Non-enzymatic platelet-activating factor formation by acetylated proteins

Paraskevi G. Thanou, Demokritos C. Tsoukatos*

Laboratory of Biochemistry, Department of Chemistry, University of Ioannina, Ioannina 45110, Greece

Received 12 May 2004; revised 1 July 2004; accepted 1 July 2004

Available online 28 July 2004

Edited by Gerrit van Meer

Abstract Substantial amounts of platelet-activating factor (PAF 1-*O*-alkyl-2-acyl-sn-glycero-3-phosphocholine), the potent phospholipid mediator of allergic and inflammatory reactions, are formed upon incubation of acetylated low-density lipoprotein, acetylated bovine serum albumin (BSA) and acetylated apolipoprotein A-I with 1-0-hexadecyl-sn-glycero-3-phosphocholine (lyso-PAF). Acetylated BSA produced 0.3 nmol PAF/mg of protein after a 6 h incubation period with 40 μM lyso-PAF. The transfer of acetate bound to acetylated proteins to lyso-PAF was non-enzymatic. Chemical PAF formation by acetylated proteins, involved in lipid metabolism and transport, could lead to complication of inflammatory and allergic events.

Keywords: Platelet-activating factor; Lyso platelet-activating factor; Acetylated proteins; Non-enzymatic acetylation

1. Introduction

Platelet-activating factor (PAF 1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a potent lipid mediator of inflammatory and allergic reactions [1]. PAF synthesis and degradation by inflammatory cells and its circulation in plasma are tightly enzymatically regulated [2].

Biological protein acetylation is confined to specific acetyl-CoA-dependent acetyltransferases [3]. However, protein acetylation by an acetyl-CoA-independent transacetylase [4] and the non-enzymatic acetylation of proteins [5] have also been reported. The latter two reactions are utilizing acetylated xenobiotics as the active acetyl donors.

The aim of the present study was to investigate a cell-independent PAF formation by acetylated proteins (bovine serum albumin (BSA) and apolipoprotein A-I (Apo A-I)) or acetylated low-density lipoprotein (acetylated LDL) involved in phospholipid transfer and metabolism in plasma. A non-specific non-enzymatic transfer of acetate from acetylated proteins to the precursor of PAF synthesis, 1-*O*-hexadecyl-sn-glycero-3-phosphocholine (lyso-PAF), was observed. PAF formation by acetylated proteins in vivo could lead to complication of inflammatory and allergic events.

* Corresponding author. Fax: +3026510-47832.
E-mail address: dtsoykat@cc.uoi.gr (D.C. Tsoukatos).

Abbreviations: PAF, platelet-activating factor (1-*O*-alkyl-2-acyl-sn-glycero-3-phosphocholine); lyso-PAF, 1-*O*-hexadecyl-sn-glycero-3-phosphocholine; C16:0 PAF, 1-*O*-hexadecyl-PAF; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BCA, bicinchoninic acid; Pefabloc, 4-[2-aminoethyl]benzenosulfonyl fluoride; apo A-I, apolipoprotein A-I; BSA, bovine serum albumin; PBS, phosphate buffered saline

2. Materials and methods

2.1. Materials

1-*O*-Hexadecyl-2-acetyl-sn-glycero-3-phosphocholine (C16:0 PAF), 1-*O*-hexadecyl-sn-glycero-3-phosphocholine phosphocreatine, creatine kinase $CaCl_2$, $MgCl_2 \cdot 6H_2O$, and $FeSO_4 \cdot 7H_20$ were from Sigma; 4-[2-aminoethyl]benzenosulfonyl fluoride (Pefabloc) SC and bicinchoninic acid (BCA) protein reagent were from Pierce; acetic anhydride was from Fluka. Solvents were from Lab-Scan. Pre-coated silica gel TLC plates were purchased from Merck. Sepharose 6B was obtained from Pharmacia.

LDL and high-density lipoprotein (HDL) were prepared from freshly isolated human plasma by sequential ultracentrifugation in a Beckman L7-65 ultracentrifuge as described in [6]. Apo A-I was purified from HDL according to [7]. The protein content was determined by the BCA method [8], using BSA as a standard. BSA and delipidated BSA were purchased from Sigma.

2.2. Acetylation of proteins

Proteins were acetylated by acetic anhydride in a saturated sodium acetate aqueous solution. 1 ml of an aqueous protein solution (approx. 1 mg/ml) and equal volume of a saturated sodium acetate aqueous solution were mixed and stirred gently at 4 °C. 25 μl of acetic anhydride was added in portions of 5 μl for every 15 min and the reaction mixture was allowed to react for 30 more min. Solutions of the acetylated proteins were dialyzed at 4 °C against two changes of 5 l of phosphate buffered saline (PBS) containing 0.05% EDTA at pH 7.4 for 24 h, then were filter-sterilized and analyzed for their protein content. The solutions were stored at 4 °C. The acetylation of proteins was determined by measuring the electrophoretic mobility relative to native proteins on agarose gels.

2.3. PAF formation

Acetylated proteins in PBS (20 mM of phosphate salts) containing 0.05% EDTA at pH 7.4 were incubated in the presence of lyso-PAF dissolved in PBS containing 0.25% BSA. The final concentrations were 0–100 μg of protein/ml, 0–160 μM lyso-PAF and 0.25 mg/ml BSA, in a reaction mixture of 1.0 ml. Reaction was performed in glass tubes for 0–10 h at 37 °C. In some experiments, 50 μg/ml of acetylated BSA was incubated with 40 μM of lyso-PAF for 6 h in PBS containing 0.05% EDTA at pH 3.0 (adjusted with 12 N HCl) and at pH 9.0 (adjusted with 10 N NaOH). In other experiments, the incubation temperatures were at 4 and 50 °C. Other experiments yet were performed in the presence of 5 mM Ca^{2+}, 5 mM Mg^{2+} or 0.15 mM Fe^{2+}. In the experiments with metal ions EDTA was omitted from the incubation buffer and in the experiment with Ca^{2+} PBS was replaced by Tris buffered saline. The reaction was stopped by extracting the lipids according to Bligh and Dyer [9]. Total lipids were then subjected to TLC on silica-gel G plates by using chloroform/methanol/water (65:35:6, v/v) as a solvent system. Lipids were identified after a brief exposure to iodine. The band corresponding to the R_f of authentic PAF was scraped off the plate and PAF was extracted from silica gel by the method of Bligh and Dyer. The TLC purification was necessary in

0014-5793/$22.00
doi:10.1016/j.febslet.2004.07.037

order to remove possible platelet aggregation lipid inhibitiors present in acetylated LDL or in acetylated BSA. The TLC-purified lipids were dissolved in 60% (v/v) ethanol and assayed for platelet-aggregating activity against aspirin-treated washed rabbit platelets as described previously [10]. Platelet aggregation assays were performed using a Chronolog aggregometer in the presence of the ADP scavenger complex phosphocreatine (1 mM)/creatine kinase (10 U/ml). The aggregating activity of PAF was expressed as nM of C16:0 PAF equivalents using a calibration curve obtained with standard solutions of C16:0 PAF. In the experiments with acetylated LDL, the lipoprotein was preincubated with 1 mM Pefabloc for 30 min at 37 °C before the addition of lyso-PAF.

In some experiments, acetylated LDL and acetylated BSA were purified by size exclusion chromatography before the assay. The solutions of proteins were applied to a Sepharose 6B column using an aqueous solution containing 10 mM Tris–base (Tris–(hydroxylmethyl)aminomethane), 1 mM EDTA, 1 mM NaCl and 0.02% NaN_3 at pH 7.4 as a solvent. The eluted protein solutions were concentrated using an Amicon concentrating apparatus and dialyzed as described above.

2.4. Purification of PAF by RP-HPLC and characterization by GC–MS

In order to determine the molecular species responsible for PAF-like bioactivity, in some experiments the TLC purified lipids were further purified by RP-HPLC and analyzed by GC–MS. Samples containing a known quantity of PAF-like bioactivity were suspended in 25 μl of HPLC mobile phase (ammonium acetate (10 mM)/acetonitrile/methanol (120:140:40, v/v/v)) before separation of the molecular species of PAF on a reversed-phase Spherisorb C6 column (Touzart et Matignon, Vitry/Seine, France). The retention time of PAF was determined using [^{3}H] labeled C16:0 standard as described in [11]. Fractions were collected, extracted with chloroform, dried and assayed for PAF content by the washed rabbit platelet bioassay. The yield of platelet-aggregating activity of PAF upon separation by reversed-phase HPLC varied between 70% and 80%.

The biologically active material, which was recovered from reversed phase-HPLC with the retention time of C16:0 PAF, was further analyzed by GC/MS as described in [12]. In brief, the samples from reversed phase-HPLC were added to tubes which contained 20 ng of the stable, isotopically labeled variant of PAF, 1-*O*-hexadecyl-2(D3)-acetyl-glycero-3-phosphocholine (D3-PAF). The samples were redissolved in ethanol and applied to silica solid phase extractor tubes (Varian, Harbor City, CA, USA). The tubes were washed with 4 ml ethanol and then eluted with 4 ml of methanol:water (4:1). The samples were then dried and subjected to phospholipase C cleavage. The diglycerides thus produced were extracted into methylene chloride, dried and then derivatized with pentafluorobenzoyl chloride. The pentafluorobenzoyl derivatives were subsequently analyzed by negative ion chemical ionization GC/MS with a Finnigan Mat (San Jose, CA) SSQ70 mass spectrometer, as described in [12].

3. Results

A lipoprotein (LDL), a HDL apolipoprotein (apo A-I), a plasma lipid carrier protein (BSA), plus its delipidated form were submitted to chemical acetylation as described in Section 2. The higher electrophoretic mobility of acetylated proteins relative to controls on agarose gel electrophoresis suggests acetylation at positive charged NH_2 containing residues, via amide bond formation. As shown in Fig. 1, upon incubation with lyso-PAF, acetylated BSA was able to produce a PAF-like bioactivity. Similar results were obtained with the other proteins or the lipoprotein of the study. RP-HPLC purification followed by GC–MS analysis revealed that the bioactive product of the reaction was C16:0 PAF (Fig. 2). The ion peak at *m/z* 552 was the $[M+H]^+$ of the pentafluorobenzoyl derivative of C16:0 PAF, after hydrolysis with phospholipase C. Its retention time on Gas Chromatography was identical with those of the internal standard D3-PAF derivative (*m/z* 555). Results with acetylated LDL are presented in Fig. 2. Similar

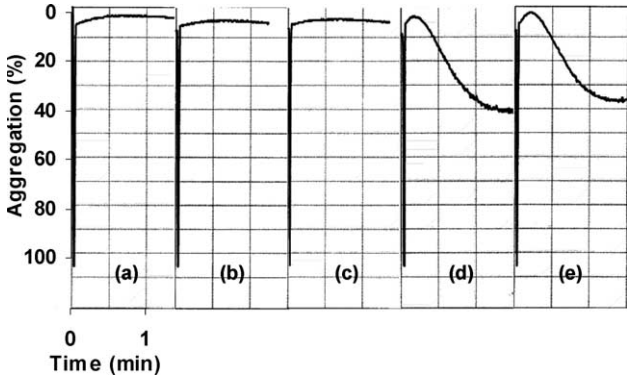

Fig. 1. PAF-like aggregating activity formation in acetylated BSA. Washed rabbit platelets were stimulated with (a) 40 μM lyso-PAF, (b) 50 μg/ml acetylated BSA, (c) sample obtained as described under Section 2 upon 6 h incubation of 50 μg/ml BSA with 40 μM lyso-PAF, (d) sample obtained as described under Section 2 upon 6 h incubation of 50 μg/ml acetylated BSA with 40 μM lyso-PAF, and (e) standard C16:0 PAF.

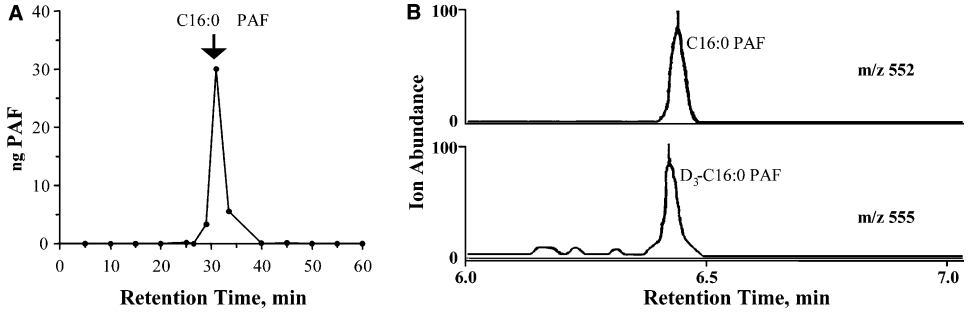

Fig. 2. (A) Reversed phase HPLC purification of PAF-like molecules formed in Pefabloc treated acetylated LDL incubated for 6 h in the presence of 40 μM lyso-PAF. Bioactive phospholipids with the R_f of PAF on TLC were separated by C6 RP-HPLC as described under Section 2. Fractions were tested for their ability to aggregate washed rabbit platelets and the bioactivity was expressed as ng of C16:0 PAF equivalents. Arrow indicates the RT of synthetic C16:0 PAF. (B) GC/MS analysis of the RP-HPLC purified PAF-like molecule. The PAF-like molecule with the RT of C16:0 PAF on RP-HPLC was analyzed by GC/MS as described under Section 2. In the figure, reconstructed ion chromatogram obtained during GC/MS analysis for the specific ions *m/z* 552 and *m/z* 555 is shown. The ion *m/z* 552 corresponds to C16:0 PAF derivative, present in the sample, and the ion *m/z* 555 to 20 ng of the internal standard D3-PAF derivative, respectively. The ratio of the ion abundance area of the elution of the D3-PAF diglyceride to that of the unlabeled PAF diglyceride was used to calculate the quantity of C16:0 PAF in the sample.

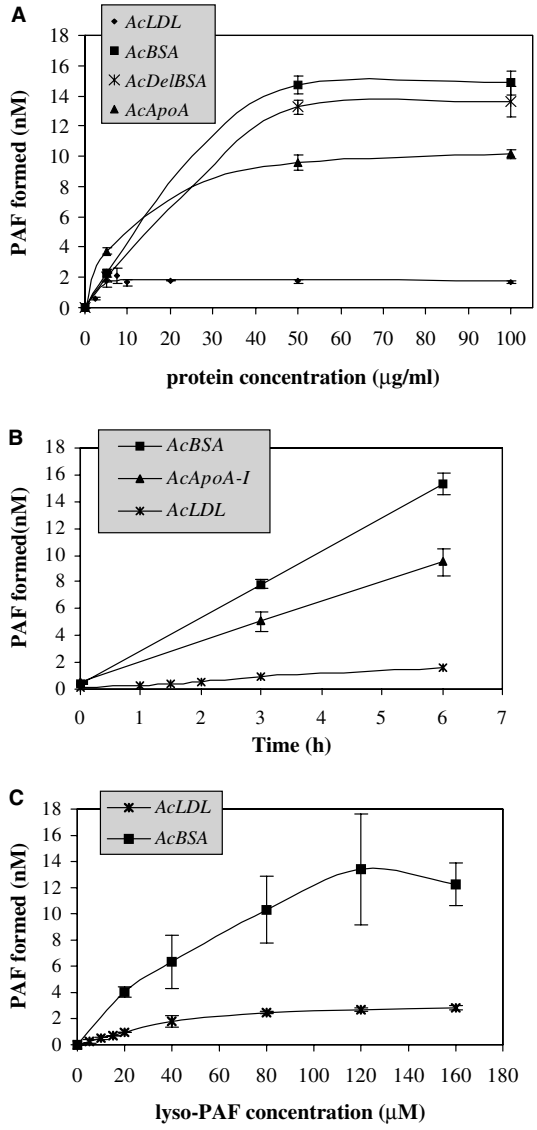

Fig. 3. (A) PAF formation by acetylated proteins. Acetylated proteins (0–100 μg/ml) were incubated with lyso-PAF (40 μM) at 37 °C for 6 h. Acetylated LDL was preincubated with 1 mM Pefabloc for 30 min at 37 °C before the addition of lyso-PAF. Values are means ± S.D. of three experiments. (B) PAF formation by acetylated proteins as a function of time. Acetylated proteins and Pefabloc treated acetylated LDL (50 μg/ml) were incubated with lyso-PAF (40 μM) at 37 °C for the indicated periods. Values are means ± S.D. of three experiments. (C) PAF formation by acetylated proteins as a function of lyso-PAF concentration. Acetylated proteins and Pefabloc treated acetylated LDL were incubated with lyso-PAF at 37 °C for 6 h. Values are means ± range of two experiments.

results were also obtained with acetylated BSA. Quantification of C16:0 PAF by the washed rabbit platelet aggregation assay, and by the RP-HPLC GC–MS analysis described in the legend of Fig. 2, gave similar results, thus throughout the study PAF quantification was performed by the bioassay. The dependence of PAF formation from the concentration of acetylated proteins is shown in Fig. 3A. PAF formation was linear up to 6 h of incubation (Fig. 3B) and increased up to 120 μM of lyso-PAF (Fig. 3C). The amount of PAF formed was the highest in acetylated BSA reaching 0.3 nmol/mg protein upon 6 h incubation of 50 μg/ml protein with 40 μM lyso-PAF. The amount of PAF was similar in acetylated delipidated BSA, but lower in acetylated apo A-I. Acetylated LDL produced the lowest amount of PAF and PAF formation was observed only upon inhibition of the LDL attached PAF acetylhydrolase by 1 mM Pefabloc treatment of LDL for 30 min at 37 °C [13]. PAF formation was not observed in acetylated LDL in the absence of Pefabloc treatment.

Table 1
Rate of PAF formation by acetylated BSA under different incubation conditions

Incubation conditions	% Of control rate
Control[a]	100 ± 32[b]
4 °C	4 ± 0
50 °C	130 ± 28
pH 3.0	4 ± 1
pH 9.0	281 ± 19
Ca^{2+} 5 mM	82 ± 15
Mg^{2+} 5 mM	113 ± 23
Fe^{2+} 0.15 mM	106 ± 24

Values represent the mean ± range of two experiments.
[a] Control incubation conditions were: 50 μg/ml acetylated BSA protein incubated with 40 μM lyso-PAF at 37 °C and pH 7.4 for 6 h.
[b] The control rate in these experiments was 6.3 nM PAF per 6 h.

In order to test the possibility that acetate bound to acetylated protein and non-free acetate of the incubation mixture are transferred to lyso-PAF, we submitted acetylated LDL and acetylated BSA to Size Exclusion Chromatography prior to the incubation with lyso-PAF. The amount of PAF bioactivity formed was similar before and after the chromatographic purification, suggesting that acetate bound to protein residues rather than free acetate was transferred to lyso-PAF.

Characterization of the reaction with acetylated BSA as the donor protein is shown in Table 1. The rate of the reaction was increased at pH 9 and was very low at pH 3. Moreover, the reaction rate was increased with rising temperature from 4 to 50 °C. The reaction was independent from the presence of metal ions at normal plasma concentrations.

4. Discussion

Our results showed that substantial amounts of PAF are formed by acetylated proteins incubated with lyso-PAF. A transfer of acetate, bound to protein, to lyso-PAF seems to be responsible for the above formation. We recently reported an enzymatic transfer of acetate from acetylated phospholipids to lyso-phospholipids, in LDL, catalyzed by the LDL attached PAF acetylhydrolase [14]. The above enzymatic transfer was sensitive to serine esterase inhibitors and was inhibited 100% by 1 mM Pefabloc. The insensitivities to Pefabloc formation of PAF in acetylated LDL and the non-specific formation of PAF by the other acetylated proteins of the study, which are not possessing enzymatic transacetylase activity, suggest that the transfer of acetate is chemical rather than enzymatic. The characteristics of the reaction and especially its pH dependence further support the above assumption. The nucleophilic attack of the sn-2 OH group of lyso-PAF on the amide bond, which may lead to acetate transfer and subsequently to PAF formation, is expected to be enhanced at high pH values.

Enzymatic and non-enzymatic biological protein acetylation have been reported. The acetyl-CoA-dependent acetylation of

histones, catalyzed by histone acetyl transferase, stands prominent [3]. Moreover, various types of proteins are known to undergo non-enzymatic acetylation when treated with aspirin [15,16]. Recently, an enzyme in rat liver microsomes that catalyzes the transfer of acetyl group from acetoxy xenobiotics to specific proteins was also described [4]. Enzyme acetylation is leading to modulation of their catalytic activities. The most familiar example is the observation that aspirin (acetylsalicylate) acetylates cyclooxygenase resulting in the inhibition of prostaglandin synthesis [5].

The protein, the lipoprotein and the apolipoprotein selected in the present study are involved in lipid transfer and metabolism in plasma. A biological function of albumin is to transport lipids by binding and solubilizing plasma fatty acids and lyso-phospholipids. A significant fraction of cellular PAF is secreted and transported by the circulation in association with albumin [17]. Apo A-I, a HDL apolipoprotein, is important in initiating reverse cholesterol transport and is the apoprotein activator of lecithin-cholesterol acyltransferase [18–20]. LDL modification by oxidation or chemical and enzymatic treatment is thought to be an initiating and sustaining event in atherogenesis [21,22]. The chemically modified LDL by acetylation is the first modified LDL discovered, which was taken up by the scavenger receptor of the monocyte derived macrophages leading to foam cell formation, the early step of atherogenesis [23]. Thus, acetylated LDL is used in experiments as a model of modified LDL [24]. PAF formation in acetylated LDL may be at least partially responsible for the inflammatory atherogenic effect of these particles.

A chemical acetylation of lyso-PAF by aspirin has been reported [25]. Moreover, aspirin is able to acetylate BSA [16,26]. PAF formation observed in the present study by acetylated BSA was several times higher than the one reported resulting from mixing lyso-PAF with aspirin in a buffer [25]. Thus mediation of a protein may enhance chemical PAF formation by a widely used acetylated xenobiotic.

It is not clear whether non-enzymatic formation of PAF from lyso-PAF and acetylated proteins takes place in vivo and further detailed studies are indispensable to clarify if non-enzymatic formation of PAF is actually of practical importance. PAF synthesis and degradation in cells and in blood are tightly enzymatically regulated. The results of the present study suggest that chemical formation of PAF by proteins acetylated by physiological metabolites or xenobiotics may break down the above regulatory system, leading to complications of inflammatory and allergic events.

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