

Inflammatory Platelet-activating Factor-like Phospholipids in Oxidized Low Density Lipoproteins Are Fragmented Alkyl Phosphatidylcholines*

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Oxidation of human low density lipoprotein (LDL) generates proinflammatory mediators and underlies early events in atherogenesis. We identified mediators in oxidized LDL that induced an inflammatory reaction *in vivo*, and activated polymorphonuclear leukocytes and cells ectopically expressing human platelet-activating factor (PAF) receptors. Oxidation of a synthetic phosphatidylcholine showed that an *sn*-1 ether bond confers an 800-fold increase in potency. This suggests that rare ether-linked phospholipids in LDL are the likely source of PAF-like activity in oxidized LDL. Accordingly, treatment of oxidized LDL with phospholipase A₁ greatly reduced phospholipid mass, but did not decrease its PAF-like activity. Tandem mass spectrometry identified traces of PAF, and more abundant levels of 1-*O*-hexadecyl-2-(butanoyl or butenoyl)-*sn*-glycero-3-phosphocholines (C₄-PAF analogs) in oxidized LDL that comigrated with PAF-like activity. Synthesis showed that either C₄-PAF was just 10-fold less potent than PAF as a PAF receptor ligand and agonist. Quantitation by gas chromatography-mass spectrometry of pentafluorobenzoyl derivatives shows the C₄-PAF analogs were 100-fold more abundant in oxidized LDL than PAF. Oxidation of synthetic alkyl arachidonoyl phosphatidylcholine generated these C₄-PAFs in abundance. These results show that quite minor constituents of the LDL phosphatidylcholine pool are the exclusive precursors for PAF-like bioactivity in oxidized LDL.

rapid inflammatory response as it is the leukocyte activating molecule produced and displayed by stimulated endothelial cells (1). PAF does not induce the bactericidal effector functions of leukocytes, but rather stimulates their adhesive and migratory behavior that allows them to transit the endothelial barrier. Leukocytes (polymorphonuclear leukocytes or PMN), monocytes, and eosinophils, as well as platelets, express the PAF receptor and accordingly are activated by PAF in concentrations ranging from picomolar to nanomolar levels. The potency of PAF, its broad actions, and the potentially deleterious events it invokes rationalize the tight regulation of PAF synthesis (2).

PAF is recognized by a single, specific receptor that is a member of the family of seven-transmembrane-spanning, G-protein-linked receptors (3, 4). Alone among this large family of receptors and related orphan sequences, the PAF receptor recognizes an intact phospholipid, and does so with a marked specificity. The PAF receptor shows a several hundredfold selectivity for the *sn*-1 ether bond of PAF, and complete specificity for the *sn*-2 acetyl residue compared with the long chain fatty acyl residue of most alkyl phosphatidylcholines (5, 6). The choline headgroup confers a several thousandfold advantage over the related phosphatidylethanolamine analog (7). Thus, compared with Edg-2 and Edg-4 receptors for lysophosphatidic acid (8), the PAF receptor has two additional, important recognition requirements; one is for a specific headgroup, and the second is for a specific, atypical *sn*-2 residue.

The PAF receptor responds to synthetic analogs that contain short *sn*-2 fatty acyl residues, and this too is relevant to inflammatory pathophysiology. PAF-like analogs with this structure are produced by oxidation of cellular (9), low density lipoprotein (10–13), or foodstuff (14) phosphatidylcholines. The predominant biologic phosphatidylcholines are lipids of the diacyl subclass, and so the oxidation products are expected to be diacyl species. These oxidatively generated PAF analogs stimulate monocytes (15), leukocytes (16), and platelets (17). Oxidation of phosphatidylcholines to PAF-like lipids also occurs *in vivo* following exposure to the strong oxidant stress of cigarette smoke (15, 18). Additionally, oxidatively fragmented phosphatidylcholines are found in atherosclerotic plaques (13), and they circulate at detectable levels in human plasma (19).

Oxidation of phosphatidylcholines generates a plethora of chemically related phosphatidylcholines and, as *sn*-1 alkyl or acyl phosphatidylcholines oxidize in a similar fashion (20), there is heterogeneity at both the *sn*-1 and *sn*-2 position. Only some of these will stimulate the PAF receptor, but identifica-

Platelet-activating factor (PAF)¹ is a phospholipid autacoid with a wide variety of actions, primarily on cells and events that comprise the inflammatory system. PAF initiates the

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¹ The abbreviations used are: PAF, platelet-activating factor; LDL, low density lipoprotein; PMN, polymorphonuclear leukocyte; HAPC, 1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; GC, gas chromatography; LC, liquid chromatography; MS, mass spectroscopy; HPLC, high performance liquid chromatography; BHT, butylated hy-

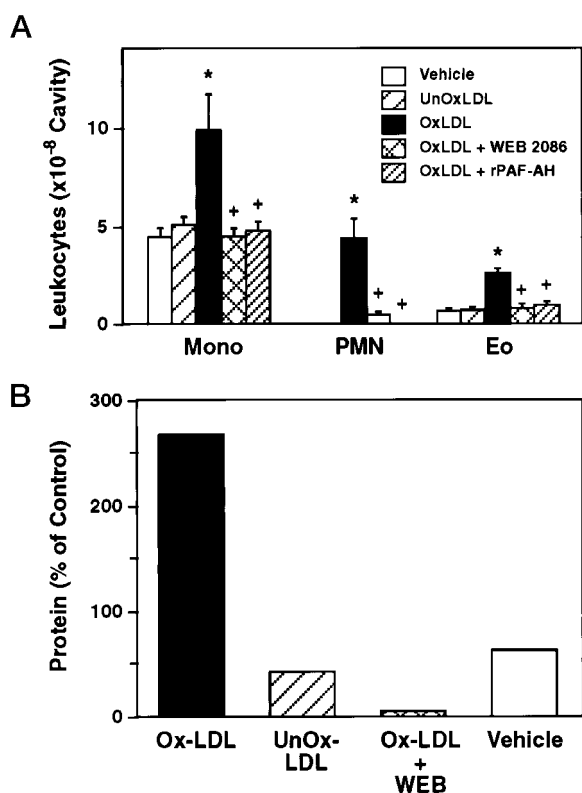


FIG. 1. Polar lipids purified from oxidized LDL are inflammatory. Lipids from native or Cu²⁺-oxidized LDL were extracted and purified by reversed phase chromatography and then pooled fractions 6–8 were injected into the pleural space of Wistar rats as described under “Materials and Methods.” Some rats were treated with the PAF receptor antagonist WEB 2086 (20 mg/kg) 1 h prior to agonist challenge, while others received lipids that had been treated with recombinant PAF acetylhydrolase (2 μ g for 20 min at 37 °C, followed by re-extraction). Pleural analysis of cell number and lavage protein content were performed 6 h after the intrathoracic injection. Statistically significant differences ($p < 0.05$) compared with control animals receiving BSA in saline are marked *, while differences compared with animals injected with lipids purified from oxidized LDL are marked +. Each bar is the mean \pm S.E. from at least four animals. *Mono*, monocytes; *PMN*, neutrophils; *Eo*, eosinophils.

oxidation products has been complicated by this heterogeneity. Here we show that one difficulty in identifying biologically active agents has been their profound dilution with related, but less active, diacyl homologs. We find that all of the PAF receptor agonists generated during the oxidation of LDL are derived from oxidation of the alkyl phosphatidylcholines found in very low abundance in LDL (21, 22). Removing the contaminating diacyl oxidation products allowed us to identify and quantitate fragmented alkyl phosphatidylcholines in oxidized LDL. While a trace amount of PAF was generated by oxidative fragmentation, major bioactive species are butanoyl- and butenoyl-PAF, which are also products of hexadecyl arachidonoyl phosphatidylcholine fragmentation. Thus, oxidation of rare phospholipid species in LDL generates bioactive, short chain PAF-analogs.

MATERIALS AND METHODS

Tissue culture grade chemicals were from Whittaker Bioproducts Inc., (Walkersville, MD), and tissue culture dishes were from Falcon Labware (Lincoln Park, NJ). Four-well multiwell dishes for PMN adhesion assays were from Nunclon (Nunc, Roskilde, Denmark). Trypsin/EDTA was from Life Technologies, Inc., fetal Bovine Serum was from Hyclone Laboratories (Logan, UT), and human albumin was from Baxter Health Care Corp. (Glendale, CA). WEB 2086 was a generous gift from Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT).

The recombinant human plasma form PAF acetylhydrolase and hPAFR293 cells expressing the human PAF receptor were from ICOS Corp. (Bothell, WA), while phospholipase A₂ (bee venom), phospholipase C (*Bacillus cereus*), phospholipase D (cabbage), and butylated hydroxytoluene (BHT) were from Sigma. Dialysis tubing (6000–8000-kDa cut-off) was from Spectrum Medical Industries, Inc. (Houston, TX), and glass fiber filter papers were from VWR Scientific (Westchester, PA). FURA-2AM ester was from Molecular Probe (Eugene, OR). All the solvents (J.T. Baker, Inc.) were HPLC grade. Lipase from *Rhizopus arrhizus* was from Roche Molecular Biochemicals. 1-*O*-Hexadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (HAPC), PAF, 1-palmitoyl-2-acetyl-*sn*-glycero-3-phosphocholine (acyl-PAF), and lysoPAF were from Biomol Research Laboratories (Plymouth Meeting, PA). The long chain phospholipids were purified by reversed phase HPLC prior to use.

Commercial lysoPAF was subjected to mild alkaline hydrolysis as described below and acetylated with acid chlorides (acetyl, butyryl, or crotonyl) in the presence of perchloric acid (23) to generate PAF and its C₄ analogs. These were then purified by reversed phase HPLC and analyzed by GC/MS as described below. The total mass of the material was determined by lipid phosphorus analysis (24).

Isolation and Oxidation of Human LDL—Human LDL was isolated by density flotation from normolipidic subjects (25) as described in detail (10), except that we employed Pebabloc (200 μ M) as a non-toxic alternative to diisopropyl fluorophosphate to inactivate PAF acetylhydrolase (26) and allow oxidized products to accumulate (10). Isolated LDL was oxidized with 10 μ M CuSO₄ for 18–24 h at 37 °C. Control LDL was not subjected to oxidation and was prevented from oxidation by 100 μ M BHT.

Separation of PAF-like Lipids—Total lipids were extracted from LDL by the method of Bligh and Dyer (27) before neutral lipids, fatty acids, and phospholipids were separated by aminopropyl chromatography (10). The phospholipid fraction was further separated on a reversed phase column (ODS silica, 250 \times 4.6-mm Microsorb MV; Rainin Instrument Co., Woford, MA) with a mobile phase of methanol/acetonitrile/H₂O (840:150:10) containing 1 mM ammonium acetate and BHT (10 μ M) at a flow rate of 1 ml/min. Fractions were collected for every minute for the first 10 min, and PAF-like lipids elute between minutes 5 and 8. Recovery of a [³H]PAF internal standard added to the LDL particle in the HPLC fractions was >75%. Fractions found to contain leukocyte agonists (as described below) were pooled, the solvent removed by a stream of N₂, reconstituted with chloroform:methanol (2:1) containing BHT (10 μ M), and stored at –20 °C. Authentic PAF and PAF-like lipids were suspended in HBSS/A and sonicated prior to use.

PAF-like lipids isolated from LDL were further purified by straight phase chromatography prior to determining their specific bioactivity. For this, a portion of the PAF-like lipids separated on reversed phase HPLC were treated with lipase from *R. arrhizus* (28) and then injected onto a 5- μ m silica column (2 \times 150 mm, Phenomenex, Torrance, CA) and the column developed with an isocratic solvent system (hexane:isopropanol:20 mM ammonium acetate, pH 7 (3:4:0.7, v/v/v)) (29) at a flow rate of 0.2 ml/min. Fractions were dried under nitrogen and used for bioassays and mass spectrometry.

PMN Adhesion—Human neutrophils were isolated by dextran sedimentation and centrifugation over Ficoll (30). CD18-dependent adhesion of activated neutrophils to a gelatin surface after 10 min of incubation at 37 °C was quantified using a video microscopy imaging system to count adherent cells. Authentic PAF was used as a positive control and to establish the daily sensitivity of the cells. In experiments where recombinant PAF acetylhydrolase was used, PAF-like lipids or PAF were treated with 4 μ g of this enzyme in HBSS/A for 1 h at 37 °C before addition of the agonist to neutrophils. The enzyme itself caused no activation at this concentration. Alternatively, neutrophils were treated with 10 μ M WEB 2086 for 20 min prior to the addition of agonist as a means to competitively block the PAF receptor.

Pleurisy Model—Wistar rats (150–200 g) were injected (0.1 ml total volume) intrathoracically with pooled HPLC fractions 6, 7, and 8 resuspended in 0.1% bovine serum albumin in sterile saline. Some animals were treated with the PAF receptor antagonist (20 mg/kg) 1 h before challenge. Some pooled HPLC aliquots were treated with recombinant PAF acetylhydrolase (2 μ g) for 20 min at 37 °C, the lipids reextracted, dried, and resuspended in injection buffer before use. The animals were euthanized 6 h after injection in a CO₂ chamber, and the thoracic cavity opened and washed with 3 ml of heparinized (Liquemine; Roche, Rio de Janeiro, Brazil) saline (10 units/ml). The pleural wash was recovered, and the volume measured with a graduated syringe. Pleural washes

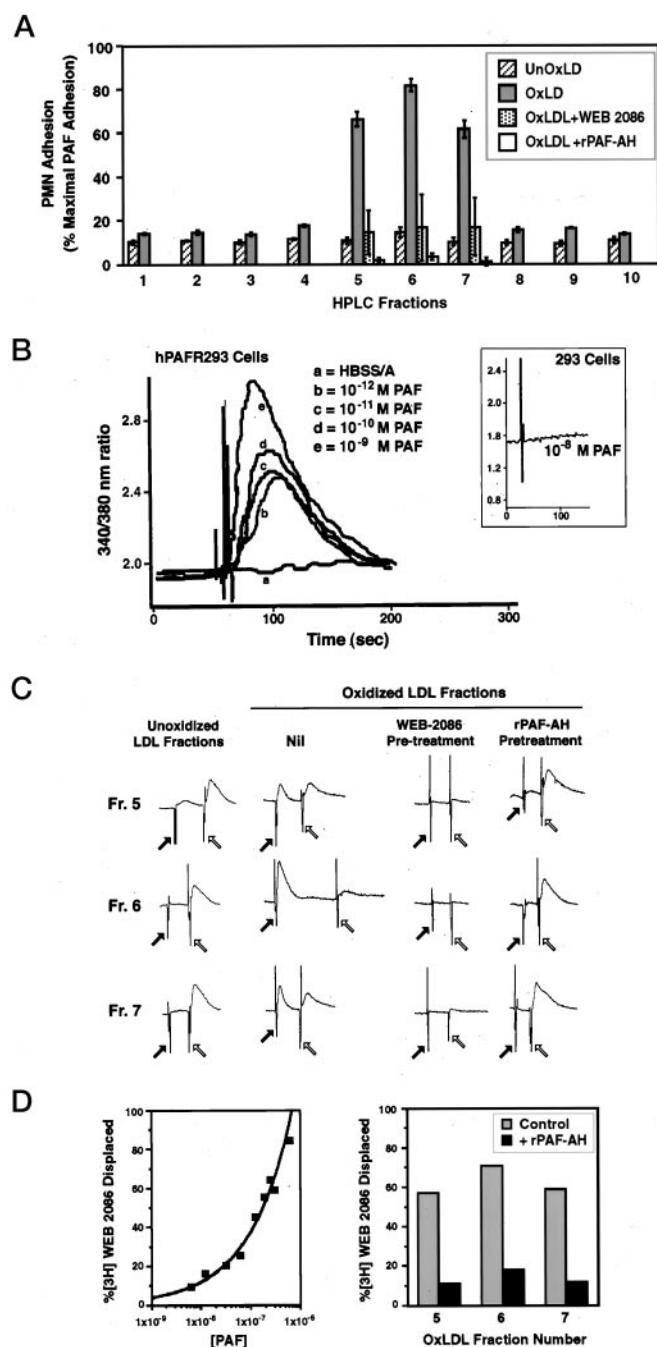


FIG. 2. Phospholipids from oxidized LDL demonstrate PAF-like activity. *A*, reversed phase HPLC purification of leukocyte agonists in oxidized LDL. Phospholipids were extracted from native or oxidized LDL, and separated by aminopropyl and C_{18} reversed phase HPLC as described under "Materials and Methods." Fractions were collected every minute, and an aliquot of this was dried under nitrogen before being reconstituted in HBSS/A. The ability of duplicate aliquots to stimulate PMN, as measured by their CD11/CD18-dependent adhesion to a gelatin-coated surface, was determined as a percentage of the maximal response to PAF by that donor's cells. The effect of the PAF receptor antagonist WEB 2086 ($10 \mu\text{M}$) on PMN adhesion, or the effect of pretreating the fractions with recombinant human PAF acetylhydrolase ($4 \mu\text{g}/\text{fraction}$) is also shown. This experiment is representative of two independent experiments. *B*, PAF-induced accumulation of intracellular Ca^{2+} in hPAFR293 cells. hPAFR293 cells were loaded with FURA2-AM and then stimulated with the stated concentration of PAF. Emission changes as fluorescence excitation jumped from 340 nm to 380 nm was captured as a function of time. The concentrations were as follows: *a*, HBSS/A buffer alone; *b*, 10^{-12} M PAF; *c*, 10^{-11} M PAF; *d*,

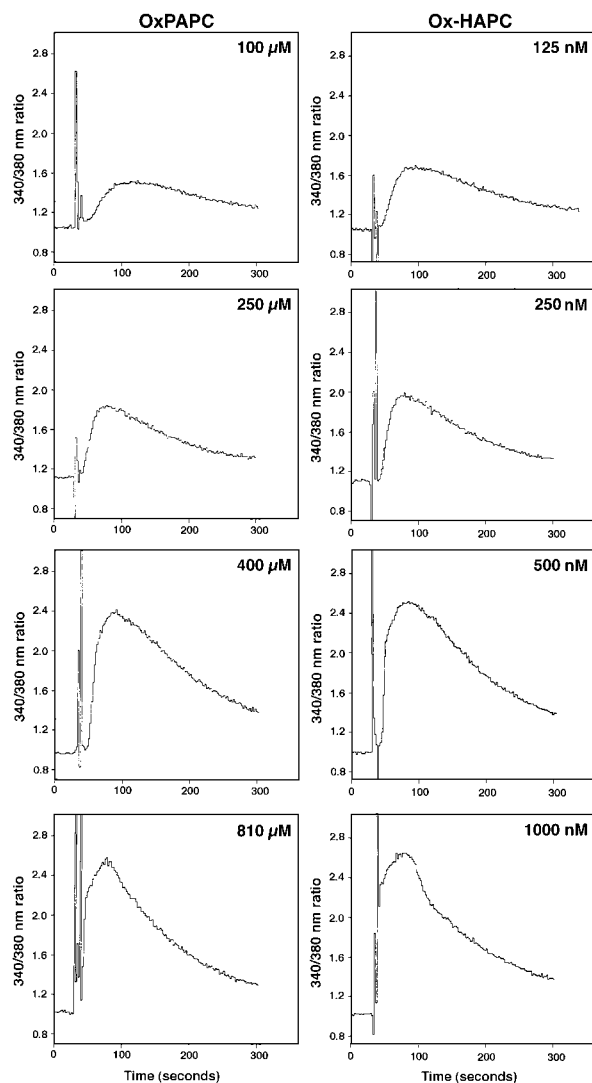


FIG. 3. Effect of an *sn*-1 ether bond on PAF-like activity of oxidized phosphatidylcholine. 1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycero-3-phospholcholine (HAPC) or 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) were oxidized with Cu^+ , the bioactive phospholipid separated by isocratic chromatography, and their concentration was determined by phosphorus analysis. Aliquots were added to FURA-2-loaded leukocytes, and the increase in intracellular Ca^{2+} was determined as described under "Materials and Methods."

loaded hPAFR293 cells were exposed to aliquots of HPLC fractions 5–7 from unoxidized LDL or Cu^+ -oxidized LDL as shown by the *filled arrow* (immediately adjacent fractions failed to alter Ca^{2+} levels in these cells and are not presented). After the fluorescence ratio returned to a stable base line, 10^{-10} M PAF was added (as shown by the *open arrow*) to measure receptor desensitization. In one series of measurements with aliquots from the same fraction, the cells were pretreated with WEB 2086 to block PAF receptor function. In a second series with material from these fractions, the aliquots were pretreated with recombinant human PAF acetylhydrolase. Individual components of this experiment were performed at least twice with similar findings. *D*, displacement of $[^3\text{H}]\text{WEB 2086}$ from hPAFR293 cell membranes. Membranes from hPAFR293 cells were purified, and their ability to bind $[^3\text{H}]\text{WEB 2086}$ was determined as described under "Materials and Methods." *Left*, PAF displacement. Increasing concentrations of PAF displace $[^3\text{H}]\text{WEB 2086}$ from hPAFR293 cell membranes. Total $[^3\text{H}]\text{WEB 2086}$ binding was 2457 ± 210 dpm, and the nonspecific binding, determined with 10^{-5} M unlabeled PAF, was 116 ± 20 dpm. *Right*, aliquots of fractions 5, 6, and

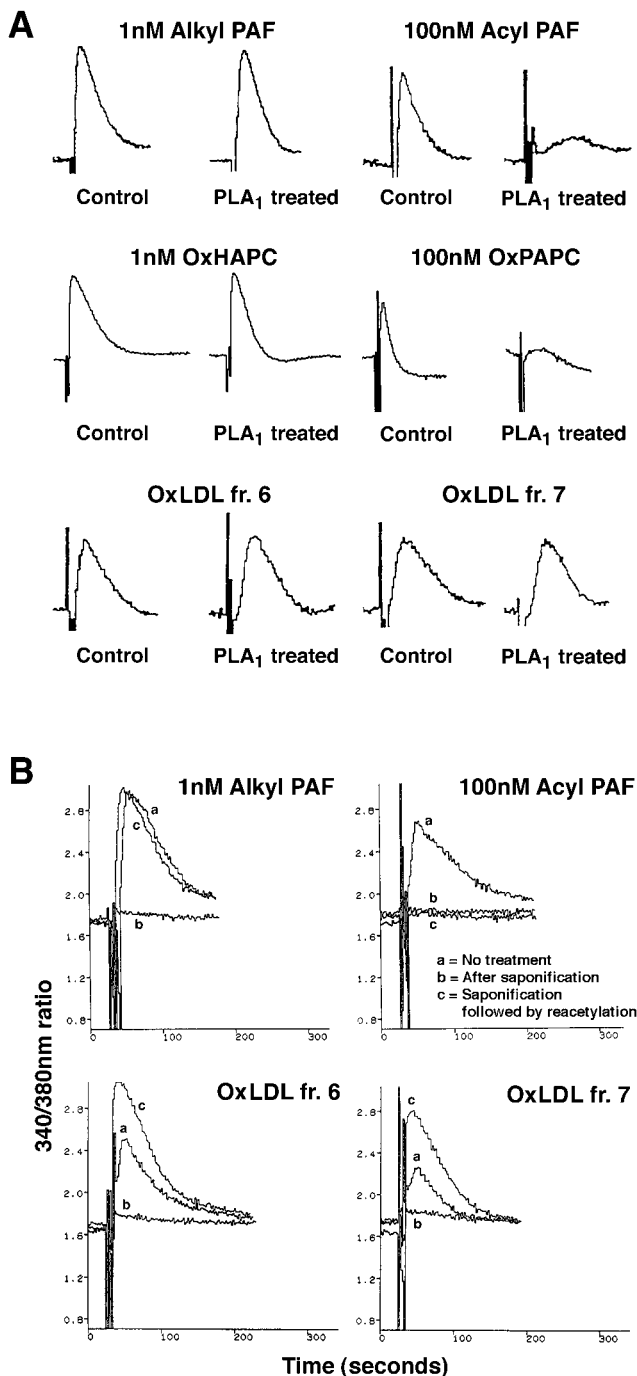


FIG. 4. Alkyl phosphatidylcholines account for the PAF-like activity found in oxidized LDL. *A*, the effect of phospholipase A_1 treatment on the Ca^{2+} flux induced in hPAFR293 cells by oxidized phospholipids. PAF and acyl-PAF (top panels) were treated, or not, with the lipase from *R. arrhizus* (5 units) in HBSS/A for 11 h at 37 °C and then added to FURA-2-loaded hPAFR293 cells. Changes in fluorescence as the excitation wavelength jumped between 340 and 380 nm was recorded as before. Synthetic phosphatidylcholines (middle panels) were oxidized, purified by isocratic HPLC, quantitated by phosphorus analysis, and treated with *R. arrhizus* lipase, or not, before adding to FURA-2-loaded hPAFR293 cells. Two maximally active fractions, fractions 6 and 7, from oxidized LDL (lower panels) were treated or not with lipase according to the above protocols, added to FURA-2-loaded hPAFR293 cells, and changes in the fluorescence ratio were determined as before. These experiments were repeated five times in different batches of LDL preparations. *B*, the effect of chemical saponification and reacylation on the Ca^{2+} flux induced in hPAFR293 cells by phospholipids from oxidized LDL. PAF, its acyl analog (upper panels)

the pleural wash was determined by a Biuret reaction after clearing by centrifugation at $500 \times g$ for 10 min.

Measurement of Intracellular Ca^{2+} in hPAFR 293 Cells—Subconfluent hPAFR293 cells (ICOS Corp., Bothell, WA) that stably express the human PAF receptor were treated with Versene (Life Technologies, Inc.) and resuspended in fresh culture medium ($\sim 1.1 \times 10^7$ cells/ml). FURA-2 AM was loaded into cells at $1 \mu M$ from a 1 mM Me_2SO stock, and after incubation in the dark for 45 min at 37 °C, the cells were washed with HBSS/A and resuspended in HBSS/A at a density of 2.25×10^6 cells/ml. Fluorescence of 1.5 ml of cells was measured at 24 °C, with dual excitation at 340 nm and 380 nm with the emission recorded at 510 nm (31). The response of each batch of cells was tested with 0.1 and 1 nM authentic PAF to generate the maximal PAF response. Control 293 cells were processed in the same way, and their response was tested with PAF, or with thrombin or lysophosphatidic acid as positive controls. For some experiments, we confirmed the results obtained with hPAFR293 cells by performing parallel experiments in FURA-2-labeled PMN. Ligand displacement of [3H]WEB 2086 from hPAFR293 cell membranes ectopically expressing the human PAF receptor was as described for Chinese hamster ovary cell membranes (32).

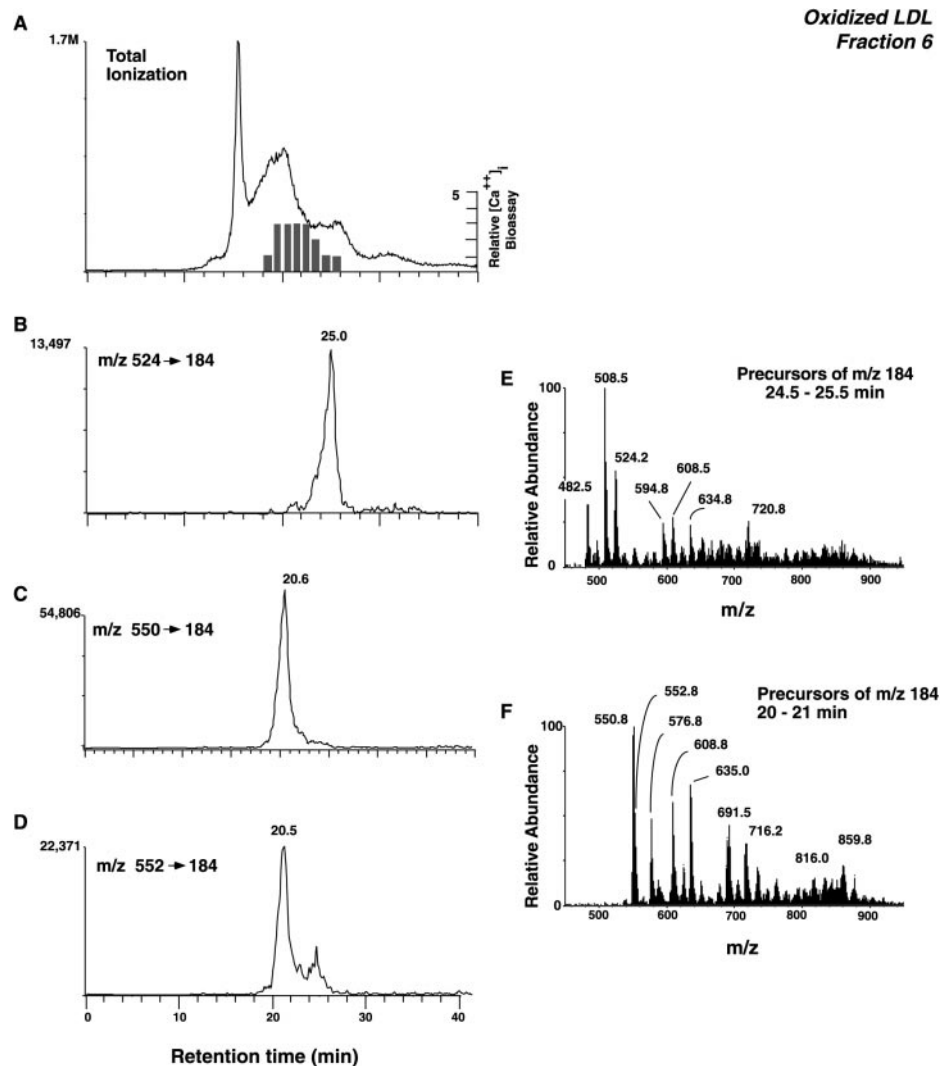
Structural Analysis—PAF-like lipids were treated with 5 units of lipase from *R. arrhizus* in HBSS/A for 11 h at 37 °C and then tested directly for their ability to mobilize Ca^{2+} in hPAFR 293 cells (28). Acyl-PAF (1-palmitoyl-2-acetyl-*sn*-glycero-3-phosphocholine) and PAF served as controls. In a similar fashion, PAF-like lipids were treated with phospholipase C (*B. cereus*), bee venom phospholipase A_2 , and cabbage phospholipase D before being tested for the ability to mobilize Ca^{2+} in PMN and hPAFR293 cells. The presence of an *sn*-1 ether bond was investigated by subjecting PAF-like lipids, PAF, or acyl-PAF to saponification with 0.5N NaOH in methanol for 2 h at 24 °C. Saponified material, containing free fatty acids and either lyso-PAF (1-*O*-hexadecyl-glycerophosphocholine) from glycerolipids with an *sn*-1 ether bond or glycerophosphocholine from diacyl phospholipids, did not induce Ca^{2+} accumulation in hPAFR293 cells. This material was reacylated with excess acetyl chloride in the presence of perchloric acid (23), and then reexamined for the ability to mobilize intracellular Ca^{2+} in the receptor-transfected cells.

Mass Spectrometric Analysis of Normal Phase HPLC Fractions—Direct LC/MS and LC/MS/MS analysis was carried out with a Sciex API-III⁺ triple quadrupole mass spectrometer (PE-Sciex, Thornhill, Ontario). For all electrospray ionization experiments, the curtain gas flow was 1.2 liter/min nitrogen with a nebulizer pressure at 38 p.s.i. The orifice potential was maintained at 75 V, and the electrospray ionization potential at +4200 V for detection of positive ions. For the analysis of negative ions, the ion spray potential was adjusted to -2800 V and purified air (zero air) was used to reduce any possibility for glow discharge at the electrospray needle. The orifice potential was maintained at -95 V. Selected ion recording experiments and multiple reaction monitoring experiments were carried out using the tandem quadrupole mass settings as indicated in the text. Normal phase HPLC was carried out in a 2×150 -mm normal phase silica column (Phenomenex, Rancho Cordova, CA) using a mobile phase of hexane/isopropanol/20 mM ammonium acetate (3/4/0.7) at the flow rate of 200 μ l/min. The GC/MS analysis of PAF-like lipids was carried out following hydrolysis of the glycerophosphocholine lipids with phospholipase C, followed by derivatization of the liberated diglycerides with pentafluorobenzoyl chloride as described previously (33). For the quantitative analysis of target molecules, [2H_3]PAF was added as internal standard (10 ng) to each aliquot taken for GC/MS analysis prior to treatment with phospholipase C.

RESULTS

Oxidation of LDL Generates Inflammatory Mediators—We extracted and purified the polar lipids from native and oxidized LDL and injected this into the pleural cavity of naive rats. The lipids isolated from oxidized LDL, but not its unoxidized counterpart, induced acute inflammation within 6 h as marked by leukocyte accumulation (Fig. 1A) and proteinaceous edema (Fig. 1B). The leukocyte accumulation was characterized by

FIG. 5. Normal phase LC/MS/MS analysis of the reverse phase HPLC fraction 6 obtained from oxidized LDL. HPLC retention times are indicated above each peak. A, elution of glycerophospholipid molecular species as indicated by the total ionization current derived from those components generating m/z 184 (phosphocholine cation) by electrospray ionization and collisional activation. Measurement of biological activity present in each HPLC fraction (0.5 min) is indicated in the bar graph as elevation of intracellular calcium ions in human polymorphonuclear leukocytes (see "Materials and Methods"). B, selected ion recording for the collisional activation of m/z 524, generating ions at m/z 184. This specific ion transition is the most abundant product ion following collisional activation of platelet-activating factor. C, selected ion recording for the collisional activation of m/z 550, generating ions at m/z 184. This specific ion transition is the most abundant product ion of collisional activation of butenoyl-PAF (16:0e/4:1-GPC). D, selected ion recording for the collisional activation of m/z 552, generating ions at m/z 184. This specific ion transition is the most abundant product ion of collisional activation of butenoyl-PAF (16:0e/4:0-GPC). E, mass spectra of all precursor ions for m/z 184 which eluted from the HPLC from 24.5 to 25.5 min. F, mass spectra of all precursor ions for m/z 184 which eluted from the HPLC from 20.0 to 21.0 min.



mononuclear cell and early eosinophil influx, but especially by a neutrophilic effusion. Treatment of the lipid preparation with recombinant human plasma acetylhydrolase (which specifically hydrolyzes phospholipids with short *sn*-2 acyl residues; Refs. 34 and 35) prior to injection into the animals blocked cellular infiltration and the edema. That the inflammatory principle was PAF or PAF-like analogs was strengthened by the potent inhibition of the inflammatory response by *in vivo* blockade of the PAF receptor with the specific antagonist WEB 2086.

Accumulation of PAF-like Lipids after Oxidation of LDL—We purified the leukocyte agonist in oxidized LDL by quantitating neutrophil adhesion, a measure of CD11/CD18 activation (36). The lipids derived from oxidized LDL that eluted between 5 and 7 min were leukocyte agonists, and these lipids were not present in native, unoxidized LDL (Fig. 2A). Like the *in vivo* events induced by the lipids isolated from oxidized LDL, *ex vivo* leukocyte activation was blocked by a specific PAF receptor antagonist WEB 2086 and by pretreating these fractions with purified, recombinant PAF acetylhydrolase. Treatment of these fractions with phospholipase A₂, phospholipase C, or phospholipase D inactivated the stimulatory compounds in fractions 5–7 (data not shown). This is an important confirmation that the biologically active species were still

cells stably transfected with the human PAF receptor that allows these cells to respond to PAF (Fig. 2B). Each fraction that activated neutrophils also induced a Ca²⁺ flux in these cells and by doing so, desensitized the ectopic PAF receptor to a second stimulus with PAF (Fig. 2C). The Ca²⁺ flux in these cells was blocked by co-incubation with WEB 2086 or by pretreatment with PAF acetylhydrolase. Lipids from unoxidized LDL did not activate these cells, showing oxidation truly generates PAF-like phospholipids. We quantitated the amount of PAF equivalents in the active fractions to determine whether this shadowed leukocyte stimulation using a competitive [³H]WEB 2086 displacement assay and purified membranes from hPAFR293 cells (Fig. 2D). We calculate that there was twice the amount of PAF-like material (equivalent to 20 nM PAF) in fraction 6 than in either fraction 5 or 7 (which contained 9 and 10 nM PAF equivalents, respectively.) Following the treatment of each fraction with recombinant PAF acetylhydrolase competition with [³H]WEB 2086 was lost, and surrounding fractions, or equivalent fractions from unoxidized LDL, also failed to displace [³H]WEB 2086.

PAF-like Lipids in Oxidized LDL Are Alkyl Phospholipids—Oxidation of synthetic diacyl phosphatidylcholines generates PAF-like activity (11, 16, 37), suggesting that some particular modification of the fragmented *sn*-2 acyl residue can overcome

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