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Preformed PAF-acether and lyso PAF-acether are bound to blood lipoproteins

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PAF-acether (PAF) is a newly formed mediator not normally present in circulating blood. A compound exhibiting all of its biological characteristics but coeluting with phosphatidylcholine (PC) in high-pressure liquid chromatography (HPLC) was unveiled ('peak X') in normal human plasma. A second HPLC run of peak X HPLC fractions revealed the presence of PAF itself with concomitant disappearance of peak X. Beside PAF, immunoreactive apolipoproteins A-I and E were found in peak X. Also lipoproteins (Ls) purified using either ultracentrifugation or immunoaffinity chromatography yielded peak X and, in a second HPLC run, authentic PAF. L-free plasma was devoid of peak X. Finally, after preincubation with plasma, labeled PAF was found associated with Ls. Thus in human blood preformed PAF is bound in high amounts to Ls, a result of interest given the role of Ls and platelets in vascular diseases and the present knowledge on PAF biosynthesis.

PAF-acether; Lyso PAF-acether; Lipoprotein

1. INTRODUCTION

Since the discovery of PAF (formerly known as platelet-activating factor) [1], several of its biological and physico-chemical features have been unveiled [2,3]. PAF is one of the most potent newly-formed mediators released from and acting on most cells and organs that participate in inflammatory diseases. It is an analog of phosphatidylcholine [4], the molecular structure of which is 1-*O*-alkyl-2-*O*-acetyl-*sn*-3-glycerophosphocholine [5,6]. In human subjects and experimental animals, the presence of PAF in blood has been reported only in pathological conditions [7–11]. Indeed, when analyzing normal plasma from various mammalian species, we did not generally find – or we detected very low levels of – PAF. Yet, after lipid extraction and HPLC, we observed what appeared as a new molecular moiety of this mediator, exhibiting all of its characteristics [1,12–14]. However, its retention time was unusual since it corresponded to that of PC. This unusual HPLC peak of platelet aggregating activity ('peak X') has already been reported by our laboratory and other investigators but not characterized [11,15,16]. We thus examined the molecular constituents of peak X and its relationship with PAF.

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Abbreviations: ADP, adenosine diphosphate; HDL, high density lipoprotein; HPLC, high-pressure liquid chromatography; LDL, low density lipoprotein; Ls, lipoproteins; PC, phosphatidylcholine; rt, retention time; VLDL, very low density lipoprotein

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2. MATERIALS AND METHODS

2.1. *Purification and characterization of Ls*

Plasma samples were obtained from 30 informed volunteers who had fasted for at least 8–12 h. In a first set of experiments, Ls were isolated using either sequential preparative ultracentrifugation, VLDL ($d < 1.006$ g/ml), LDL (d 1.006–1.063 g/ml) and HDL (d 1.063–1.21 g/ml) [17], or an affinity column of goat anti-apoprotein B antibody coupled to Sepharose 4B [18]. In the latter method, plasma (20 ml) was applied to the immunosorber. After the unretained fractions were eluted with PBS, pH 7.3, and the absorbance returned to the baseline, 3 M sodium thiocyanate (20 ml) followed by PBS, pH 7.3, was applied to the column to desorb the retained L containing apoprotein B (a mixture of LDL and VLDL). Ls obtained using both methods were dialyzed against 0.15 M NaCl, pH 7.5, and submitted to the Folch extraction procedure [19]. In some experiments, [^{3}H]PAF (1.06 pmol, Amersham, England, 100 Ci/mmol) was incubated for 1 h at 4°C with 3 ml of normal human plasma, which was then submitted to gradient ultracentrifugation [20]: VLDL ($d < 1.017$ g/ml); IDL (d 1.017–1.023 g/ml); LDL (d 1.023–1.061 g/ml); intermediate fraction (d 1.061–1.070 g/ml); HDL (d 1.070–1.115 g/ml); proteins (d 1.115–1.253 g/ml). Each fraction was dialyzed against 0.15 M NaCl, pH 7.5, to remove unbound PAF. Samples of protein fractions were assessed for radioactivity and protein determination. Sandwich immunoassay for human apoproteins was performed as described using polyclonal antibodies against apoprotein A-I, A-II, B, C-III, E and a monoclonal antibody against apoprotein E [21].

2.2. *HPLC of L fractions*

Human plasma (1 ml) was mixed with ethanol (4 ml) for 2 h at room temperature and then centrifuged at 900 × g for 15 min. The precipitate was discarded and the supernatant was dried. The dried residue or the L dried extracts from the Folch procedure (see above) were dissolved in 500 μl of HPLC solvent (dichloromethane/methanol/water, 400:333:50). In some experiments 5 nCi of L-3-phosphatidylcholine-1-stearoyl-2-[1-^{14}C]arachidonyl (Amersham, 1 mCi/mmol was added to plasma immediately before ethanol extraction). Samples were applied to a Microporasil column 3.9 mm ID × 300 mm length (Waters Associates, Milford, MA), which was then eluted at a flow rate of 1 ml/min. PC, sphingomyelin, PAF and lysophosphatidylcholine were used as markers. 1 ml fractions were analyzed by differential refractometry, collected, dried and resuspended in 100 μl of 60% ethanol and then assayed for platelet aggregating activity. A sample of each fraction (100 μl) was mixed with scintillation fluid for measurement of radioactivity. In some experiments, peak X fractions (11–13 min) from a first HPLC were pooled, dried and dissolved in 80% ethanol and kept at room temperature for at least 7 days. The fraction pool was then dried and redissolved in the HPLC solvent system for a second HPLC run. In other experiments, the second run was performed using a reverse HPLC procedure. A Spherisorb C_6 column was used with a fluid phase of 10 mM ammonium acetate, acetonitrile, methanol, 10:45:45, at a flow rate of 1 ml/min. Standards were labeled PAF and labeled PC.

2.3. *Lyso PAF and PAF assay*

Fractions corresponding to peak X were submitted to chemical acetylation using acetic anhydride and pyridine [22]. Rabbit washed platelets were prepared as in [1,13]. Aspirinated platelets (1.6×10^8) in Tyrode's (300 μl) containing 2.5% gelatin and the ADP scavenger mixture, creatine phosphate (1 mM)/creatine phosphokinase (10 U/ml) were stirred in an aggregometer (Icare, Marseille, France). Aggregating activity of the samples before and after chemical acetylation was measured over the linear portion of the calibration curve obtained with 5 to 50 pg synthetic PAF. Other criteria for PAF (native or following chemical acetylation) were used as detailed in section 3 below.

3. RESULTS AND DISCUSSION

Results are given as means ± 1 SD.

3.1. *Presence of peak X and PAF in human blood and plasma*

The peak X (i.e. the aggregating activity eluted from standard phase HPLC with a rt similar to PC, 11–13 min) was observed in all ethanolic ex-

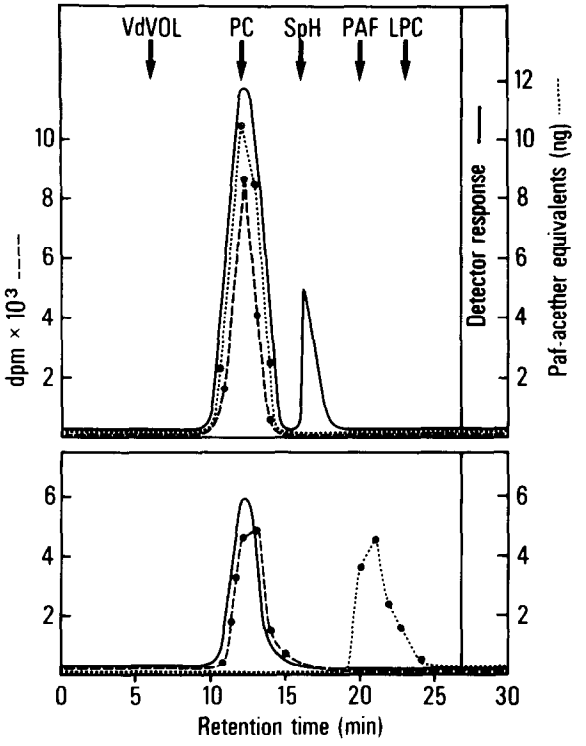

Fig.1. HPLC (standard phase) elution profile of peak X and PAF-acether from plasma. One experiment representative of three. Arrows indicate the void volume (VdV01) and the rt of phosphatidylcholine (PC), sphingomyelin (SpH), PAF-acether (PAF) and lysophosphatidylcholine (LPC). (Top) First HPLC run of plasma ethanolic extracts. (Bottom) Second HPLC run of peak X recovered from the first HPLC. [^{14}C]Phosphatidylcholine was added to ethanolic extracts before HPLC. Detector response was monitored during HPLC and radioactivity and platelet aggregation were assessed on each HPLC fraction.

tracts from plasma and whole blood (fig.1, top). The aggregating activity in the peak X recovered from 1 ml of plasma (n = 4) or blood (n = 4) was 19.46 ± 10.30 and 9.37 ± 2.19 ng, respectively. Given a hematocrit of around 0.4, the peak X appears predominantly associated with the plasma. In some samples, we observed a platelet aggregating activity of fractions comigrating on HPLC with synthetic PAF (rt = 20–24 min). However, this activity was much lower than in peak X, i.e. 0.23 ± 0.29 ng and 0.23 ± 0.28 ng in fractions from 1 ml of blood and plasma, respectively. Lyso PAF, the deacetylated precursor/derivative of PAF, was present in peak X in high amounts: for 1 ml of plasma, 478.8 ± 91.0 ng, n = 3.

When the PC-X peak was rechromatographed after prolonged storage in 80% ethanol, a PC peak devoid of aggregating activity was detected and a typical PAF activity peak with a rt of 20–24 min was now present (fig.1, bottom). Labeled PC incorporated in the starting plasma ethanolic extract co-chromatographed with the PC-X peak in the first and with the PC peak in the second HPLC run (fig.1, top, bottom). Thus, the PAF-like activity present in the peak X could be dissociated from PC, yielding in the second HPLC run a substance that shared the same characteristics with authentic PAF. Furthermore, peak X migrated in reverse-phase HPLC with a rt between 42 and 52 min, whereas labeled PAF and PC were eluted at 5–14 and 22–30 min, respectively. Taken together, these results show that peak X and PC, although comigrating in the first HPLC run, are different compounds.

3.2. *Nature of the aggregating substance present in peak X*

Apart from its chromatographic behaviour, this substance appeared strictly undistinguishable from PAF itself, since it shared all of its known characteristics: (i) peak X, as well as PAF, induced aggregation of rabbit and human platelets pretreated with aspirin in the presence of the creatine phosphate/creatine phosphokinase complex, thus ruling out the participation of arachidonic acid and ADP, respectively [13]. (ii) The aggregating activity – native or following acetylation – found in peak X was totally suppressed following treatment with phospholipase A_2, C and D but not with lipase from *R. arrhizus* [4]. (iii) Both peak X and PAF induced platelet desensitization to a second challenge by PAF, suggesting that the structural requirements for activity were the same for peak X as for PAF [12]. (iv) BN 52021 (a gift from IPSEN-Beaufour, Le Plessis-Robinson, France), a specific antagonist of the PAF putative receptor [14,23], inhibited aggregation induced by submaximal concentrations of peak X and PAF (50 pg/ml). The antagonist IC_{50} values were: 0.35 ± 0.19 and 0.45 ± 0.23 μM (n = 3) respectively. Using gas chromatography with electrocapture detection, the PAF isolated af-

ter the second HPLC run was identified as being mainly 1-*O*-hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (not shown).

3.3. *Molecular composition of peak X*

The above results suggested that PAF was associated with a complex that modified its HPLC rt and that was dissociated in the second HPLC run. Ls, that bind phospholipids, were likely candidates as PAF carrier. The sandwich enzyme-immunoassay for human apoproteins revealed some immunoreactivity of peak X with polyclonal anti A-I and E antibodies and monoclonal anti-E antibody. Thus peak X appeared to contain low amounts (about 5 ng/ml) of a material immunoreactive with apolipoprotein A-I and E. In other words, peak X was a complex composed of apolipoproteins, PAF, lyso PAF and probably other unidentified lipids. This complex was not dissociated during extraction and during a first HPLC run. However, it is highly plausible that most of the apolipoproteins were denatured during organic extraction and HPLC explaining the low amount of proteins detected using the immunoassay.

We then recovered peak X following HPLC of plasma L, purified using either sequential preparative ultracentrifugation or immunoaffinity chromatography. Almost the same amount of peak X was present in LDL and HDL, whereas a small activity was measured in VLDL (table 1). A minute activity exhibiting the rt of PAF was also extracted from LDL and HDL. A second HPLC run of peak X from purified L again yielded a platelet aggregating activity with the typical rt and biological features of PAF. These experiments demonstrated that circulating Ls were a source of peak X that was dissociated to yield PAF itself in the second HPLC run. Another strong argument in favour of Ls was that in three experiments, plasmas freed from L by ultracentrifugation yielded 0.16 ± 0.17 ng/ml of peak X (vs 19.46 ± 10.30 in 4 normal plasmas) and 0.071 ± 0.12 ng/ml PAF (vs 0.23 ± 0.28 in normal plasmas). This experiment again indicated a strong link between the presence of peak X and L, and ruled out serum albumin as a carrier for PAF in peak X, since the L-free plasmas contained a normal amount of albumin but yet practically neither peak X nor PAF activity was found.

Labeled PAF incubated with plasma at 4°C bound to protein fractions obtained by gradient ultracentrifugation (table 2). 24 and 65% of the radioactivity was recovered in the L fractions and in the fraction containing albumin and other plasma proteins, respectively. However, when these results are expressed in pg PAF bound per mg protein, preferential incorporation of the radioactivity is observed in Ls. In two preliminary experiments, it was investigated whether labeled PAF was incorporated either as such or as a metabolite. In the first experiment, one third of the radioactivity coeluted with the void volume, one third with peak X, and one third with PAF. In the other experiment, most of the label coeluted with lyso PAF (not shown). While this article was in preparation, binding of labeled PAF to lipoproteins was reported [24].

The binding pattern of labeled PAF to plasma described above is reminiscent of the well-established binding of PAF to serum albumin [1]. The question thus arises whether the latter is also a circulatory carrier for PAF. The answer is negative since in several attempts we never recovered PAF from serum albumin either from Sephadex G-200-fractionated normal human serum or from commercial sources. Therefore, even if serum albumin does bind PAF, the endogenous PAF final distribution in the plasma is the result of a long-term equilibrium which depends on presently undefined factors. We know since many years that, if 2.5 mg serum albumin per ml of buffer allows for an optimal yield of PAF, raising this concentration to 25 mg/ml abolishes PAF recovery and, in parallel, inactivates exogenously added PAF (Benveniste, unpublished). Therefore, physiological concentrations of albumin might help in controlling the level of free PAF in plasma but cannot act as a PAF carrier, a role which appears devoted to Ls.

The latter finding is not surprising in view of the known capability of L to bind phospholipids. An interaction of PAF with HDL has been suggested since esterification of plasma cholesterol was inhibited by PAF [25]. Yet, this report is the first to establish that L and especially LDL and HDL carry high amounts of a mediator that is considered as a most potent agonist in inflammatory and shock states. Recovery of PAF from L is in apparent contradiction with the association to HDL

Table 1

Peak X and PAF-acether in lipoprotein fractions (n = 3)

	Sequential ultracentrifugation			Immunoaffinity chromatography
	LDL	HDL	VLDL	Lipoproteins containing apoprotein B
Peak X	3.43 ± 1.79[a]	2.60 ± 1.40	0.80 ± 0.10	4.19
PAF-acether	0.29 ± 0.50	0.14 ± 0.24	0	0

[a] ng equivalent PAF-acether/mg protein

Ls were isolated from plasma using either sequential ultracentrifugation [17] or affinity chromatography with Sepharose 4B-coupled anti-apoprotein B antibody [18]. Isolated Ls were then submitted to HPLC and platelet aggregation was assessed on fractions. Peak X and PAF-acether corresponded to rt 11–13 min and 20–24 min, respectively

and LDL of the PAF catalytic enzyme, acetylhydrolase [24,26]. We lack information on the site(s) and the mode of incorporation of PAF into the lipoprotein complex. However, since we recovered both the intact molecule and its lyso precursor/derivative, we can hypothesize that: (i) PAF escapes hydrolysis when buried in the complex, most probably in a compartment separated from acetylhydrolase; and (ii) lyso PAF is either directly bound to the complex or results from the hydrolysis of part of the PAF within the complex. Whether the L PAF originates from the de novo or the remodeling pathway [2] remains to be investigated. Nevertheless, binding of PAF to L may favour release of this mediator from cell sources, prevent its degradation by plasma acetylhydrolases and also influence the L/cell interactions. It is noteworthy that Ls which play a major role in atherosclerosis, carry a compound (PAF) that is capable both of suddenly increasing local vascular permeability and of powerfully attracting and activating platelets and polymorphonuclear leucocytes, i.e. the major cell participants in vascular lesions.

Table 2

Incorporation of [^{3}H]PAF-acether in plasma proteins (n = 4)

Protein fractions	Incorporated radioactivity	
	(% of total added)	(pg bound/mg protein)
VLDL	2.8 ± 1.2	24.2 ± 6.1
IDL	1.5 ± 0.4	33.1 ± 20.3
LDL	9.0 ± 1.7	21.2 ± 3.2
Intermediate fraction	1.6 ± 0.9	15.4 ± 3.8
HDL	10.8 ± 1.1	9.5 ± 1.5
Proteins	65.0 ± 14.0	1.1 ± 0.2

[^{3}H]PAF-acether (1.06 pmol) was incubated with normal plasma (3 ml). Ls were obtained using gradient ultracentrifugation [20]. Radioactivity counts and protein determination were performed on L and protein fractions

In addition, these results might change our current view on PAF biosynthesis and release. PAF should now be considered both as newly-formed upon cell activation via classical biosynthetic pathways [2] and as a preformed circulating mediator. Preformed PAF is also bound to L in various cell types and organs in the form of peak X. We have recently performed clinical studies indicating that, in inflammatory states, it is mandatory to assess the amount of L PAF in the blood and biological fluids where free PAF is generally absent (Benveniste et al., to be published). It is clear from the present data that when PAF is 'hidden' within the L complex, it cannot reach its molecular or cellular targets. However, should it

be liberated from its plasma or cellular carrier, a blood concentration of about 9 nM could be reached, thus causing severe intravascular lesions or even death. The presence in blood of high amounts of PAF bound to L and its potential release in pathological states might lead to reconsider the pathophysiological role of this mediator, especially in acute and chronic vascular diseases.

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