Molecular and mechanistic characterization of platelet-activating factor-like bioactivity produced upon LDL oxidation

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Abstract **Oxidation of LDL is thought to be involved in both initiating and sustaining atherogenesis through the formation of proinflammatory lipids and the covalent modification of LDL particles. Platelet-activating factor (PAF; 1-0-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent phospholipid mediator involved in inflammation. Upon oxidation of LDL, oxidized phospholipids with PAF-like structure are generated, and some of them may act via the PAF receptor. We evaluated the contribution of 1-0-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C16:0 PAF) and of other PAF analogs on the PAF-like bioactivity formed upon Cu^{2+}-initiated oxidation of LDL. Reverse-phase HPLC purification and electrospray ionization-MS analyses showed that upon oxidation of LDL with inactivated PAF-acetylhydrolase (PAF-AH), C16:0 PAF accounted for >30% of PAF-like biological activity and its *sn*-2 butenoyl analog accounted for >50%. However, upon LDL oxidation in the presence of exogenous 1-0-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF) without PAF-AH inactivation, C16:0 PAF formation accounted for >90% of the biological activity recovered. We suggest that the C16:0 PAF, despite being a minor constituent of the LDL peroxidation products, may contribute substantially to the bioactivity formed in oxidized LDL.** **The higher bioactivity of C16:0 PAF, and the higher selectivity of the LDL-attached lyso-PAF transacetylase toward very short acyl chains [acetate (C2) vs. butanate (C4)], may explain the contribution described above.**—Androulakis, N., H. Durand, E. Ninio, and D. C. Tsoukatos. **Molecular and mechanistic characterization of platelet-activating factor-like bioactivity produced upon LDL oxidation.** ***J. Lipid Res.*** **2005.** 46: **1923–1932.**

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Atherosclerosis is a chronic inflammatory disease (1). Free radical-mediated oxidation of cholesterol-rich LDL plays a key role in the development of fatty streaks and the subsequent formation of lipid-rich, atheromatous plaques (2). The preferential retention of LDL in the intima upon interaction with extracellular matrix components, such as proteoglycans, exposes these particles to oxidative stress, involving the action of reactive oxygen species and of transition metals (3). The atherogenic effect of oxidative modified LDL is thought to be mediated through the formation of inflammatory lipids and the covalent modification of the particle (4, 5).

Platelet-activating factor (PAF; 1-0-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent phospholipid mediator involved in inflammatory reactions (6) that may play an equally crucial role in atherogenesis (reviewed in 7). Its actions, on cells and processes that constitute the inflammatory system, are mediated via the PAF receptor (8). The PAF receptor is a single specific receptor belonging to the family of seven transmembrane-spanning G-protein-linked receptors and has two important recognition requirements: one is for a specific head group (choline) and the second is for a specific atypical short-chain *sn*-2 residue (8, 9).

Upon oxidation of LDL, myriad oxidized phospholipids, with structures resembling the structure of PAF,

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Abbreviations: acetyl-PC, 1-hexadecanoyl-2-acetyl-*sn*-glycero-3-phosphocholine; butanoyl-PAF, 1-0-hexadecyl-2-butanoyl-*sn*-glycero-3-phosphocholine; butanoyl-PC, 1-hexadecanoyl-2-butanoyl-*sn*-glycero-3-phosphocholine; butenoyl-PAF, 1-0-hexadecyl-2-butenoyl-*sn*-glycero-3-phosphocholine; CP, creatine phosphate; CPK, creatine phosphokinase; D3-PAF, 1-0-hexadecyl-2(D3)-acetyl-glycero-3-phosphocholine; ESI, electrospray ionization; lyso-PAF, 1-0-alkyl-*sn*-glycero-3-phosphocholine; C16:0 lyso-PAF, 1-0-hexadecyl-*sn*-glycero-3-phosphocholine; C16:0 lyso-PC, 1-hexadecanoyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor (1-0-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); C16:0 PAF, 1-0-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF-AH, platelet-activating factor-acetylhydrolase; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; Pefabloc, 4-[2-aminoethyl]benzenesulfonyl fluoride; propionyl-PAF, 1-0-hexadecyl-2-propionyl-*sn*-glycero-3-phosphocholine; RP, reverse-phase.

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are generated by the fragmentation of the *sn*-2 esterified PUFAs. The reaction is free radical-mediated and triggered by pro-oxidants such as metal ions (10–12). Some of the oxidatively generated PAF analogs may act via the PAF receptor (13, 14).

PAF and PAF analogs are hydrolyzed by platelet-activating factor-acetylhydrolase (PAF-AH; EC 3.11.48), which is a Ca^{2+}-independent enzyme belonging to group 7 of the family of phospholipases A_2 (15). This enzyme inactivates PAF and PAF analogs by hydrolyzing its *sn*-2 short-chain groups (16–18); additionally, it is capable under certain conditions of transacylase activity (19, 20). PAF-AH in plasma is associated mainly with LDL and to a lesser extent with HDL (17). Upon oxidative modification of unfractionated LDL, the acetylhydrolase activity of PAF-AH decreases dramatically and the oxidized LDL loses its anti-inflammatory property (21, 22). When the residual PAF-AH is totally abolished by chemical manipulation, a PAF-like bioactivity accumulates in oxidized LDL as a consequence (21).

The association of PAF-AH with LDL particles is heterogeneous, as the majority of the enzyme is associated with small, dense LDLs (d = 1.050–1.063 g/ml) (23, 24). We have shown previously that upon oxidation of the intermediate LDL subfraction (d = 1.029–1.039 g/ml) with low PAF-AH activity, a substantial amount of PAF could be formed in vitro without the need for chemical inactivation of PAF-AH (25).

It was also reported that the LDL-attached PAF-AH possesses the transacetylase activity that catalyzes a transfer of acetate between phospholipids and leads to the formation of PAF (20). Such transacetylase activity may exceed the acetylhydrolase activity in the presence of exogenously added lyso-phospholipids (20). As a consequence, a PAF-like bioactivity is formed transiently during the first hour of LDL oxidation in the presence of exogenous 1-0-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF) without chemical inactivation of PAF-AH (20).

The PAF-like biological activity generated in the oxidized LDL particles is derived from the ether-linked phosphatidylcholines (21, 25, 26). Regarding the molecules that contribute to this PAF-like bioactivity, we have shown that upon 3 h of Cu^{2+}-initiated oxidation of the intermediate LDL subfraction, without PAF-AH inactivation, 1-0-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C16:0 PAF) was the main molecule responsible for the PAF-like bioactivity (25). Marathe et al. (26) have shown that upon Cu^{2+}-induced oxidation of LDL in the course of 24 h, with chemically inactivated PAF-AH, the butenoyl and butanoyl PAF analogs were the main components of the bioactivity. Thus, the contribution of C16:0 PAF to the PAF-like bioactivity formed in oxidized LDL deserved further investigation.

In the present study, we have focused on the contribution of C16:0 PAF and other PAF analogs to the PAF-like biological activity formed upon LDL oxidation under various conditions. The aim was to elucidate the mechanism of formation of the bioactive phospholipids upon oxidation of LDL.

MATERIALS AND METHODS

Materials

C16:0 PAF, 1-0-hexadecyl-*sn*-glycero-3-phosphocholine (C16:0 lyso-PAF), 1-hexadecanoyl-*sn*-glycero-3-phosphocholine (C16:0 lyso-PC), fatty acid-free BSA, creatine phosphate (CP), creatine phosphokinase (CPK), and butyric anhydride were from Sigma; valeric anhydride and propionic anhydride were from Aldrich; acetic anhydride was from Fluka; 4-[2-aminoethyl]benzenesulfonyl fluoride (Pefabloc) and bicinchoninic acid protein reagent were from Pierce. Liquid scintillation fluid (Optiphase Hi-Safe 3) was supplied by Zinsser Analytic, Berkshire, UK. BN 52021 was kindly provided by Dr. P. Braquet (Institut Henri Beaufour, Paris, France), and WEB 2086 was a gift from Boehringer Ingelheim. Solvents, HPLC grade, were from Lab-Scan. 1-0-Hexadecyl-2-[^{3}H-acetyl]*sn*-glycerophosphocholine (10 Ci/mmol), 1-0-[^{3}H]hexadecyl-*sn*-glycerophosphocholine (58.3 mCi/mmol), and 1-[palmitoyl-1-^{14}C]phosphatidylcholine (50.5 mCi/mol) were from Du Pont-New England Nuclear. 1-Octadecyl-2-[^{3}H-acetyl]*sn*-glycerophosphocholine (80–180 Ci/mmol) was from Amersham International. Lipase from *Rhizopus arrhizus* was supplied by Boehringer Mannheim.

Preparation of PAF analogs

The ether- and ester-linked analogs of PAF were prepared by acylation of C16:0 lyso-PAF or C16:0 lyso-PC with the appropriate anhydride dissolved in anhydrous pyridine, as described (27). The products were purified by TLC on silica-gel G plates purchased from Merck using chloroform-methanol-water (65:35:6, v/v) as a solvent system. In this TLC system, the relative mobility (R_f) values of lyso-PAF and PAF were 0.11 and 0.17, respectively. The propionyl, butanoyl, and valeroyl PAF analogs showed the following R_f values: 0.21, 0.24, and 0.28, respectively. The corresponding acyl analogs showed similar R_f values as their ether counterparts. The purified phospholipids were quantified by phosphorus determination (28, 29). The radiolabeled analogs were prepared by acylation of [^{3}H]C16:0 lyso-PAF or [^{14}C]C16:0 lyso-PC with the appropriate anhydride dissolved in anhydrous pyridine.

Preparation and oxidation of LDL

Plasma LDL (d = 1.019–1.063 g/ml) was 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 40,000 rpm at 14°C for 10 h with a type NVT 65 rotor as described previously (30). Subjects were healthy, normolipidemic volunteers. Lipoproteins were washed by a second centrifugation step and dialyzed at 4°C against two changes of 5 liters of PBS (10 mmol/l) containing 0.01% EDTA at pH 7.4 for 24 h. LDL was then filter-sterilized and analyzed for its protein content. Lipoproteins were stored at 4°C and used within 2 weeks from their preparation. The protein content of the lipoproteins was determined by the bicinchoninic acid method using BSA as a standard (31).

Before oxidation, LDL was dialyzed extensively against 10 mM PBS, pH 7.4, at 4°C to remove EDTA. A portion of the LDL was treated for 30 min with 1 mM Pefabloc to inactivate PAF-AH (32). LDL, both treated and untreated with Pefabloc, was oxidized by incubating LDL (0.1 mg protein/ml) with 5 μM $CuSO_4$ in 10 mM PBS, pH 7.4, at 37°C for 3, 6, or 24 h in both the presence and absence of 40 μM exogenous C16:0 lyso-PAF. Oxidation was terminated by the addition of 0.01% EDTA and refrigeration. The rate of oxidation was deduced from changes in relative electrophoretic mobility on agarose gels compared with native LDL and by the content of thiobarbituric acid-reactive substances (33).

Measurement of PAF-like biological activity in oxidized LDL

Total lipids of oxidized LDL were extracted according to Bligh and Dyer (34) and separated by TLC on silica-gel G plates using chloroform-methanol-water (65:35:6, v/v) as a solvent system. Lipids were identified after brief exposure in iodine vapors, and the band corresponding to the R_f of PAF and its analogs (R_f = 0.17–0.28) was scraped off the plate. The TLC-purified lipids were dissolved in 60% ethanol and assayed for platelet-aggregating activity using aspirin-treated washed rabbit platelets (35). Platelet aggregation assays were performed on a Chronolog aggregometer in the presence of the ADP scavenger complex, CP (1 mM) and CPK (10 U/ml). PAF aggregating activity was expressed as picomoles of C16:0 PAF equivalents per milligram of LDL protein using a calibration curve with standard solutions of PAF (35). Aggregation was characterized as PAF-like by two factors: *a*) its inhibition by the specific PAF receptor antagonist BN 52021 or WEB 2086; and *b*) its resistance in the treatment of lipase from *Rhizopus arrhizus* as described (36).

PAF and PAF-like analogs were tested for their ability to increase intracellular Ca^{2+} concentration in THP-1 cells. THP-1 cells (4×10^6 cells in HBSS buffer supplemented with 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mM HEPES; referred to as HBSS) were used for each measurement and were loaded for 45 min with Fura-2 in the presence of pluronic acid (0.02%). They were then washed by centrifugation and resuspended in HBSS buffer. PAF or its analogs was added into the cell suspension after 2 min to permit stabilization. The Ca^{2+} concentration fluxes were quantified by a dual-wavelength spectrofluorometer (SAFAS) measuring the ratio of the fluorescence emitted at 510 nm by the cells excited at 340 and 380 nm. Ca^{2+} concentration was calculated using the formula of Grynkiewicz, Poenie, and Tsien (37).

PAF and PAF analog analysis by reverse-phase HPLC and characterization by electrospray ionization-MS, and quantification of PAF by GC-MS analysis

The dry residues containing PAF bioactivity were suspended in 25 μl of methanol before separation of the molecular species of PAF on a reverse-phase (RP) Spherisorb C6 column (Waters). The HPLC mobile phase consisted of 55% methanol-ammonium acetate (10 mM) (1:3, v/v) and 45% acetonitrile; the flow rate was 1 ml/min. The retention times of PAF-like molecules were determined using ^{3}H-labeled lyso-PAF, ^{3}H-labeled C16:0 or C18:0 PAF, as well as its radiolabeled ester and ether analogs as standards. Fractions were collected, extracted with chloroform, dried, and assayed for PAF biological activity.

The biologically active fractions recovered from RP-HPLC were analyzed by electrospray ionization (ESI)-MS (Plotform LS; Micromass). The curtain gas flow was 4.5 l/min nitrogen. Phospholipids were introduced into the mass spectrometer by flow injection analysis. The flow injection analysis solvent consisted of methanol-ammonium acetate (10 mM) (70:30). Phospholipids suspended in flow injection analysis solvent were injected at a rate of 50 μl/min. The orifice potential was maintained at 75 V and the ESI potential at 3.5 kV for the detection of positive ions.

The biologically active material that was recovered from RP-HPLC with the retention time of C16:0 PAF was further analyzed and quantified by GC-MS as described (38). In brief, the samples from RP-HPLC were added to tubes that contained 2 ng of the stable, isotopically labeled variant of PAF, 1-0-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). The tubes were washed with 4 ml of 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 derivatized with pentafluorobenzoyl chloride. The pentafluorobenzoyl derivatives were analyzed subsequently by negative ion chemical ionization GC-MS with a Finnigan Mat (San Jose, CA) SSQ70 mass spectrometer, as described previously (38).

Enzymatic PAF and PAF analog formation in oxidized LDL

The enzymatic assay was performed by incubating 6 h oxidized LDL in 10 mM PBS, pH 7.4, with 1-hexadecanoyl-2-acetyl-*sn*-glycero-3-phosphocholine (acetyl-PC) or 1-hexadecanoyl-2-butanoyl-*sn*-glycero-3-phosphocholine (butanoyl-PC) and [^{3}H]lyso-PAF dissolved in 2.5 mg/ml BSA/PBS. Reactions were performed in polypropylene tubes for 60 min at 37°C. The final concentrations were 5 μg of oxidized lipoprotein per milliliter, 80 μM acetyl-PC or butanoyl-PC, 40 μM [^{3}H]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 (34). Total lipids were then subjected to TLC on silica-gel G plates using chloroform-methanol-water (65:35:6, v/v) as a solvent system. Lipids were identified after brief exposure to iodine. The band corresponding to the R_f of standard PAF or 1-0-hexadecyl-2-butanoyl-*sn*-glycero-3-phosphocholine (butanoyl-PAF) was scraped off the plate, and the radioactivity was measured by liquid scintillation counting. In some experiments, the lipoprotein was preincubated with 1 mM Pefabloc for 30 min at 37°C before the assay.

Statistical analysis

Results are expressed as means ± SD. Mean values were compared with Student's *t*-test, with significance defined at $P < 0.05$.

RESULTS

Formation of PAF-like bioactivity upon LDL oxidation

We investigated the formation of PAF-like bioactivity on LDL upon Cu^{2+} oxidation under two conditions: *a*) LDL oxidation with inactivated PAF-AH; and *b*) LDL oxidation in the presence of exogenous lyso-PAF.

As shown in **Table 1** and in accordance with our earlier

TABLE 1. PAF-like bioactivity formed in LDL oxidized under different conditions

Native LDL	0	0	0.1 ± 0.1	0.2 ± 0.2
Oxidized LDL, 3 h	0.3 ± 0.2	2.5 ± 1.0[a]	3.1 ± 1.1[b]	3.0 ± 1.5
Oxidized LDL, 6 h	0.5 ± 0.1	7.1 ± 1.1[c]	2.0 ± 0.7[b]	10.2 ± 3.1
Oxidized LDL, 24 h	0.5 ± 0.1	10.1 ± 1.1[d]	0.8 ± 0.1[e]	13.0 ± 4.2
Pefabloc (1 mM)	−	+	−	+
Lyso-PAF (40 μM)	−	−	+	+

Lyso-PAF, 1-0-alkyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor; Pefabloc, 4-[2-aminoethyl]benzenesulfonyl fluoride. PAF-like bioactivity was measured by the washed rabbit platelet aggregation assay and is expressed as picomoles of 1-0-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine equivalent per milligram of LDL protein. LDL (100 μg of protein) was oxidized with 5 μM $CuCl_2$ for 3, 6, or 24 h in both the presence and absence of the PAF-acetylhydrolase inhibitor Pefabloc (1 mM) and both the presence and absence of 40 μM 1-0-hexadecyl-*sn*-glycero-3-phosphocholine. Results are means ± SD for four LDL preparations.

[a] $P < 0.02$ compared with 3 h of oxidized LDL without Pefabloc.

[b] $P < 0.02$ compared with 3 h of oxidized LDL without Pefabloc and without lyso-PAF.

[c] $P < 0.01$ compared with 3 h of oxidized LDL with Pefabloc.

[d] $P < 0.01$ compared with 6 h of oxidized LDL with Pefabloc.

[e] $P < 0.05$ compared with 6 h of oxidized LDL without Pefabloc plus lyso-PAF.

observations (20, 21), PAF-like bioactivity was formed in LDL upon oxidation with chemically inactivated PAF-AH ($P < 0.02$). The amount of biological activity increased relative to the time of incubation from 3 to 24 h ($P < 0.01$). Moreover, without the need of PAF-AH inhibition, a PAF-like biological activity was observed in oxidized LDL in the presence of exogenous lyso-PAF. A substantial amount of activity was observed in the first 3–6 h of oxidation ($P < 0.02$), but PAF-like bioactivity decreased after 24 h of oxidation ($P < 0.05$).

Our results lead us to suggest that the amount of PAF-like biological activity formed in oxidized LDL depends on the oxidation conditions (i.e., the time of oxidation, the presence or absence of PAF-AH inhibitor, and the presence or absence of exogenously added lyso-PAF).

Analysis of PAF-like bioactivity by RP-HPLC

Subsequently, we asked whether the amount of PAF-like bioactivity and its molecular composition varied depending on the oxidation conditions.

The isocratic RP-HPLC separation system described in Materials and Methods permitted the separation of the most active C16:0 PAF analogs from the less active C18:0 analogs (39). The retention times of C16:0 lyso-PAF and C16:0 PAF were 17–18 min and 22–24 min, respectively. The retention times of C18:0 lyso-PAF and C18:0 PAF were 33–38 min and 45–50 min, respectively. The RP-HPLC system also permitted the separation of the *sn*-1 ester from the ether-linked PAF analogs. C16:0 acetyl-PC showed a retention time of 18–21 min. Moreover, our HPLC analysis offered a good separation of the bioactive C16:0 ether analogs with increasing *sn*-2 chain length, as shown by the arrows in the upper part of **Fig. 1**.

Thus, the TLC-purified PAF-like bioactivity was analyzed in this RP-HPLC system. Fractions were collected, and the C16:0 PAF equivalents were measured in each fraction by the washed rabbit platelets assay. After elution from the RP column, 60–70% of the bioactivity injected was recovered at the retention time of bioactive PAF analogs (Fig. 1). Upon 6 h of LDL oxidation with inactivated PAF-AH, a substantial amount of bioactivity (35.1 ± 4.6%; n = 3) was recovered by RP-HPLC with the retention time of C16:0 PAF. Higher amounts of bioactivity (50.0 ± 5.4%; n = 3) ($P < 0.05$) were recovered from the RP-HPLC column with the retention time of 1-0-hexadecyl-2-propionyl-*sn*-glycero-3-phosphocholine (propionyl-PAF) and 1-0-hexadecyl-2-butenoyl-*sn*-glycero-3-phosphocholine (butenoyl-PAF). Similar results were obtained upon 3 h of LDL oxidation. A low amount of bioactivity was recovered from the RP-HPLC setting with higher retention time than that of propionyl- and butenoyl-PAF in the 24 h oxidation experiment. In LDL oxidized for 3 or 6 h in the presence of exogenous C16:0 lyso-PAF, almost 90% of the bioactivity was recovered from the RP-HPLC column with the retention time of C16:0 PAF.

We thus conclude that the molecular species contributing to the PAF-like bioactivity depended on the conditions of LDL oxidation.

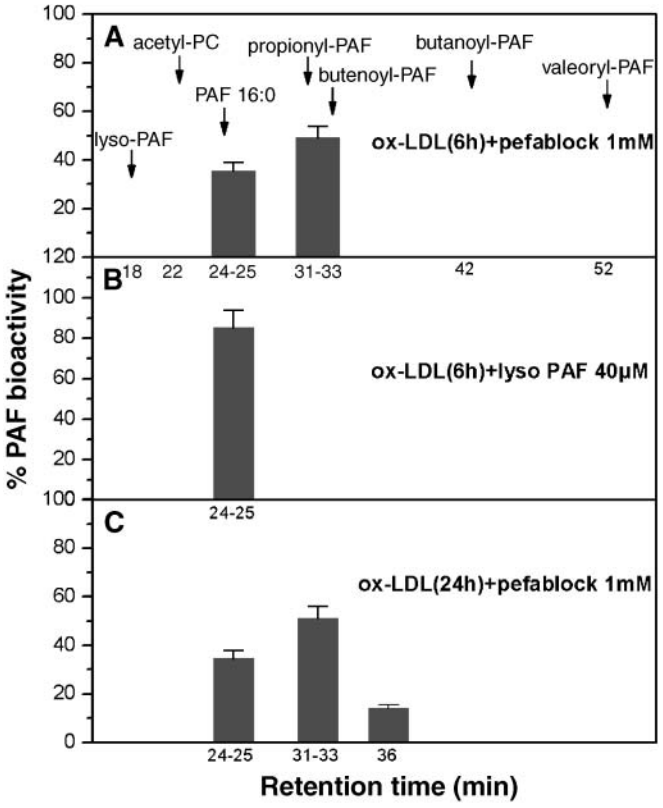

Fig. 1. Separation of platelet-activating factor (PAF)-like bioactivity by reverse-phase (RP)-HPLC. The TLC-purified PAF-like bioactive molecules were separated by RP-HPLC using a Spherisorb C6 column and a mobile phase consisting of 55% methanol-ammonium acetate (10 mM) (1:3, v/v) and 45% acetonitrile. Graphs show the percentage contribution of each HPLC fraction to the PAF-like bioactivity in oxidized LDL (ox-LDL), measured by washed rabbit platelet aggregation. A: RP-HPLC separation of PAF-like bioactivity formed upon 6 h of LDL oxidation in the presence of the platelet-activating factor-acetylhydrolase (PAF-AH) inhibitor 4-[2-aminoethyl]benzenesulfonyl fluoride (Pefabloc). B: RP-HPLC separation of PAF-like bioactivity formed upon LDL oxidation in the presence of exogenous C16:0 1-0-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF). C: RP-HPLC separation of PAF-like bioactivity formed upon 24 h of LDL oxidation in the presence of the PAF-AH inhibitor Pefabloc. Arrows indicate the retention times of synthetic radiolabeled standards. The bars show means ± SD of three LDL preparations. See the text for statistical significance. acetyl-PC, 1-hexadecanoyl-2-acetyl-*sn*-glycero-3-phosphocholine.

Mass spectrometric analysis of the biologically active material

The biologically active material recovered from the RP-HPLC column was analyzed by ESI-MS using the positive ion mode (**Fig. 2**). MS results for the molecular species with the retention time of C16:0 PAF in 6 h oxidized LDL in the presence of exogenous lyso-PAF are presented in Fig. 2A. MS revealed a major ion at *m/z* 524–525, corresponding to the protonated molecule that was the expected $[M+H]^+$ for C16:0 PAF. Moreover, two other diagnostic ions were observed: one at *m/z* 546 corresponding to the $[M+Na]^+$ of C16:0 PAF and one at *m/z* 562 corresponding to the $[M+K]^+$ of C16:0 PAF. A similar result was observed for the molecular species with the retention time

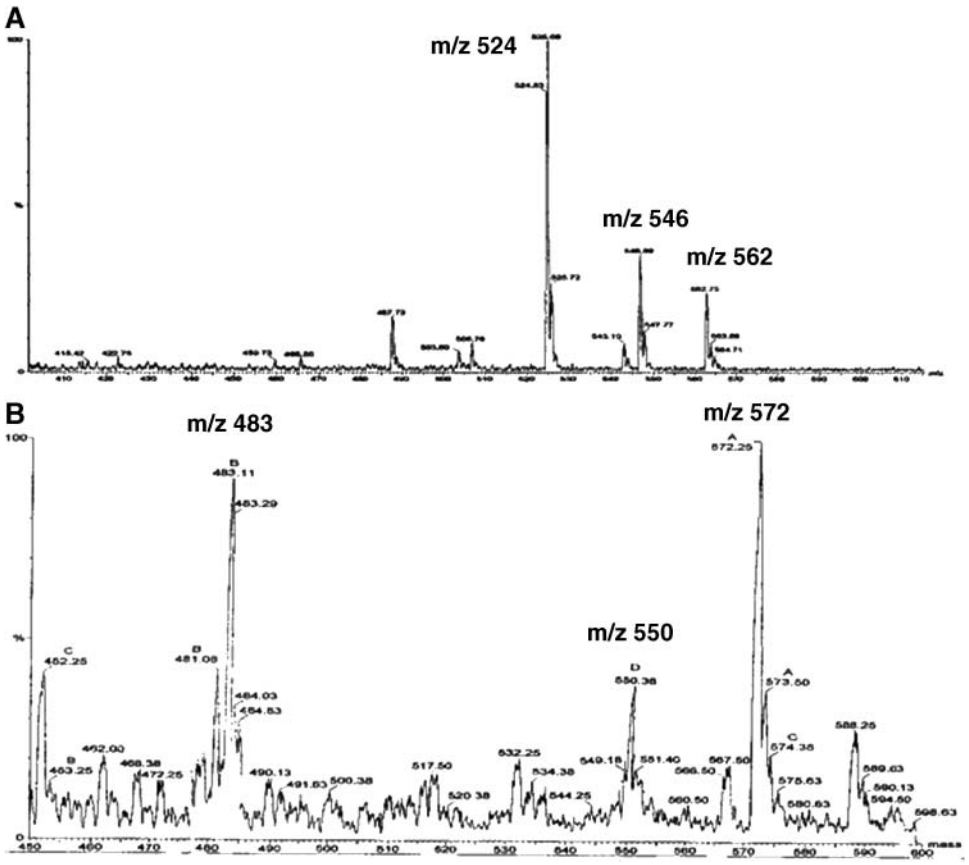

Fig. 2. A: The molecules that were recovered from the RP-HPLC column with the retention time of 1-0-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C16:0 PAF) were introduced into the mass spectrometry ion source dissolved in methanol-ammonium acetate (10 mM) (70:30) and were analyzed by positive ion flow injection electrospray mass spectrometry. *m/z* 524–525 corresponds to the $[M+H]^+$ of C16:0 PAF. B: The bioactive material that was recovered from the RP-HPLC column with the retention time of 1-0-hexadecyl-2-propionyl-*sn*-glycero-3-phosphocholine (propionyl-PAF) and 1-0-hexadecyl-2-butenoyl-*sn*-glycero-3-phosphocholine (butenoyl-PAF) was introduced into the mass spectrometry ion source dissolved in methanol-ammonium acetate (10 mM) (70:30) and was analyzed by positive ion flow injection electrospray mass spectrometry. *m/z* 572–573 corresponds to the $[M+Na]^+$ of butenoyl-PAF, *m/z* 551–552 corresponds to the $[M+H]^+$ of butenoyl-PAF, and *m/z* 483–484 corresponds to the $[M+H]^+$ of lyso-PAF.

of C16:0 PAF in 3 h oxidized LDL or in 6 h oxidized LDL with inactivated PAF-AH.

Further ESI-MS analysis of the biologically active material with the retention times of propionyl- and butenoyl-PAF showed the absence of the diagnostic ion for propionyl-PAF (Fig. 2B). The MS analysis revealed a major ion at *m/z* 572–573, which possibly corresponds to the $[M+Na]^+$ of butenoyl-PAF. It is known that when the sodium or other alkali metals are present in the sample, their attachment to the analyzed molecule is frequently observed. The appearance of these ions is dependent on the concentration of alkali metals in the sample (40). In addition, two other diagnostic ions were observed: one at *m/z* 551–552, corresponding to the $[M+H]^+$ of butenoyl-PAF, and another at *m/z* 483–484, corresponding to the $[M+H]^+$ of lyso-PAF. ESI-MS gave no diagnostic information on the constituents of the biologically active material formed upon 24 h of LDL oxidation, because abundant ions shorter than lyso-PAF were observed. The diagnostic ions observed by ESI-MS and the retention time upon RP-HPLC separation constitute evidence to suggest, in accordance with previous findings (26), that the butenoyl-PAF analog may be the major molecule with PAF-like bioactivity, which was formed upon LDL oxidation with chemically inactivated PAF-AH.

When the exogenous C16:0 lyso-PAF was supplied to the LDL oxidation assay, however, a substantial formation of C16:0 PAF was observed without PAF-AH inactivation. GC-MS analysis of the HPLC fraction with the retention time of C16:0 PAF revealed that the bioactive product of the reaction was C16:0 PAF (**Fig. 3**). 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 to that of the internal standard D3-PAF derivative (*m/z* 555). The quantity of C16:0 PAF measured by GC-MS, as described in the legend to Fig. 3, was 80% of that measured by the washed rabbit platelet aggregation assay. Thus, the important contribution of specific C16:0 PAF in the bioactivity, formed in oxidized LDL without PAF-AH inactivation and

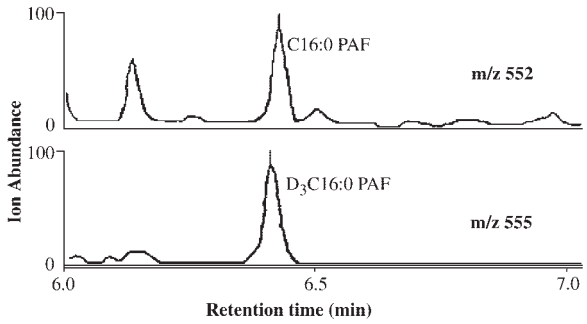

Fig. 3. GC-MS analysis of the RP-HPLC fraction with the retention time of C16:0 PAF. The PAF-like molecule with the retention time of C16:0 PAF on RP-HPLC, formed in oxidized LDL in the presence of exogenous lyso-PAF and without PAF-AH inactivation, was analyzed by GC-MS as described in Materials and Methods. A reconstructed ion chromatogram obtained during GC-MS analysis is shown for the specific ions *m/z* 552 and *m/z* 555. The ion at *m/z* 552 corresponds to the C16:0 PAF derivative, present in the sample, and the ion at *m/z* 555 corresponds to 2 ng of the internal standard 1-0-hexadecyl-2(D3)-acetyl-glycero-3-phosphocholine (D3-PAF) derivative. 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.

in the presence of exogenous C16:0 lyso-PAF (Fig. 1B), was further confirmed by GC-MS quantification.

Estimation of the amount of C16:0 PAF upon LDL oxidation with inactivated PAF-AH

To estimate from the RP-HPLC/ESI-MS analysis the quantity of C16:0 PAF formed upon LDL oxidation with inactivated PAF-AH, we synthesized PAF analogs with increasing *sn*-2 chain lengths and tested their biological activity. The EC_{50} values in the aggregation test with washed rabbit platelets are shown in **Table 2**. The C16:0 PAF and its propionyl analog showed similar bioactivity. The butanoyl analog, bearing a slightly longer acyl chain (one additional methyl group) at the *sn*-2 position, showed 40-fold lower activity; the valeoryl analog was 10,000 times less active. The ester-linked analogs were 400 times less active than their ether-linked counterparts. It is important to note that the addition of an aldehydic or carboxylic group, or the addition of a double bond to the *sn*-2 acyl chain, did not change the biological activity (our unpublished observations). The functionality of certain PAF analogs was further tested for their ability to induce Ca^{2+} fluxes in PAF receptor-bearing THP1 cells. PAF, propionyl-PAF, and butanoyl-PAF, at 50-fold higher concentrations, induced Ca^{2+} fluxes that were of similar amplitude (**Fig. 4**). However, the concentrations required to stimulate the Ca^{2+} fluxes in THP-1 were higher than those used in the platelet aggregation bioassay. We estimate that the mass of C16:0 PAF constituted <1% of the mass of the butenoyl-PAF analog. Our reasons for this estimation result from three observations: *a*) the bioactivity of C16:0 PAF is 40-fold greater than that of its butanoyl or butenoyl analog; *b*) the recovery of 30–40% of the bioactivity with the retention time of C16:0 PAF upon RP-HPLC separation; and *c*) the molecular species characterization by ESI-MS analysis. Similar results were obtained by Marathe et al. (26) upon LDL oxidation with inactivated PAF-AH.

Thus, we conclude that C16:0 PAF, despite being an important contributor to the PAF bioactivity, is a relatively minor phospholipid peroxidation product of LDL.

Enzymatic PAF and PAF analog formation in oxidized LDL

We also investigated the possibility of enzymatic PAF and PAF analog formation in oxidized LDL. As shown in **Table 3**, using 6 h oxidized LDL protein as the source of the enzyme, a transacetylase activity was able to produce PAF and butanoyl-PAF without the need for PAF-AH inhibition by Pefabloc treatment. Acetyl-PC or butanoyl-PC was the acyl donor, and lyso-PAF was the acceptor molecule. One millimolar Pefabloc treatment totally inhibited the activity. It is important to note that by increasing the chain length at the *sn*-2 position of the donor molecule from the acetate (C2) to the butanate (C4) moiety, the activity of transacylation decreased two to three times ($P < 0.02$). Exogenous lyso-PAF is possibly a preferential acceptor of acetate rather than butanate upon transacylation in oxidized LDL. Thus, PAF may be the main product of the enzymatic formation.

DISCUSSION

The results of the present work show that the most active PAF analog, C16:0 PAF, makes a substantial contribution to the PAF bioactivity formed in LDL upon Cu^{2+}-induced oxidation; however, its mass compared with other bioactive molecules is extremely low, reaching ~1% of the total.

PAF-like bioactivity was formed in oxidized LDL when the LDL-associated PAF-AH had been inhibited by the serine esterase inhibitor Pefabloc (Table 1). Under these oxidation conditions, the formation of PAF analogs results from the free radical oxidation and decomposition of PUFAs esterified at the *sn*-2 position of ether-linked 1,2-diacyl-*sn*-glycero-3-phosphocholine (PC) (41). A possible mechanism of PAF analog formation is shown in **Fig. 5A**. It is caused by autoxidation and decomposition of the *sn*-2 arachidonoyl ether-linked PC (I). Alkyl radicals of esterified unsaturated fatty acids (II) react nonenzymatically

TABLE 2. EC_{50} values of PAF and PAF analogs

	PAF	Propionyl-PAF	Butanoyl-PAF	Valeoryl-PAF	Acetyl-PC	Propionyl-PC
EC_{50}	$[4.3 \pm 2.0] \times 10^{-11}$	$[4.5 \pm 2.5] \times 10^{-11}$	$[1.8 \pm 2.0] \times 10^{-9}$	$[4.0 \pm 3.0] \times 10^{-7}$	$[2.0 \pm 1.0] \times 10^{-8}$	$[4.0 \pm 1.5] \times 10^{-8}$

The EC_{50} values were calculated using the washed rabbit platelet aggregation assay as described in Materials and Methods. Results are means ± SD of four experiments.

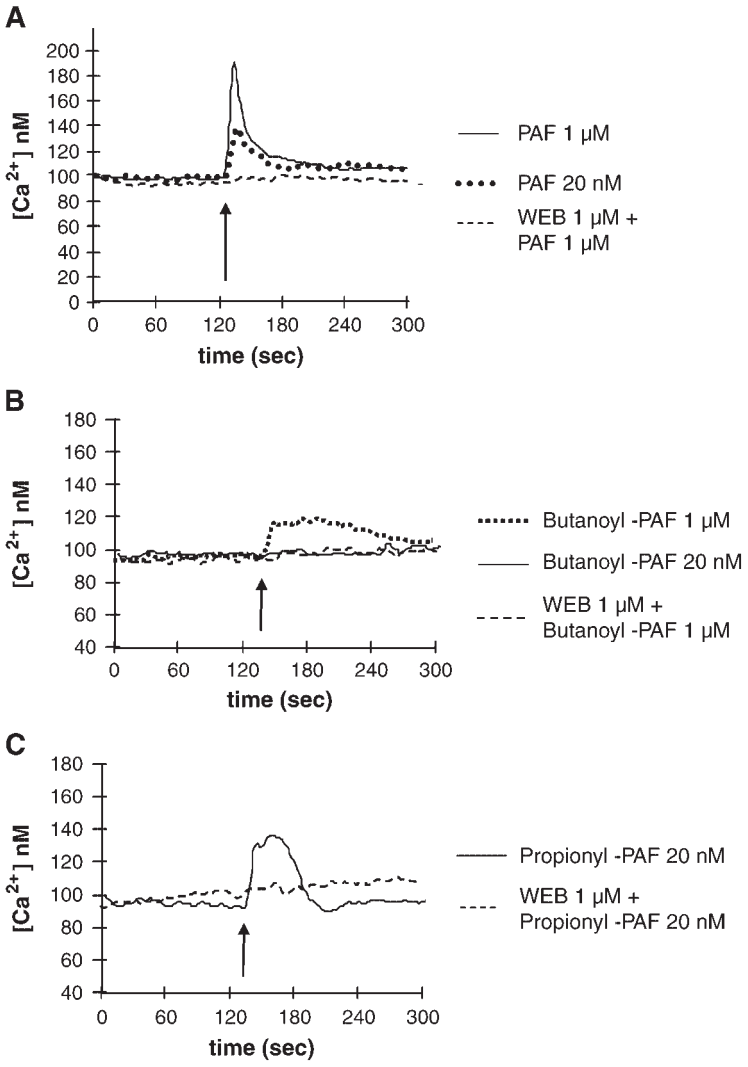

Fig. 4. Ca^{2+} concentration fluxes induced in THP-1 cells by the bioactive PAF analogs. A: 1 μM PAF, 20 nM PAF, and 1 μM PAF + 1 μM WEB 2086. B: 1 μM butanoyl-PAF, 20 nM 1-0-hexadecyl-2-butanoyl-*sn*-glycero-3-phosphocholine (butanoyl-PAF), and 1 μM butanoyl-PAF + 1 μM WEB 2086. C: 20 nM propionyl-PAF and 20 nM propionyl-PAF + 1μM WEB 2086.

with molecular oxygen to yield peroxyl radicals (III), and the resulting hydroperoxides (IV) can decompose in the presence of divalent metal ions into the corresponding alkoxyl radicals (V). 4,5β-Scission by homolytic cleavage between the alkoxyl radical carbon and an adjacent C-C bond produces aldehydes and secondary radicals (VI). Double bond formation, by H atom rearrangement of the secondary radical intermediate, forms the unsaturated butenoyl species (VII). Our results suggest that this scheme is the main route of PAF-like bioactivity formation upon the oxidation of LDL in the presence of Cu^{2+} ions and with inactivated PAF-AH.

TABLE 3. Enzymatic PAF and PAF analog formation

Acyl Donor	Acyl Acceptor	Enzymatic Activity	
		−1 mM Pefabloc	+1 mM Pefabloc
		nmol product formed/min/mg protein	
Acetyl-PC	Lyso-PAF	4.0 ± 1.0	0
Butanoyl-PC	Lyso-PAF	1.5 ± 0.3[a]	0

Five micrograms of 6 h oxidized LDL protein was incubated with 80 μM acyl donor and 40 μM [^{3}H]lyso-PAF for 1 h, and the enzymatic activity was measured as described in Materials and Methods. Results are means ± SD for three LDL preparations.

[a] $P < 0.02$ compared with acetyl-PC as the acyl donor.

The formation of C16:0 PAF may be explained by a side route of the same chain of reactions (Fig. 5A). Dioxygenated secondary products from PUFA free radical autoxidation may occur by activated hydrogen abstraction of the hydroperoxides (42) and by cyclization of the peroxyl radicals (43). Figure 5A shows the presence of the dioxygenated products, which may be formed by the intramolecular abstraction of a hydrogen atom through the peroxyl radical. As mentioned above, the amount of C16:0 PAF was estimated to be ~1% of the amount of the butenoyl analog; thus, this side chain of reactions has a low probability of incidence compared with the reactions that lead to the formation of butenoyl-PAF.

Tokumura et al. (44) reported the formation of a few minor fragmentation products upon the oxygenation of PUFAs containing PC, which possess a shortened *sn*-2 acyl group, frequently one or two methylene groups minus those of the major species. Thus, in parallel with C16:0 PAF, its propionyl analog with similar bioactivity should be expected to be formed by the peroxidation of the *sn*-2 arachidonoyl ether-linked PC. However, we were unable to

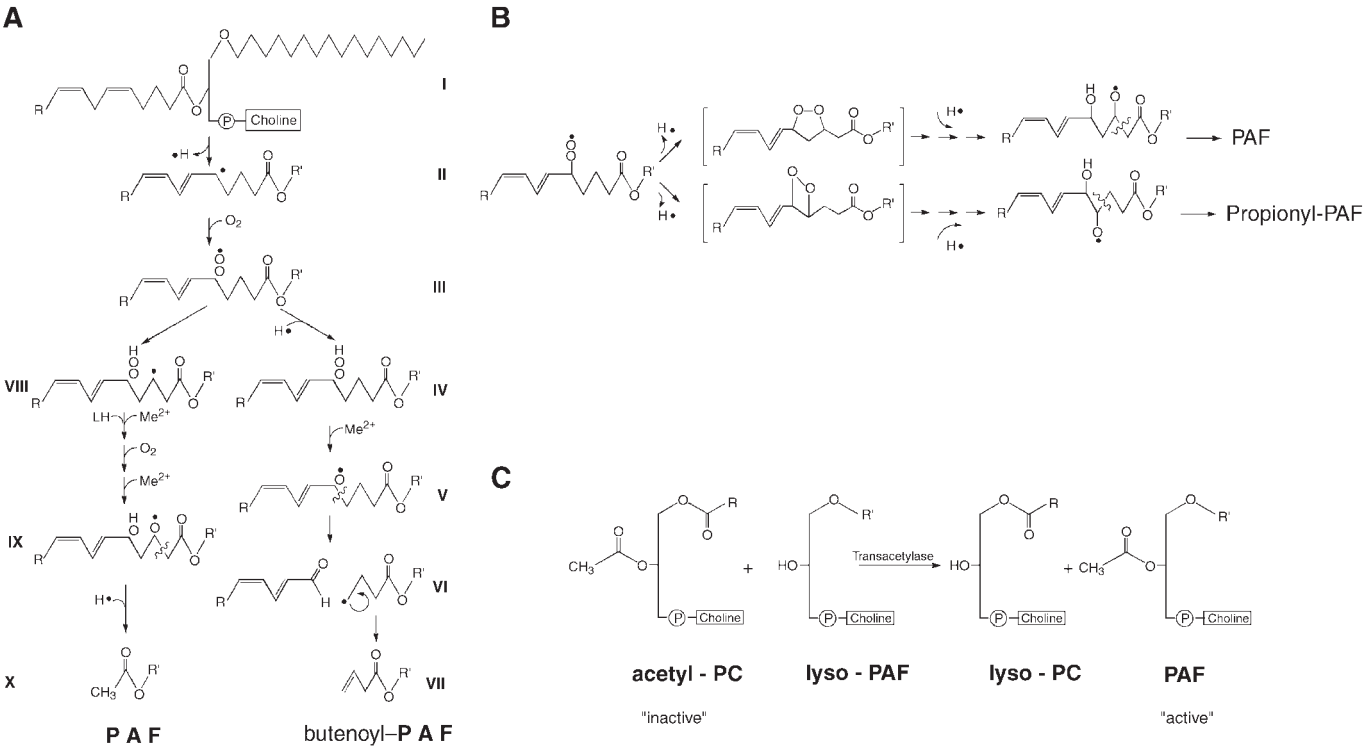

Fig. 5. A: Proposed mechanism of butenoyl-PAF and PAF formation by peroxidation and decomposition of the *sn*-2 arachidonoyl ether-linked 1,2-diacyl-*sn*-glycero-3-phosphocholine (PC). B: Proposed mechanism of the intramolecular hydrogen atom abstraction by the peroxyl radical, resulting in PAF and propionyl-PAF formation. C: Scheme of the transacetylation reaction driven by PAF-AH between acetyl-PC, formed upon chemical PC peroxidation of LDL, and exogenously added lyso-PAF, leading to the formation of highly active PAF.

detect by ESI-MS analysis any diagnostic ion characteristic for propionyl-PAF. An explanation could be the formation of a five-membered ring endoperoxide (XI; 1,2-dioxolane) (Fig. 5B) (45) via 1,3-cyclization as a possible intermediate product for the intramolecular hydrogen abstraction presented in the scheme shown in Fig. 5A. The 1,3-cyclization appears more plausible than the more condensed 1,2-cyclization, which would lead to the proximal hydrogen abstraction (Fig. 5B).

A question arises regarding the mechanism of C16:0 PAF formation upon oxidation of LDL in the presence of exogenously added lyso-PAF. It is important to note that under the oxidation conditions described above, chemical PAF-AH inactivation was not required, and the addition of exogenous lyso-phospholipids may mimic their local accumulation in the atherosclerotic lesions (46). We and others have reported that PAF-AH displays both transacetylase and acetylhydrolase activities (19, 20). The transacetylase activity on LDL may exceed the acetylhydrolase activity in the presence of exogenous lyso-phospholipids. Under such conditions, the transacetylase may acylate extracellular lyso-PAF to form PAF analogs (20). In accordance with the observations described above, the results of this work show that a transacylase enzymatic reaction in oxidized LDL should produce PAF and PAF analogs.

Through the phospholipid autoxidation, shown at the side route in Fig. 5A, the much less active acetyl-PC will be formed preferentially rather than the highly bioactive PAF, because the ester-linked PC is 100 times more abundant than the ether-linked PC in LDL (25). Thus, in the presence of exogenous lyso-PAF (Fig. 5C), the transfer of acetate from the inactive acetyl-PC to lyso-PAF may increase PAF bioactivity. By increasing the chain length at the *sn*-2 position of the donor molecule from acetate (C2) to butanate (C4), the specific activity of transacylation decreased two to three times. The bioactivity of C16:0 PAF was 40 times greater than that of its *sn*-2 C4 analogs. The high contribution of C16:0 PAF to the bioactivity observed upon LDL oxidation without PAF-AH inactivation may be explained by this fact. C16:0 PAF formation, observed in the first hour of oxidation of the intermediate LDL subfraction without PAF-AH inactivation, could be explained by a similar mechanism (25).

We conclude that the C16:0 PAF, although a minor constituent among the LDL peroxidation products, makes a substantial contribution to the PAF bioactivity formed in oxidized LDL. C16:0 PAF is the main bioactivity constituent, especially upon LDL oxidation, in which PAF-AH is still active and exogenous lyso-PAF is present. This contribution may be explained by the higher bioactivity of C16:0 PAF and the higher selectivity of the LDL-attached lyso-PAF transacetylase toward very short acyl chains [acetate (C2) vs. butanate (C4)].

The in vitro experiments described here suggest that in the atherosclerotic lesions and in other sites of chronic inflammatory damage, there may be a critical imbalance among three factors: *a*) the generation of bioactive oxidized phospholipids by various pro-oxidants; *b*) the level

of PAF-AH activity that hydrolyzes the active phospholipids; and *c*) the level of PAF-AH transacetylase activity that catalyzes PAF formation. This imbalance could contribute to the formation of atherogenic LDL particles via the accumulation of PAF and PAF-like bioactivity.

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REFERENCES

1. Ross, R. 1999. Mechanism of disease: atherosclerosis—an inflammatory disease. *N. Engl. J. Med.* **340:** 115–126.
2. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
3. Camejo, G., E. Hurt-Camejo, U. Olsson, and G. Bondjers. 1993. Proteoglycans and lipoproteins in atherosclerosis. *Curr. Opin. Lipidol.* **4:** 385–391.
4. Steinberg, D. 1997. Low density lipoprotein oxidation and its pathobiological significance. *J. Biol. Chem.* **272:** 20963–20966.
5. Berliner, J. A., M. Navab, A. M. Fogelman, J. S. Frank, L. L. Demer, P. A. Edwards, A. D. Watson, and A. J. Lusis. 1995. Atherosclerosis: basic mechanisms, oxidation, inflammation, and genetics. *Circulation.* **91:** 2488–2496.
6. Prescott, S. M., G. A. Zimmerman, D. M. Stafforini, and T. M. McIntyre. 2000. Platelet-activating factor and related lipid mediators. *Annu. Rev. Biochem.* **69:** 419–445.
7. Ninio, E. 2005. Phospholipid mediators in the vessel wall: involvement in atherosclerosis. *Curr. Opin. Clin. Nutr. Metab. Care.* **8:** 123–131.
8. Honda, Z., M. Nakamura, I. Miki, M. Minami, T. Watanabe, Y. Seyama, H. Okado, H. Toh, K. Ito, T. Miyamoto, et al. 1991. Cloning by functional expression of platelet-activating factor receptor from guinea-pig lung. *Nature.* **349:** 342–346.
9. Izumi, T., and T. Shimizu. 1995. Platelet-activating factor receptor: gene expression and signal transduction. *Biochim. Biophys. Acta.* **1259:** 317–333.
10. Kamido, H., A. Kuksis, L. Marai, and J. J. Myher. 1995. Lipid ester-bound aldehydes among cooper-catalyzed peroxidation products of human plasma lipoproteins. *J. Lipid Res.* **36:** 1876–1886.
11. Tokumura, A., M. Toujima, Y. Yoshioka, and K. Fukuzawa. 1996. Lipid peroxidation in low density lipoproteins from human plasma and egg yolk promotes accumulation of 1-acyl analogues of platelet activating factor like lipids. *Lipids.* **31:** 11251–11258.
12. Heery, J. M., M. Kozak, D. M. Stafforini, D. A. Jones, G. A. Zimmerman, T. M. McIntyre, and S. M. Prescott. 1995. Oxidatively modified LDL contains phospholipids with platelet activating factor like activity and stimulates the growth of smooth muscle cells. *J. Clin. Invest.* **96:** 2322–2330.
13. Smiley, P. L., K. E. Stremler, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1991. Oxidatively fragmented phosphatidylcholines activate human neutrophils through the receptor for platelet-activating factor. *J. Biol. Chem.* **266:** 11104–11110.
14. Subbanagunder, G., N. Leitinger, P. T. Shih, K. F. Faull, and J. A. Berliner. 1999. Evidence that phospholipid oxidation products and/or platelet-activating factor play an important role in early atherogenesis: in vitro and in vivo inhibition by WEB 2086. *Circ. Res.* **85:** 311–318.
15. Six, D. A., and E. A. Dennis. 2000. The expanding superfamily of phospholipase A2 enzymes: classification and characterization. *Biochim. Biophys. Acta.* **1488:** 1–19.
16. Blank, M. L., T. C. Lee, V. Fitzgerald, and F. Snyder. 1981. A specific acetylhydrolase for 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (a hypotensive and platelet-activating lipid). *J. Biol. Chem.* **256:** 175–178.
17. Stafforini, D. M., T. M. McIntyre, M. E. Carter, and S. M. Prescott. 1987. Human plasma platelet-activating factor acetylhydrolase: association with lipoprotein particles and role in the degradation of platelet-activating factor. *J. Biol. Chem.* **262:** 4215–4222.
18. Stremler, K. E., D. M. Stafforini, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1989. An oxidized derivative of phosphatidylcholine is a substrate for the platelet-activating factor acetylhydrolase from human plasma. *J. Biol. Chem.* **264:** 5331–5334.
19. Liu, M., and P. V. Subbaiah. 1994. Hydrolysis and transesterification of platelet-activating factor by lecithin-cholesterol acyltransferase. *Proc. Natl. Acad. Sci. USA.* **91:** 6035–6039.
20. Tsoukatos, D. C., T. A. Liapikos, A. D. Tselepis, M. J. Chapman, and E. Ninio. 2001. Platelet-activating acetylhydrolase and transacetylase activities in human plasma low-density lipoprotein. *Biochem. J.* **357:** 457–464.
21. Liapikos, T. A., S. Antonopoulou, S-A. P. Karabina, D. C. Tsoukatos, C. A. Demopoulos, and A. D. Tselepis. 1994. Platelet-activating factor formation during oxidative modification of low-density lipoprotein when PAF-acetylhydrolase has been inactivated. *Biochim. Biophys. Acta.* **1212:** 353–360.
22. Dentan, C., P. Lesnik, M. J. Chapman, and E. Ninio. 1994. PAF-acether-degrading acetylhydrolase in plasma LDL is inactivated by cooper- and cell-mediated oxidation. *Arterioscler. Thromb.* **14:** 353–360.
23. Karabina, S. A. P., T. A. Liapikos, G. Grekas, J. Goudevenos, and A. D. Tselepis. 1994. Distribution of PAF-acetylhydrolase activity in human plasma low-density lipoprotein subfractions. *Biochim. Biophys. Acta.* **1213:** 34–38.
24. Tselepis, A. D., C. Dentan, S. A. P. Karabina, M. J. Chapman, and E. Ninio. 1995. PAF-degrading acetylhydrolase is preferentially associated with dense LDL and VHDL-1 in human plasma. Catalytic characteristics and relation to the monocyte-derived enzyme. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1764–1773.
25. Tsoukatos, D. C., M. Arborati, T. A. Liapikos, K. L. Clay, R. C. Murphy, J. M. Chapman, and E. Ninio. 1997. Cooper-catalyzed oxidation mediates PAF formation in human LDL subspecies: protective role of PAF acetylhydrolase in dense LDL. *Arterioscler. Thromb. Vasc. Biol.* **17:** 3505–3512.
26. Marathe, G. K., S. S. Davies, K. A. Harrison, A. R. Silva, R. C. Murphy, H. Castro-Faria-Neto, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1999. Inflammatory platelet-activating factor-like phospholipids in oxidized low density lipoproteins are fragmented alkyl phosphatidylcholines. *J. Biol. Chem.* **274:** 28395–28404.
27. Lekka, M. E., D. C. Tsoukatos, A. D. Tselepis, and V. M. Kapoulas. 1988. Semisynthetic preparation of 1-O-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (platelet-activating factor). *Z. Naturforsch.* **43c:** 665–670.
28. Barlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
29. Marinetti, G. V. 1962. Chromatographic separation, identification and analysis of phosphatidates. *J. Lipid Res.* **3:** 1–28.
30. Kelley, J. L., and P. Alaupovic. 1976. Lipid transport in the avian species. Part I. Isolation and characterization of apolipoproteins and major lipoprotein density classes of male turkey serum. *Atherosclerosis.* **24:** 155–175.
31. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150:** 76–85.
32. Dentan, C., A. D. Tselepis, M. J. Chapman, and E. Ninio. 1996. Pefabloc, 4-[2-aminoethyl]benzenesulfonyl fluoride, is a new, potent nontoxic and irreversible inhibitor of PAF-degrading acetylhydrolase. *Biochim. Biophys. Acta.* **1299:** 353–357.
33. Morel, D. W., J. R. Hessler, and G. M. Chilsom. 1983. Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipids. *J. Lipid Res.* **24:** 1070–1076.
34. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
35. Bossant, M. J., E. Ninio, D. Delautier, and J. Benveniste. 1990. Bioassay of paf-acether by rabbit platelet aggregation. *Methods Enzymol.* **187:** 125–130.
36. Benveniste, J., J-P. Le Couedic, J. Polonsky, and M. Tence. 1977. Structural analysis of purified platelet-activating factor by lipases. *Nature.* **269:** 170–171.
37. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260:** 3440–3450.
38. Clay, K. L., C. Johnson, and S. Worthen. 1991. Biosynthesis of

platelet activating factor and 1-0-acyl analogues by endothelial cells. *Biochim. Biophys. Acta.* **1094:** 43–50.
39. Mac Manus, L. M., D. S. Woodward, S. I. Deavers, and R. N. Pinckard. 1993. Biology of disease. PAF molecular heterogeneity: pathobiological implications. *Lab. Invest.* **69:** 639–650.
40. Voelkel, N. F., J. Simpson, S. Worthen, J. T. Reeves, P. M. Henson, and R. C. Murphy. 1983. Platelet-activating factor causes pulmonary vasoconstriction and edema via platelet-independent leukotriene formation. *Adv. Prostaglandin Thromboxane Leukot. Res.* **12:** 179–183.
41. Frankel, E. N. 1998. Lipid Oxidation. The Oily Press, Dundee, UK.
42. Mlakar, A., and G. Spiteller. 1994. Reinvestigation of lipid peroxidation of linolenic acid. *Biochim. Biophys. Acta.* **1214:** 209–212.
43. Frankel, E. N. 1987. Secondary products of lipid oxidation. *Chem. Phys. Lipids.* **44:** 73–85.
44. Tokumura, A., T. Sumida, M. Toujima, K. Kogure, K. Fukuzawa, Y. Takahashi, and S. Yamamoto. 2000. Structural identification of phosphatidylcholines having an oxidatively shortened linoleate residue generated through its oxygenation with soybean or rabbit reticulocyte lipoxygenase. *J. Lipid Res.* **41:** 953–962.
45. Balci, M. 1981. Bicyclic endoperoxides and synthetic applications. *Chem. Rev.* **81:** 91–108.
46. Yla-Herttuala, S., H. Sumuvuori, K. Karkola, M. Mottonen, and T. Nikk. 1987. Atherosclerosis and biochemical composition of coronary arteries in Finish men. *Atherosclerosis.* **65:** 109–115.