Platelet-activating factor acetylhydrolase and transacetylase activities in human plasma low-density lipoprotein

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In this study, we demonstrate the presence of a transacetylase activity in human plasma low-density lipoprotein (LDL) that transfers short-chain fatty acids from platelet-activating factor (PAF) and its close ether- and ester-linked analogues to ether/ester-linked lysophospholipids (lyso-PL). We show evidence that both PAF acetylhydrolase (PAF-AH) and transacetylase activities are inhibited to the same extent by serine esterase inhibitors, are resistant to heat treatment, and exhibit identical distributions in lipoprotein classes and in LDL subfractions. Additionally, the competitive inhibition of PAF-AH by lyso-PL, and the evidence that the recombinant PAF-AH also showed a similar transacetylase activity, suggest that PAF-AH is responsible for both activities. Using PAF as a donor molecule and lyso-PAF (1-*O*-alkyl-*sn*-glycero-3-phosphocholine) as an acceptor, the transacetylase activity showed typical allosteric kinetics, due to the positive co-operativity of the substrates, with apparent $V_{\max} = 19.6 \pm 3.4$ nmol/min per mg of protein, apparent $h = 2.0 \pm 0.3$ and apparent $[S]_{0.5} = 9.4 \pm 2.3$ μ M at saturation for the concentration of lyso-PAF. The substrate specificity of the

donor molecules was decreased by increasing the chain length of the acyl moiety in the *sn*-2 position of the glycerol. The ether linkage in the *sn*-1 position of the substrate was 30 % more effective than the ester bond; cholesteryl acetate was inactive as an acetyl donor. The two acceptors tested, lyso-PAF and the ester-linked lyso-PC (1-acyl-*sn*-glycero-3-phosphocholine), showed similar specificity. Addition of exogenous lyso-PAF induced the transient formation of PAF-like aggregating activity predominately in small dense LDL subfractions upon oxidation. We conclude that PAF-AH possesses both transacetylase and acetylhydrolase activities which remove PAF and its ether-linked analogues from LDL particles upon LDL oxidation. However, in atherogenic small dense LDL-5 particles, the transacetylase activity may acetylate extracellular lyso-PAF into biologically active PAF.

Key words: atherosclerosis, inflammation, lipoprotein subfractions, oxidized LDL.

INTRODUCTION

Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent lipid mediator involved in inflammatory reactions [1] that may equally play a crucial role in atherogenesis (reviewed in [2]). PAF is synthesized by a wide variety of pro-inflammatory cells, including endothelial cells, platelets and macrophages (reviewed in [1]), all of which are known to play essential roles in the development of atherosclerotic plaques (reviewed in [3]). PAF activates target cells via a receptor-mediated process [1].

In plasma, PAF is rapidly hydrolysed and converted into its inactive metabolite, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF), by hydrolysis of its acetate group. This reaction is catalysed by a Ca^{2+} -independent acetylhydrolase (PAF-AH; EC 3.1.1.47) [4], a hydrophobic enzyme that is associated with lipoproteins, mostly low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs) [5]. It was also shown that lecithin-cholesterol acyltransferase (LCAT; EC 2.3.1.43) was able to

hydrolyse PAF in plasma [6], and that LCAT and semi-purified PAF-AH were able to transfer acetate from PAF to 1-acyl-*sn*-glycero-3-phosphocholine (lyso-PC). This reaction was considered to be an additional mechanism in the inactivation of PAF, since its acyl analogue is biologically inactive [6].

It is well established that the oxidation of LDL plays a key role in the initiation and progression of atherosclerosis [7]. The oxidative modification of LDL involves the hydrolytic transformation of its content of oxidized phospholipids into lyso-phospholipids (lyso-PL), and such hydrolysis is mediated by the LDL-associated PAF-AH [8,9]. Upon oxidative modification of LDL, however, PAF-AH activity decreases dramatically, and thus oxidized LDL is devoid of anti-inflammatory properties [9,10]. Indeed, we showed that PAF is produced upon copper-mediated oxidation of LDL subspecies of the intermediate subclass with low PAF-AH activity; by contrast, PAF did not accumulate in small dense LDL as a result of its elevated content of PAF-AH [11]. In addition, we have shown that PAF was produced upon the copper-mediated oxidation of LDL in which

Abbreviations used: PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); lyso-PAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; lyso-PC, 1-acyl-*sn*-glycero-3-phosphocholine; lyso-PL, lysophospholipids; C16:0 PAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; C16:0 lyso-PAF, 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; C16:0 lyso-PC, 1-hexadecanoyl-*sn*-glycero-3-phosphocholine; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; PAF-AH, PAF acetylhydrolase; DFP di-isopropylfluorophosphate; Pefabloc, 4-(2-aminoethyl)benzenesulfonyl fluoride.

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PAF-AH was irreversibly inhibited [9]. In a recent study, Marathe et al. [12] showed that the ether-linked phospholipids of oxidized LDL were the main compounds among phospholipids endowed with pro-inflammatory activities, including PAF and its butanoyl and butenoyl *sn*-2 analogues.

The aim of the present study was to investigate the identity of the transacetylase activity present in human LDL. Our results suggest that PAF-AH possesses both transacetylase and acetylhydrolase activities, which remove PAF and its ether-linked analogues from LDL particles upon oxidation. However, in atherogenic small dense LDL-5 particles, the transacetylase may acylate extracellular lyso-PAF into biologically active PAF and its analogues.

EXPERIMENTAL

Materials

1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C16:0 PAF), 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (C16:0 lyso-PAF), 1-hexadecanoyl-*sn*-glycero-3-phosphocholine (C16:0 lyso-PC), fatty-acid-free BSA, di-isopropylfluorophosphate (DFP), phosphocreatine, creatine kinase and butyric anhydride were from Sigma; valeric anhydride and propionic anhydride were from Aldrich; acetic anhydride was from Fluka; Pefabloc SC [4-(2-aminoethyl)benzenesulphonyl fluoride] and BCA protein reagent were from Pierce. Liquid scintillation fluid (Opti-phase Hi-Safe 3) was supplied by E.G.G. BN 52021 was kindly provided by Dr P. Braquet (Institut Henri Beaufour, Paris, France). Solvents were from Lab-Scan. 1-*O*-Hexadecyl-2-[³H]acetyl-*sn*-glycero-3-phosphocholine (10 Ci/mmol), 1-*O*-[³H]hexadecyl-*sn*-glycero-3-phosphocholine (58.3 mCi/mmol), 1-[³H]palmitoyl-1-¹⁴C]phosphatidylcholine (50.5 mCi/mol) and [³H]acetic acid, sodium salt (86.4 mCi/mmol) were from Du Pont–New England Nuclear. Recombinant PAF-AH contained in the PAF-AH assay kit (cat. no. 760901) was obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.).

Preparation of PAF analogues

The ether- and ester-linked analogues of PAF were prepared by acylation of C16:0 lyso-PAF or C16:0 lyso-PC, dissolved in anhydrous pyridine, by the appropriate anhydride, as described in [13]. The products were purified by TLC on silica-gel G plates (Merck) by using chloroform/methanol/water (65:35:6, by vol.) as a solvent system. In this TLC system, the R_F values of lyso-PAF and PAF were 0.11 and 0.17 respectively. The propionyl, butyryl and valeroyl analogues of PAF had R_F values of 0.21, 0.24 and 0.28 respectively. The corresponding acyl analogues had R_F values similar to those of their ether counterparts. The purified phospholipids were quantified by phosphorus determination [14,15].

Isolation of plasma lipoproteins

Plasma lipoproteins [very-low-density lipoprotein (VLDL) + intermediate-density lipoprotein (IDL), density < 1.019 g/ml; LDL, 1.019–1.063 g/ml; HDL, 1.063–1.210 g/ml) and lipoprotein-deficient proteins (> 1.210 g/ml) were prepared from freshly isolated human plasma containing 0.01% EDTA and 5 mg/ml gentamicin by sequential ultracentrifugation in a Beckman L7-65 ultracentrifuge at 125000 *g* at 14 °C for 10 h with a type NVT 65 rotor, as described previously [16]. Lipoproteins were washed by a second centrifugation step and dialysed at 4 °C against two changes of 5 litres of 10 mmol/l PBS containing 0.01% EDTA at pH 7.4 for 24 h. All lipoproteins

were then filter-sterilized and analysed for their protein content. Lipoproteins were stored at 4 °C and used within 2 weeks of preparation. The protein content of the lipoproteins was determined by the method of Lowry et al. [17], or by the BCA (bicinchonic acid) method [18], using BSA as a standard.

Preparation of lipoprotein subfractions

Lipoproteins were fractionated by isopycnic density-gradient ultracentrifugation using a Beckman SW 41 Ti rotor at 200000 *g* for 44 h in a Beckman XL70 centrifuge at 15 °C, by a slight modification of the method described by Chapman et al. [19]. Briefly, plasma density was increased to 1.21 g/ml by the addition of dry, solid KBr. Construction of a discontinuous density gradient at ambient temperature was initiated by pumping 2 ml of an NaCl/KBr solution of density 1.24 g/ml into the bottom of the tube. The following solutions were then layered above: 3 ml of plasma at 1.21 g/ml; 2 ml of an NaCl/KBr solution at 1.063 g/ml; 2.5 ml of an NaCl/KBr solution at 1.019 g/ml; and 2.5 ml of an NaCl solution at 1.006 g/ml. All density solutions contained EDTA (0.3 mmol/l) and 50 µg/ml gentamicin at pH 7.4. After ultracentrifugation, gradients were collected by the successive aspiration of 24 fractions, of 0.4 ml each, from the meniscus downward. All fractions were analysed for their protein content. Subsequently, equal volumes of certain gradient fractions were pooled to constitute the lipoprotein subfractions, as follows: fractions 1 and 2 (VLDL + IDL; density < 1.019 g/ml); 3 and 4 (LDL-1; 1.019–1.023 g/ml); 5 and 6 (LDL-2; 1.023–1.029 g/ml); 7 and 8 (LDL-3; 1.029–1.039 g/ml); 9 and 10 (LDL-4; 1.039–1.050 g/ml); 11 and 12 (LDL-5; 1.050–1.063 g/ml); 13–16 (HDL-2; 1.063–1.100 g/ml); 17–22 (HDL-3; 1.100–1.167 g/ml); 23 and 24 [very-high-density lipoprotein-1 (VHDL); 1.167–1.190 g/ml]. All subfractions were dialysed in Spectrapor membrane tubing (exclusion limit 12000–14000 Da) against three changes of 5 litres of 10 mmol/l PBS containing EDTA (2 mmol/l), pH 7.4, at 4 °C for 24 h. All subfractions were then filter-sterilized and analysed for their protein content. Lipid analysis was also performed using commercially available kits (BioMerieux).

Transacetylase assay

The transacetylase assay was performed by incubating each lipoprotein fraction in 10 mM PBS, pH 7.4, with PAF and [³H]lyso-PAF dissolved in PBS containing 2.5 mg/ml BSA. Reactions were performed in polypropylene tubes for 60 min at 37 °C. The final concentrations were 5 µg of protein/ml lipoprotein, 80 µM PAF, 30 µM [³H]lyso-PAF (0.1 µCi) and 250 µg/ml BSA, in a reaction mixture of 0.4 ml. The reaction was stopped by extracting the lipids according to Bligh and Dyer [20]. Total lipids were then subjected to TLC on silica-gel G plates by using chloroform/methanol/water (65:35:6, by vol.) as a solvent system. Lipids were identified after a brief exposure to iodine. The band corresponding to the R_F of standard PAF was scraped off the plate and the radioactivity was measured by liquid scintillation counting. In some experiments, the lipoprotein was preincubated with 20 mM DFP or 5–1000 µM Pefabloc for 30 min at 37 °C, or was preheated at 60 °C for 60 min before the assay. In selected experiments, the acyl analogue of PAF or its alkyl and acyl analogues with longer chains at the *sn*-2 position, dissolved in PBS containing 2.5 mg/ml BSA, were used as substrate instead of PAF, with 30 µM [¹⁴C]lyso-PC (0.1 µCi) as the acceptor molecule. In other experiments PAF, 1-hexadecanoyl-2-acetyl-*sn*-glycero-3-phosphocholine or cholesteryl acetate, in micellar form, was used as substrate. Recombinant PAF-AH was used instead of LDL protein in some experiments.

Acetylhydrolase assay

The acetylhydrolase assay was performed by incubating each lipoprotein fraction in 10 mM PBS, pH 7.4, with [*acetyl*-³H]PAF and lyso-PAF, dissolved in PBS containing 1.25 mg/ml BSA, in an Eppendorf polypropylene tube for 60 min at 37 °C. Final concentrations were 5 µg of protein/ml lipoprotein, 80 µM [*acetyl*-³H]PAF (0.1 µCi), 30 µM lyso-PAF and 250 µg/ml BSA in a reaction mixture of 0.1 ml. The reaction was stopped in an ice bath. Unreacted [*acetyl*-³H]PAF was bound to an excess of BSA (final concentration 16.7 mg/ml) for 10 min and precipitated by the addition of trichloroacetic acid (final concentration 8%, v/v), as described [21]. The samples were then centrifuged in an Eppendorf centrifuge for 5 min and the [³H]acetate released into the aqueous phase was measured by liquid scintillation counting. In some experiments, the lipoprotein was preincubated with 20 mM DFP or 5–1000 µM Pefabloc for 30 min at 37 °C, or was preheated at 60 °C for 60 min before the assay. Recombinant PAF-AH was used instead of LDL protein in some experiments.

Kinetic studies

Kinetic studies were performed in the presence of 2.5–100 µM PAF or [*acetyl*-³H]PAF and 10–200 µM [³H]lyso-PAF or lyso-PAF. In some experiments recombinant PAF-AH was used instead of LDL. The data were fitted to Michaelis–Menten or Hill plots by using Ultrafit, a non-linear curve-fitting program for Macintosh (Biosoft, Cambridge, U.K.). Kinetic constants were determined from the Michaelis–Menten or Hill plots by using the Lineweaver–Burk or the Hill representation of the data.

Oxidation of LDL

In order to study the effects of oxidation on the two enzyme activities, we used, as enzyme source, LDL oxidized in the presence of Cu²⁺ for various time intervals. Before oxidation, LDL was dialysed extensively against 10 mM PBS, pH 7.4, at 4 °C to remove EDTA. Oxidized LDL was prepared by incubating LDL (0.1 mg of protein/ml) with 5 µM CuSO₄ in 10 mM PBS, pH 7.4, at 37 °C for various time intervals up to 24 h. Oxidation was terminated by the addition of 0.01% EDTA and refrigeration. The rate of oxidation was deduced from changes in the relative electrophoretic mobility of oxidized LDL on agarose gels as compared with native LDL, and by the content of thiobarbituric acid-reactive substances [22].

Characterization of PAF and its analogues in oxidized LDL

Total lipids of oxidized LDL were extracted according to Bligh and Dyer [20], and separated by TLC on silica-gel G plates by using chloroform/methanol/water (65:35:6, by vol.) as a solvent system. Lipids were identified after brief exposure in iodine vapour, and the band corresponding to the *R_f* value of PAF and its analogues (*R_f* 0.17–0.28) was scraped off the plate. The TLC-purified lipids were dissolved in 60% (v/v) ethanol and assayed for platelet-aggregating activity using aspirin-treated washed rabbit platelets [23]. Platelet aggregation assays were performed using a Chronolog aggregometer in the presence of the ADP scavenger complex phosphocreatine (1 mM)/creatine kinase (10 units/ml). The aggregating activity of PAF was expressed as pmol of C16:0 PAF equivalents/mg of LDL protein using a calibration curve obtained with standard solutions of PAF [23]. Aggregation was characterized as 'PAF-like' by its inhibition by the specific PAF receptor antagonist BN 52021, and its resistance

to treatment with lipase from *Rhizopus arrhizus*, as described previously [24].

Statistical analysis

Results are expressed as means ± S.D. Mean values were compared by Student's *t* test, with significance defined at a level of *P* < 0.05. Preferential fitting of the kinetic data to a Michaelis–Menten curve or to allosteric kinetics was tested by obtaining the reduced Chi-squared value and the *h* value.

RESULTS

Transacetylase reaction catalysed by human plasma LDL

We established a method to measure the transacetylase activity in human plasma LDL. In preliminary experiments we found that the radiolabelled substrates PAF and lyso-PAF were incorporated quantitatively into LDL particles within 10 min. In the presence of substrate concentrations of 30 µM [³H]lyso-PAF and 80 µM PAF, the transacetylase activity was linear with LDL protein concentration up to 5 µg/ml (Figure 1) and with time up to 60 min (results not shown), and reached an activity of 15.2 ± 1.9 nmol/min per mg of protein (three independent LDL preparations). When sodium [³H]acetate (0.1 mM, 0.1 µCi) and unlabelled lyso-PAF were used in the transacetylase assay at different LDL concentrations, we did not detect significant radioactivity corresponding to the *R_f* of the PAF standard by TLC. Thus a direct transfer of acetate from sodium acetate to lyso-PAF is not implicated in PAF formation in LDL.

Next we modified the conditions of the standard acetylhydrolase assay described previously [21], in order to measure both acetylhydrolase and transacetylase activities under the same incubation conditions. In this assay the acetylhydrolase activity was linear with protein concentration up to 20 µg/ml (Figure 1) and with time up to at least 90 min (results not shown).

A concentration of 30 µM lyso-PAF was saturating for the transacetylase activity; higher concentrations up to 120 µM did not increase the activity further (Figure 2A), whereas concentrations above 150 µM were inhibitory, possibly due to the detergent effect of lyso-PAF. Under similar conditions, the acetylhydrolase activity was significantly decreased in the presence of 120 µM lyso-PAF (*P* < 0.05) (Figure 2B). We calculated the ratio of transacetylase to acetylhydrolase activity, and found

Figure 1 Effects of LDL protein concentration on transacetylase and acetylhydrolase activities

Transacetylase (●) and acetylhydrolase (○) activities are expressed as pmol of product formed/min per ml. Values represent the means ± S.D. of three independent LDL preparations.

Figure 2 Effects of lyso-PAF concentration on transacetylase and acetylhydrolase activities

(A) Effect of lyso-PAF concentration on transacetylase activity. Activity was determined as described in the Experimental section in the presence of 80 μ M PAF. Values represent means $\pm$ S.D. of four independent LDL preparations. (B) Transacetylase (■) and acetylhydrolase (□) activities in the presence of various concentrations of lyso-PAF. Both activities were determined as described in the Experimental section in the presence of lyso-PAF and 80 μ M PAF. Values represent means $\pm$ S.D. of three independent LDL preparations.

Table 1 Effects of serine esterase inhibitors and of heat treatment on transacetylase and acetylhydrolase activities

Both activities were determined as described in the Experimental section, in the presence of 30 μ M lyso-PAF and 80 μ M PAF. Values represent means $\pm$ S.D. of four LDL preparations. The transacetylase activity in the absence of any inhibitor was 15.2 ± 1.9 nmol/min per mg of protein, and the acetylhydrolase activity in the absence of any inhibitor was 21.6 ± 5.9 nmol/min per mg of protein.

Inhibitor	Transacetylase activity (%)	Acetylhydrolase activity (%)
None	100	100
Heat treatment (60 °C, 60 min)	90.2 ± 36.4	106.5 ± 2.9
DFP (20 mM)	0	0
Pefabloc (5 μ M)	57.6 ± 11.9	65.0 ± 3.8
Pefabloc (10 μ M)	17.3 ± 5.8	34.6 ± 0.1
Pefabloc (50 μ M)	0	0
Pefabloc (1000 μ M)	0	0

that it increased in proportion to the lyso-PAF concentration present in the assay, from 0.73 ± 0.16 (30 μ M lyso-PAF) to 1.30 ± 0.21 (80 μ M lyso-PAF) and to 2.43 ± 0.86 (120 μ M lyso-PAF) ($P < 0.03$ and $P < 0.03$ respectively). Thus, clearly, the

Figure 3 Effect of LDL oxidation on transacetylase and acetylhydrolase activities

Transacetylase (●) and acetylhydrolase (○) activities were determined as described in the Experimental section, in the presence of 30 μ M lyso-PAF and 80 μ M PAF. Oxidation was performed in the presence of 5 μ M Cu^{2+} and 100 μ g of LDL protein/ml. Values represent the means of six independent LDL preparations (see text for statistics).

transacetylation reaction becomes predominant when the concentration of the lyso-PL is raised above 100 μ M.

Characteristics of transacetylase activity

We next asked whether the serine esterase inhibitors DFP and Pefabloc, which inhibit the acetylhydrolase activity [25,26], were also effective against the transacetylase activity. As shown in Table 1, the two activities associated with LDL were inhibited comparably by these agents. By contrast, both activities were resistant to heat treatment at 60 °C for 1 h, a procedure that completely inactivates LCAT activity [31].

As LDL-associated acetylhydrolase activity is decreased during LDL oxidation [9,10], we performed LDL oxidation with Cu^{2+} *in vitro* and assessed both acetylhydrolase and transacetylase activities. Both activities decreased upon oxidation (Figure 3). The ratio of transacetylase to acetylhydrolase activity was 0.79 ± 0.11 before the initiation of the oxidation, and it increased continuously during oxidation, reaching values of 1.03 ± 0.29 after 12 h ($P < 0.04$) and 1.16 ± 0.11 after 24 h ($P < 0.003$) of oxidation.

These results show that the two activities share the same characteristics upon treatment with serine esterase inhibitors or heating, and were both decreased upon LDL oxidation. It is interesting to note that before oxidation the acetylhydrolase activity dominated, whereas after 12 h of oxidation the two activities attained similar values.

Apparent kinetic characteristics of the transacetylase and acetylhydrolase activities

We subsequently performed studies of transacetylase and acetylhydrolase activities in order to calculate the apparent kinetic constants using LDL as the source of the enzymes. As mentioned above, acetylhydrolase activity was inhibited by lyso-PAF in a dose-dependent manner (Figure 2B). Thus, using two different lyso-PAF concentrations (30 μ M and 120 μ M), we performed kinetic studies of acetylhydrolase activity. As the curves fitted best to the Michaelis–Menten equation (results not shown), we used Lineweaver–Burk plots of the data, and found that the apparent K_m value of the enzyme increased from 24.7 ± 3.7 to

Table 2 Substrate specificity of the transacetylase reaction

Lipids were dissolved in BSA (0.25%, w/v). Cholesteryl acetate was used in micellar form. Activity was determined as described in the Experimental section, in the presence of 30 μ M lyso-PAF and 80 μ M acyl-donor. GPC, *sn*-glycero-3-phosphocholine. Values represent the means of three independent LDL preparations. The transacetylase activity with C16:0 PAF (1-*O*-hexadecyl-2-acetyl-GPC) as donor (100%) was 17.2 ± 3.4 nmol/min per mg of protein.

Acyl donor	Transacetylase activity (%)
C16:0 PAF	100
1- <i>O</i> -Hexadecyl-2-propionyl-GPC	73
1- <i>O</i> -Hexadecyl-2-butanoyl-GPC	40
1- <i>O</i> -Hexadecyl-2-valeroyl-GPC	17
Cholesteryl acetate	0

75.2 ± 0.4 μ M ($P < 0.001$), whereas the apparent $V_{\max}$ decreased from 31.8 ± 7.5 to 13.2 ± 2.2 nmol/min per mg of protein ($P < 0.004$). Results were means $\pm$ S.D. of six independent LDL preparations. These results suggest a mixed type of inhibition of acetylhydrolase by lyso-PAF.

Next we performed kinetic studies of transacetylase activity in the presence of the saturating concentration (30 μ M) of lyso-PAF. The enzyme in LDL exhibited allosteric kinetics that fitted best to the Hill equation (results not shown). Using the Hill plot, we calculated the apparent h , $[S]_{0.5}$ and $V_{\max}$ values to be 2.0 ± 0.3 , 9.4 ± 2.3 μ M and 19.6 ± 3.4 nmol/min per mg of protein respectively. Results were means $\pm$ S.D. of seven independent LDL preparations.

The ratio of transacetylase to acetylhydrolase activity will become greater than 1 due to the inhibitory effect of a high concentration of lyso-PAF, as shown in Figure 2(B), but also due to the sigmoidal kinetics of the transacetylase. In the presence of 30 μ M lyso-PAF and 25 μ M PAF, the transacetylase/acetylhydrolase activity ratio was 1.1 ± 0.06 , whereas in the presence of 80 and 100 μ M PAF the ratio was 0.74 ± 0.06 ($P < 0.03$) and 0.80 ± 0.08 ($P < 0.03$) respectively.

Substrate specificity of transacetylase activity

We studied the specificity of LDL-associated transacetylase activity towards different donor molecules. On increasing the chain length at the *sn*-2 position of the donor molecule from an acetate (C_2) to a valerate (C_5) moiety, the transacylation activity decreased severalfold, whereas cholesteryl acetate (in micellar form) did not serve as a donor of acetate (Table 2). The ether-linked phospholipids were 30% more efficient than their ester-linked analogues. In contrast, no difference was observed in transacetylase activity when the ether-linked lyso-PAF or the ester-linked lyso-PC was used as the acceptor molecule. When lyso-PAF or lyso-PC was used as the acceptor molecule, the transacetylase activity was 17.2 ± 3.4 and 18.9 ± 5.1 nmol/min per mg of protein respectively (means $\pm$ S.D. of three independent LDL preparations). Since in LDL the amount of lyso-PC is at least 100-fold greater than the amount of lyso-PAF [11,27], lyso-PC is likely to be a preferential acceptor of short-chain fatty acids upon transacylation or transacylation.

Distribution of transacetylase activity among lipoprotein subfractions

Using an analytical isopycnic density-gradient ultracentrifugation method, we fractionated plasma lipoproteins into nine subfractions (VLDL+IDL, LDL-1, LDL-2, LDL-3, LDL-4, LDL-5, HDL-2, HDL-3 and VHDL) and we studied both the

Figure 4 Distribution of transacetylase and acetylhydrolase activities in human plasma lipoprotein subfractions

Transacetylase (■) and acetylhydrolase (□) activities were determined as described in the Experimental section, in the presence of 30 μ M lyso-PAF and 80 μ M PAF. Values represent means $\pm$ S.D. of three independent LDL subfraction preparations.

Table 3 Apparent kinetic constants of acetylhydrolase and transacetylase activities in LDL-4 and LDL-5 subfractions

Lipoproteins were fractionated by isopycnic density-gradient ultracentrifugation. Acetylhydrolase and transacetylase activities were determined as described in the Experimental section, in the presence of 30 μ M lyso-PAF and 80 μ M PAF. Apparent kinetic constants were calculated from the Lineweaver–Burk plots for the acetylhydrolase activity, and from the Hill plots for the transacetylase activity. Values represent means $\pm$ S.D. of three independent LDL subfraction preparations. Significance of differences compared with LDL-5 subfraction: * $P < 0.002$, ** $P < 0.03$, *** $P < 0.04$, **** $P < 0.05$.

Subfraction	Transacetylase activity			Acetylhydrolase activity	
	$V_{\max}$	h	$[S]_{0.5}$	$V_{\max}$	K_m
LDL-4	$16.1 \pm 3.3^{***}$	1.9 ± 0.4	$11.0 \pm 2.8^{****}$	$20.7 \pm 3.1^*$	$17.9 \pm 0.7^{**}$
LDL-5	33.5 ± 2.8	2.7 ± 0.6	14.1 ± 2.3	69.6 ± 5.5	31.8 ± 3.4

transacetylase and acetylhydrolase activities associated with each subfraction. The distributions of the two activities among the subfractions were similar, and there was a preferential association with the LDL-5 subfraction (Figure 4).

Based on the observation that the acetylhydrolase activity associated with subfractions LDL-1–LDL-4 possesses different kinetic constants (lower apparent K_m and $V_{\max}$ values) as compared with that in the LDL-5 subfraction [21], we studied the apparent kinetic constants of the transacetylase and acetylhydrolase activities associated with the LDL-4 and LDL-5 subfractions. Using Hill plots, we calculated the apparent $V_{\max}$, h and $[S]_{0.5}$ values of the transacetylase. The apparent $V_{\max}$ and $[S]_{0.5}$ values were significantly higher in the LDL-5 subfraction than in the LDL-4 subfraction (Table 3). Furthermore, the apparent K_m and $V_{\max}$ values of acetylhydrolase (calculated by Lineweaver–Burk plots) were significantly higher in the LDL-5 than in the LDL-4 subfraction (Table 3).

Transacetylase activity of recombinant PAF-AH

To ascertain that PAF-AH is endowed with transacetylase activity, we performed experiments with recombinant PAF-AH, and showed that PAF-AH does indeed carry two enzymic

Figure 5 Effects of recombinant PAF-AH concentration on the transacetylase and acetylhydrolase activities of the enzyme

Transacetylase (●) and acetylhydrolase (○) activities of recombinant PAF-AH were determined as described in the Experimental section, in the presence of 30 μ M lyso-PAF and 80 μ M PAF. Results are means $\pm$ S.D. of three experiments.

Table 4 Kinetic constants for transacetylase and acetylhydrolase activities of recombinant PAF-AH

The kinetic studies were performed as described in the Experimental section. Results are means $\pm$ S.D. of three experiments.

Activity	$V_{\max}$ (pmol/min per ng)	h	$[S]_{0.5}$ (μ M)	K_m (μ M)
Transacetylase	105.2 $\pm$ 4.3	1.7 $\pm$ 0.2	22.2 $\pm$ 0.3	—
Acetylhydrolase	244.3 $\pm$ 5.3	—	—	39.2 $\pm$ 2.0

Table 5 Effects of serine esterase inhibitors on transacetylase and acetylhydrolase activities of recombinant PAF-AH

Results are means $\pm$ S.D. of three experiments. The transacetylase activity without inhibition was 105.2 $\pm$ 4.3 pmol/min per ng of protein, and the acetylhydrolase activity without inhibition was 244.3 $\pm$ 5.3 pmol/min per ng of protein.

Inhibitor	Transacetylase activity (%)	Acetylhydrolase activity (%)
DFP (20 mM)	0	0
Pefabloc (0.1 μ M)	100	100
Pefabloc (0.5 μ M)	97.2 $\pm$ 3.4	87.5 $\pm$ 5
Pefabloc (1 μ M)	39.5 $\pm$ 1.9	27.4 $\pm$ 2.1
Pefabloc (50 μ M)	5.4 $\pm$ 0.4	0.9 $\pm$ 0.9
Pefabloc (100 μ M)	1.2 $\pm$ 0.6	0.1 $\pm$ 0.2
Pefabloc (1000 μ M)	0	0

activities (Figure 5), namely acetylhydrolase and transacetylase. The kinetic characteristics of the two activities, as well the effects of treatment with serine esterase inhibitors, are shown in Tables 4 and 5.

Exogenously added lyso-PAF induces the formation of PAF-like biological activity upon LDL oxidation

Upon LDL oxidation, PAF and its ether-linked analogues with short chains at the *sn*-2 position are formed in LDL or in LDL subfractions with low PAF-AH activity, e.g. LDL-3 [9,11,12]. Addition of 40 μ M synthetic lyso-PAF induced a transient PAF-like aggregating activity between 3 h and 6 h of oxidation in total

Figure 6 Generation of PAF and PAF-like products upon LDL oxidation in the presence of synthetic lyso-PAF

LDL (100 μ g of protein) was oxidized with 5 μ M CuCl_2 for various time intervals up to 24 h. ●, LDL supplemented with 40 μ M lyso-PAF; ○, LDL in the absence of lyso-PAF. Results are means $\pm$ S.D. of six independent LDL preparations.

Figure 7 Formation of PAF and PAF-like analogues in LDL subfractions

LDL-3 and LDL-5 subfractions prepared by isopycnic density-gradient ultracentrifugation, as described in the Experimental section, were oxidized with 5 μ M CuCl_2 per 100 μ g of protein for 3 h in the absence of lyso-PAF (■) or in the presence of 40 μ M C16:0 lyso-PAF (□). Results are means $\pm$ S.D. for each LDL subfraction prepared by density-gradient ultracentrifugation from plasma samples obtained from three volunteers.

LDL (Figure 6). Next we added 40 μ M lyso-PAF to LDL-3 and to LDL-5, and allowed oxidation with copper to proceed for 3 h. We observed a moderate, but statistically insignificant, increase in PAF-like aggregating activity in LDL-3, compared with a huge increase ($P < 0.01$) in LDL-5 (Figure 7). As the latter subfraction contains the majority of PAF-AH associated with lipoproteins [21], we postulate that the latter enzyme may be responsible for the transacetylation or transacylation of lyso-PAF added to the oxidation assay.

DISCUSSION

We show here for the first time that human plasma PAF-AH is clearly endowed with two activities: one that degrades PAF and a second that is involved in the transacetylation or transacylation of short (up to C_5) chains of lyso-PAF and lyso-PC. Several findings support the above conclusion: (i) similar behaviour of acetylhydrolase and transacetylase towards serine esterase inhibitors and heat treatment, (ii) their sensitivity to Cu^{2+} -induced oxidation, (iii) their similar distributions in lipoprotein classes and LDL subspecies, (iv) competitive inhibition of acetylhydrolase activity by lyso-PAF (increased K_m), and (v)

expression of acetylhydrolase and transacetylase activities by recombinant PAF-AH.

Our results agree with former studies showing that purified transacetylase from rat lung was bifunctional, catalysing both the deacylation and the transacylation of lyso-PL [28]. Additionally, rat kidney intracellular transacetylase was shown to be the same enzyme as bovine PAF-AH II [29]. LCAT and semi-purified PAF-AH were also shown to catalyse the transfer of short-chain fatty acid chains from PAF to lyso-PC [6]. LCAT protein, which possesses various activities, including phospholipase A₂ [30], acetylhydrolase [6], acyltransferase (LAT) [31] and transacetylase (LAT II) [6], could account, at least partially, for the transacetylase activity observed in the present study. However, the pattern of distribution of LCAT (which is associated mainly with HDL) [32], its heat sensitivity [31] and its resistance to Pefabloc [33] indicate that LCAT is not involved in the transacylation reaction observed in LDL.

In the absence of oxidative stress, only low levels of lyso-PL are detectable in LDL [27], and thus acetylhydrolase activity will predominate and play a strategic role in the hydrolytic removal of PAF and its biologically active analogues from LDL. However, upon LDL oxidation, as lyso-PL formation increases [9,27] and PAF and its analogues are formed in LDL particles [9,11,12], the importance of the transacetylase reaction may increase. We showed that, at high lyso-PL concentrations, the transacetylase activity became higher than that of acetylhydrolase. Additionally, we showed that the sigmoidal kinetics favour transacetylase over acetylhydrolase in the presence of low PAF concentrations.

The ether-linked phospholipids are responsible for the majority of PAF and PAF-like biological activity upon LDL oxidation [9,11,12]. In the present study, we showed that lyso-PAF and lyso-PC possess similar affinities in the transacetylase reaction. As generation of lyso-PC upon LDL oxidation is 100 times more efficient than generation of lyso-PAF [11,27], we can predict that the transacetylase reaction, by transferring short-chain fatty acids from biologically active ether-linked phospholipids to the biologically less active ester-linked phospholipids, may contribute to the removal of pro-inflammatory mediators from LDL particles upon oxidation.

However, as shown in our experiments with exogenously added lyso-PAF in the LDL oxidation assay, it is possible that transacetylase may produce substantial amounts of PAF, and presumably other PAF-like species. PAF [1], 1-hexadecanoyl-2-acetyl-*sn*-glycero-3-phosphocholine [34–36] and lyso-PAF [37] are released at sites of inflammation from pro-inflammatory cells. Therefore the transacetylase reaction associated with LDL may transform lyso-PAF into the biologically active PAF and its analogues with longer chains at the *sn*-2 position of the molecule. The latter reaction may occur in the atherogenic small dense LDL-5 particles [38], which are endowed with the highest acetylhydrolase/transacetylase activity, and may confer on these particles higher pro-inflammatory potential in atherosclerosis-prone areas. This could be a partial explanation for the pro-atherogenic effects of LDL-associated PAF acetylhydrolase activity described in recent studies [39–41].

We conclude that PAF-AH is endowed with both transacetylase and acetylhydrolase activities. The transacetylase activity in LDL, together with the acetylhydrolase, acts to remove PAF and its analogues from the LDL particles upon oxidation. However, in atherogenic small dense LDL-5 particles, this activity may transform extracellular lyso-PAF into biologically active molecules with pro-inflammatory activity.

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