 15 min failed to suppress the reduction.

On the basis of these experiments, we can conclude that the thiol groups are not solely responsible for the reduction of free radicals in the cells. Nevertheless, it should be emphasized that *N*-ethylmaleimide activates the platelets, as seen by changes in their shape and by the reducing power observed in the extracellular medium. The same phenomena were observed when the platelets ($2 \cdot 10^9$cells/ml) were treated with thrombin (1 U/ml) a well-known agonist of these cells, which provokes the activation, change in shape and secretion of granular constituents, under the experimental conditions used (absence of Ca^{2+}; data not shown). Because of this, we were unable to determine whether the reduction of free radicals observed when the platelets were treated with *N*-ethylmaleimide was related to cell activation or reflects the existence of reducing agents other than the thiol groups. The only point that can be clarified is that the reducing power was in the soluble internal contents of the cells, since membranes obtained by centrifugation did not reduce the amplitude of the ESR signals of either (0,2)A or (0,2)PAF. Thus, the decrease in the ESR signal was closely correlated with the internalization of the spin probe and its interactions with the cytosol of the cells.

2. Interaction of (0,2)PAF with human blood components

2a. Platelets. Since platelets are the main target cells for PAF-acether, they were studied first. As already described [11,12] PAF-acether binds at two distinct types of sites on blood platelets. The first is a saturable specific binding site. In addition, there is a close correlation between the abilities of PAF-acether analogues to bind at the specific site and their abilities to cause the platelet aggregation. The other category of sites consists of the so-called nonspecific binding sites. They are related to the hydrophobic nature of PAF-acether, making it a component that easily penetrates biological membrane. The specific binding site was found only in PAF-acether-responsive cells, unlike the nonspecific site [11]. At low temperature and a PAF-acether concetration of 1 nM, at least 30–40% of the binding appeared to be specific. Above 1 nM PAF-acether, total binding increased progressively, which was entirely due to nonspecific binding, suggesting the uptake of PAF-acether in the platelets. At 37°C, PAF-acether uptake was several times greater than receptor binding, making receptor studies at this temperature quite difficult [12]. (0,2)PAF did not provoke any platelet aggregation

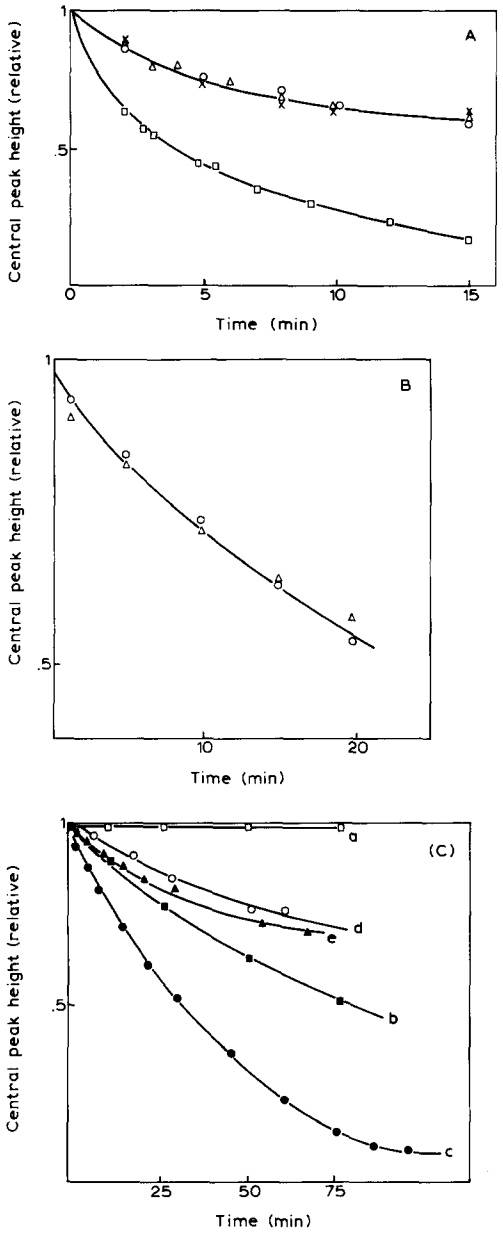


Fig. 1. (A) Rate of (0,2)PAF (10 μM) reduction by sodium ascorbate (1 mM) at 4°C. Experiments were performed in the presence of 1 mM (×), 5 mM (○), or 10 mM (△) sonicated egg lecithin (pH 7.4). Reduction of 10 μM (0,2)A by the same ascorbate concentration is shown for comparison (□).
(B) Comparison of the internalization and reduction of (0,2)A by platelets. $1.4 \cdot 10^9$ platelets/ml were incubated with 36 μM (0,2)A. The amplitudes of ESR spectra are shown for the whole medium (△), the supernatant after centrifugation at $10000 \times g$ for 5 min (○) and sonicated medium (×) (less than 5% living cells remaining as determined by counting).
(C) Rate of (0,2)A (10 μM) reduction by different media.

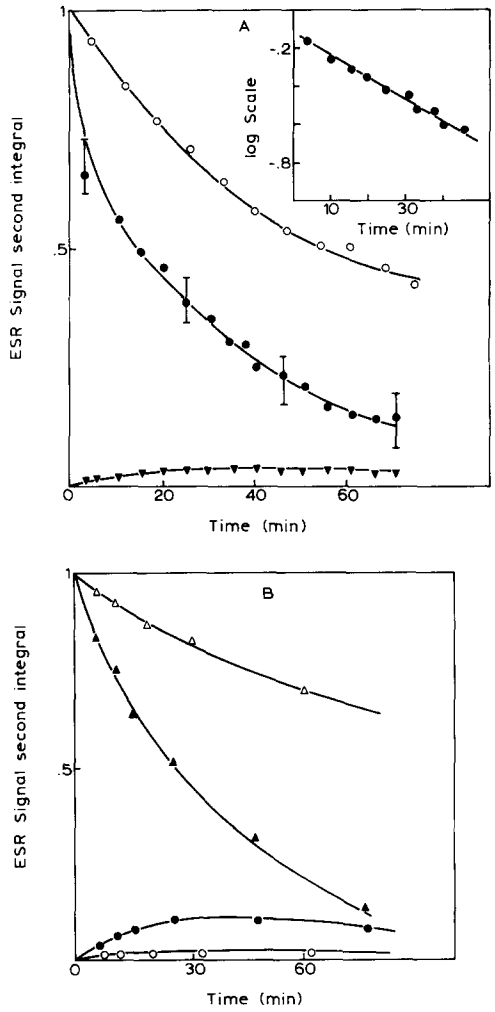


Fig. 2. (A) Interaction of (0,2)PAF (20 μM) with platelets ($2 \cdot 10^9$ cells/ml) (●) and erythrocytes (10^9 cells/ml) (○). Bars represent the maximum (interindividual) deviation in three experiments. Hydrolysis of the ester bond at position 2 in the presence of platelets, as measured by the (0,2)A concentration (▼) in water phase (see Materials and Methods). Insert: logarithmic conversion of the reduction rate by platelets showing first-order kinetics.
(B) Effect of preincubating (15 min at 37°C) platelets ($2 \cdot 10^9$ cells/ml) with PMSF (1 mM) on the reduction and hydrolysis rates of (0,2)PAF. Reduction: (▲) without PMSF (control); (△) with PMSF. Hydrolysis: (●) without PMSF; (○) with PMSF.

Curves a and b: 1 mM glutathione without Fe^{2+} (□) or in the presence of 500 μM Fe^{2+} (■) at 37°C. Curves c and d: platelets ($2 \cdot 10^9$ cells/ml) pretreated (○) or not (●) with 0.2 mM *N*-ethylmaleimide. Curve e: the extracellular medium obtained by centrifugation of $2 \cdot 10^9$ platelets/ml, treated 15 min by 0.2 mM *N*-ethylmaleimide (▲).

at concentrations less than 1 μM. It is thus a useful tool for studying nonspecific interactions between PAF-acether and blood components. Fig. 2A shows the reduction of the (0,2)PAF ESR signal by platelets, depending on the interaction of the probe with the cytosol, which exhibited a half-reaction time of about 15–25 min. At the same time, the ESR signal of the (0,2)A moiety, obtained by (0,2)PAF hydrolysis, was very weak. However, the fast reduction of (0,2)A by the internal content of the cells must be considered. In order to reduce the hydrolysis rate, some experiments were performed after preincubation of the platelets with 1 mM PMSF, which inhibits acetyl hydrolase [17]. Fig. 2B shows that both reduction and hydrolysis were considerably reduced by this treatment, so that we were unable to dissociate hydrolysis from reduction.

2b. Other components. It is well known [18] that PAF-acether is rapidly hydrolyzed by a partially identified serum component. The reaction is not specific for an acetyl ester bond since more than 80% of the (0,2)A can be released into the serum in 2 h. This result is significant since Blank et al. [19] and particularly Farr et al. [20] have shown that at position 2, fatty esters no more than 4 carbon atoms long can be cleaved by an acid-labile factor contained in serum. On the other hand, (0,2)PAF can also penetrate red blood cells, as seen in Fig. 2A, but more slowly than it penetrates platelets. After a 40 min reaction time, the platelets internalized 70% of (0,2)PAF whereas at the same time only 40% of the paramagnetic compound was reduced by the internal content of the erythrocytes (Fig. 2A). Finally, in whole blood, the membrane component of the (0,2)PAF ESR signal disappeared, as in the presence of red blood cells. Moreover, the (0,2)A component was very noticeable in this case because of the hydrolase activity of the serum noted above. Table I shows the results obtained after a 30 min incubation of (0,2)PAF with whole blood or different blood components. They indicate that (0,2)PAF entered into platelets more efficiently than into erythrocytes.

TABLE I

BALANCE (%) IN REDUCTION AND HYDROLYSIS OF (0,2)PAF BY DIFFERENT MEDIA AFTER 30 min OF INCUBATION AT 37°C

Water-soluble and membrane-soluble ESR spectra were computed by electronic subtraction. Their relative concentrations were measured after double-integrating the spectra obtained and comparing them with known concentrations of spin labels. The percentages are expressed relative to the initial concentration of (0,2)PAF in the different media.

	Platelets ($2 \cdot 10^9$/ml)	Eryhtrocytes (10^9/ml)	Serum	Blood
Reduction	80	35	0	29
Hydrolysis	< 2	< 2	100	39

Discussion

The following main conclusions can be drawn from our results.

The internalization rate of (0,2)PAF in cells can be followed, because of the reduction reaction between the nitroxide probe and cytosol (Fig. 1B). This reaction, probably related to the metabolic state of cells, is not solely due to glutathione, since the latter is only slightly reactive, and since *N*-ethylmaleimide treatment failed to suppress the reduction completely (Fig. 1C). On the contrary, the treatment stimulated the cells: the external medium of the platelets became a reducing agent by itself, showing that at least a part of the nitroxide-reactive compounds were enclosed in secretion granules. On the other hand, the reaction even occurred when the (0,2)PAF reached the inner leaflet of the plasma membrane, since a water-soluble reagent, like ascorbate, can react with the probe linked to a membrane.

(0,2)PAF enters platelets and erythrocytes, with a relative specificity for the former (Fig. 2A). Drug internalization is fast, since about $5 \cdot 10^6$ (0,2)PAF molecules entered one platelet in the first two minutes of incubation (Fig. 2A), whereas only about half this amount entered erythrocytes in the same time. This difference appeared also in the long-term value (30 min) obtained with these cells: $5 \cdot 10^7$ (0,2)PAF molecules entered one platelet cell but only about $(1–2) \cdot 10^7$ molecules entered one erythrocyte cell (Table I). Thus, platelet membrane seems to have a greater transverse diffusion rate for phospholipids such as (0,2)PAF.

(0,2)PAF is readily hydrolyzed by plasma (see

Table I). This may be related to the presence of an acetylhydrolase in the medium, which is not very specific for the acetyl ester bond. Moreover, platelet acetylhydrolase seems to be much more specific for acetyl bonds since (0,2)PAF was poorly cleaved in the presence of platelets and erythrocytes (Table I). This is in agreement with Blank et al. [19] who showed that many tissues contain acetylhydrolase activity specific for acetyl ester bonds at position 2.

It is important to consider whether the internalization rate of (0,2)PAF in different cells has any physiological meaning. First, it should be noted that many physicochemical properties related to the hydrophobic balance of (0,2)PAF and PAF-acether are nearly identical, e.g. binding to serum albumin [21] or CMC (0.8 and 1.5 μM for the two molecules (0,2)PAF and PAF-acether, respectively) [14,22]. Second, the internalization rate of (0,2)PAF corresponds to first-order kinetics (Fig. 2A). This means that the half-reaction time is independent of the actual label concentration, as seen with other spin-labeled phospholipids (Sune, A. et al., unpublished results). Thus, it seems reasonable to assume that the transverse diffusion rate for the two molecules, (0,2)PAF and PAF-acether, were nearly identical, independently of their actual concentration. Third, although the local concentration of PAF-acether around a stimulated cell, such as polymorphonucleocytes, is not yet accurately known, it could be much higher than the mean concentration in whole blood. For example, it has been reported that 10^6 stimulated polymorphonucleocytes and macrophages can release an amount in PAF-acether corresponding to an external concentration of 10–100 nM [23–25]. It is interesting to note that platelets and erythrocytes can incoroporate similar quantities of (0,2)PAF in two minutes.

Thus, it appears that (0,2)PAF mimics the non-specific interactions of PAF-acether with blood components. Consequently, we can conclude that apart from being trapped by serum albumin and catabolised by cytoplasmic or serum acetylhydrolase, PAF-acether can be removed by blood cells. Considering their high concentration, red blood cells may release PAF acether as efficiently as platelets or serum. Blood cells may thus play an important role in the clearing rate of PAF-acether, possibly by the numerous reactions in which lyso-PAF-acether is subsequently involved. This could be related to the rapid clearance of [^{3}H]PAF-acether after intravenous injection [19,25]. In accordance, a high portion of the radioactivity taken up by tissues is still found as intact PAF acether, despite the presence of acetyl hydrolase in plasma, PAF-acether being probably protected in erythrocytes.

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