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 atherosclerosis. 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-PAF analogs 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.

Platelet-activating factor (PAF)† is a phospholipid autacoid with a wide variety of actions, primarily on cells and events that comprise the inflammatory system. PAF initiates the 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 identification of the biologically active species in the mix of similar
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 Da cut-off) was from Spectrum Medical Industries, Inc. (Houston, TX), and glass fiber filter papers were from VWR Scientific (Westchester, PA). PURA-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-aranachidonoyl-sn-glycerol-3-phosphocholine (HAPC), PAF, 1-palmitoyl-2-acyl-Haparachidonoylethanolamine (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 lysophosphatidylcholine 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 Pefabloc (200 μg) as a non-toxic alternative to diisopropyl fluorophosphate to inactivate PAF acetylhydrolase (26). Lipids and oxidized products to accumulate (10). Oxidized LDL was oxidized with 10 μM CuSO4 for 18–24 h at 37 °C. Control LDL was not subjected to oxidation and was prevented from oxidation by 100 μM 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 × 4.6-mm Microsorb, Rainin Instrument Co., Woford, MA) with a mobile phase of methanol/acetonitrile/ 

H2O (840:150:10) containing 1 mM ammonium acetate and BHT (10 μM) 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 [14C]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 μM), 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 activity. 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-μm silica column (2 × 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)) at a flow rate of 0.2 ml/min. Fractions were dried under nitrogen and used for assays 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 μg 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 μM 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) intratracheally with pooled HPLC fractions 6, 7, and 8 and 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 μg) for 20 min at 37 °C, the lipids reextracted, dried, and resuspended in injection buffer before use. The animals were essentially killed 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 were diluted in Turk fluid (2% acetic acid) for total cell counts in Neubauer chambers. Differential analysis was performed in cytosmears stained by the May Grunwald-Giemsa method. The protein content of

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 μg 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 ± S.E. from at least four animals. Mono, monocytes; PMN, neutrophils; Eo, eosinophils.
Figure 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 C8 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 (10⁻⁶ M) on PMN adhesion, or the effect of pretreating the fractions with recombinant human PAF acetylhydrolase (4 mg/fraction) is also shown. This experiment is representative of two independent experiments.

B, PAF-induced accumulation of intracellular Ca²⁺ 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, 10⁻¹⁰ M PAF; e, 10⁻⁹ M PAF. Inset, fluorescence ratio of FURA2-loaded untransfected 293 cells exposed to 10⁻⁶ M PAF. C, activation of hPAFR293 cells by aliquots of purified LDL phospholipids. FURA-2-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 Ca²⁺ levels in these cells and are not presented). After the fluorescence ratio returned to a stable baseline, 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 [³H]WEB 2086 from hPAFR293 cell membranes. Membranes from hPAFR293 cells were purified, and their ability to bind [³H]WEB 2086 was determined as described under “Materials and Methods.” Left, PAF displacement. Increasing concentrations of PAF displace [³H]WEB 2086 from hPAFR293 cell membranes. Total [³H]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 7 were used to displace bound [³H]WEB 2086. Some aliquots of fractions 5, 6, and 7 were treated with recombinant human PAF acetylhydrolase prior to addition. This experiment is representative of one other.
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Measurement of Intracellular Ca\(^{2+}\) in hPAFR293 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 (~1.1 × 10^6 cells/ml). FURA-2 AM was loaded into cells at 1 μM from a 1 mM Me\(_2\)SO 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 FURA2-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 hPAFR293 cells (28). Acyl-PAF (1-palmitoyl-2-acetyl-sn-glycero-3-phosphocholine) and PAF were used 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 0.5 N 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 reacetylated 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 flux induced in hPAFR293 cells by 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 the pleural wash was determined by a Biuret reaction after clearing by centrifugation at 500 × g for 10 min.

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 saponified material was chemically acetylated with acetyl chloride before addition to FURA2-loaded hPAFR293 cells. The tracings are as follows: a, untreated material; b, after saponification; c, after acetylation of saponified material.
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 activated the stimulatory compounds in fractions 5–7 (data not shown). This is an important confirmation that the biologically active species were still phospholipids, and were not simply fragments released from oxidizing polyunsaturated acyl residues. We established that the active agent(s) acted through the PAF receptor using 293 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 Ca$^{2+}$ flux in these cells and by doing so, desensitized the ectopic PAF receptor to a second stimulus with PAF (Fig. 2C). The Ca$^{2+}$ flux in these cells was blocked by co-incubation with WEB 2086 or by pre-treatment 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 $[^3]$HWEB 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 $[^3]$HWEB 2086 was lost, and surrounding fractions, or equivalent fractions from unoxidized LDL, also failed to displace $[^3]$HWEB 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 the normally strong preference for an sn-1 ether bond. We tested this prediction by oxidizing 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine and its sn-1 ether homolog 1-O-
hexadecyl-2-arachidonoyl-sn-glycero-3-phosphocholine, purifying the oxidation products, and quantitating their mass by phosphorus analysis. When the concentration of the two homologous oxidation products was adjusted to give equivalent amounts of Ca\(^{2+}\) release in leukocytes, we found (Fig. 3) that 800-fold more diacyl products were required. This suggests that there is no highly preferred sn-2 residue in oxidized diacyl phosphatidylcholines that can overcome the requirement for an sn-1 ether bond.

In light of this information, we determined the nature of the sn-1 bond of the bioactive phospholipids in oxidized LDL. This was done by hydrolyzing diacyl phosphatidylcholines with phospholipase A\(_1\) before analysis in the hPAFR293 cell Ca\(^{2+}\) flux assay. Control experiments (Fig. 4A) showed the acyl analog of PAF (which is about 1% as potent as PAF in this assay) was destroyed by this digestion, while PAF with its sn-1 ether bond was unaffected. An identical result was obtained when the oxidation products of 1-palmitoyl-2-arachidonoyl-glycerophosphocholine and 1-hexadecyl-2-arachidonoyl-glycerophosphocholine were digested. Similarly, phospholipase A\(_2\) digestion destroyed nearly all of the phospholipid mass in fractions 5 through 7 derived from oxidized LDL as determined by phosphorus staining of the lipids resolved by TLC (not shown). In contrast, phospholipase A\(_2\) did not detectably reduce the PAF-like bioactivity in these fractions (Fig. 4A). We confirmed this result using chemical saponification to completely hydrolyze diacyl compounds, which abolished PAF-like activity of both PAF and acyl-PAF (Fig. 4B). Chemical acetylation returned the PAF sample to its original level of activity (compare traces a and c), but did not have a similar effect with acyl-PAF. Saponification of fractions 6 and 7 from oxidized LDL, also completely inactivated the PAF-like activity. Acetylation of the hydrolysis products restored PAF-like activity, a result not possible if fractions 6 and 7 just contained oxidation products derived from diacyl phospholipids.

Identification of PAF-like Lipids in Oxidized LDL—We took advantage of the above findings to obtain highly purified PAF-like lipids from oxidized LDL for tandem mass spectrometry. Direct analysis of the phospholipids isolated from oxidized LDL following treatment with phospholipase A\(_1\), as well as analysis of oxidized synthetic 1-hexadecyl-2-arachidonoyl-glycerophosphocholine, was carried out with online liquid chromatography directed into a tandem quadrupole mass spectrometer using electrospray ionization. Extracts from oxidized LDL or synthetic phospholipid were first separated by reverse phase HPLC, and the biologically active fractions were then separated by normal phase HPLC during the LC/MS/MS experiment. Elution of glycerophosphocholine components was detected by precursor ion scanning in a positive ion mode by measuring those ions that could be collisionally activated to yield m/z 184 (Fig. 5A), the phosphocholine ion (38). Using this approach, specific molecular species could be detected as they eluted at the appropriate HPLC retention times, including m/z 524 → 184 for PAF (Fig. 5B) and m/z 552 → 184 for butanoyl-PAF (Fig. 5D). Additional glycerophospholipid species also eluted in the region of PAF (24.5-25.5 min) as seen by numerous abundant phosphocholine ions between m/z 480 and 900 (Fig. 5E). This was also true for the elution position of 1-hexadecyl-2-butanoyl-glycerophosphocholine (16:0e/4:0-GPC; m/z 552.8 → 184, 20.5 min) from the HPLC (Fig. 5F). The most abundant species, with the transition m/z 550.8 → 184, eluted at 20.6 min (Fig. 5C). This species was tentatively identified as 16:0e/4:1-GPC because it contained one additional degree of unsaturation compared with 16:0e/4:0-GPC (m/z 552.8). The
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Quantitation of PAF and PAF analogs in oxidized LDL fractions

LDL was oxidized, a [3H]PAF internal standard was added, and fractions containing PAF-like activity were identified using a leukocyte adhesion assay. These fractions were digested with phospholipase C and the free hydroxy group derivatized with pentafluorobenzoyl chloride before analysis by GC/MS as described under "Materials and Methods."

| Analog         | Fraction 6 | Fraction 7 |
|----------------|------------|------------|
| PAF            | 1.0        | <0.3       |
| Butanoyl-PAF   | 10.5       | 31.7       |
| Butenoyl-PAF   | 20.5       | 16.2       |

DISCUSSION

The PAF receptor is a G protein-linked heptaspanning receptor (4) that specifically recognizes the sn-2 ether bond, the...
short \( sn-2 \) acetyl residue, and the choline headgroup of PAF (5, 6). Oxidation of phosphatidylcholine from various sources (9–11, 13, 14) generates PAF mimetics and a host of related phospholipid products with fragmented \( sn-2 \) residues. Because of the many products, it has been difficult to identify those that account for the bioactivity, especially when starting with a biologic source that contains a mixture of \( sn-1 \) bonds and various \( sn-2 \) fatty acyl residues. Further complicating the issue is the generation of PAF mimetics from synthetic diacyl phosphatidylcholines (12, 16, 17, 39). These lack the \( sn-1 \) ether bond that confers potency, suggesting that an unusual modified \( sn-2 \) residue(s) produced by oxidative fragmentation might overcome the preference of the PAF receptor for an \( sn-1 \) ether bond. Here we find that, in fact, nearly all of the PAF mimetics produced by oxidation of LDL as a source of mixed starting phospholipids are derived from its rare alkyl acyl phosphatidylcholines. Identification and quantitation of these shows that two C4 homologs of PAF, apparently derived from the fragmentation of an \( sn-2 \) arachidonoyl residue, account for much of the inflammatory activity in this atherogenic particle. The functionality that increases the potency of the LDL oxidation products is in fact the \( sn-1 \) ether bond, not unusual \( sn-2 \) oxidation products.

Oxidation of LDL is now thought to be an initiating and sustaining event in atherogenesis through the creation of inflammatory lipids and the covalent modification of the particle (40, 41). Injection of oxidized LDL results in a systemic inflammatory reaction where leukocytes adhere to the walls of the microvascular system (42) after activation of the PAF receptor on leukocytes and platelets (43). We show that the lipids present in oxidized LDL not only function as chemoattractants for neutrophils in \textit{vivo}, but that these lipids cause a significant monocytic and eosinophilic influx within 6 h. Recruitment of these inflammatory cells was also associated with considerable edema. Each of these cell types contain functional PAF receptors, and one consequence of its activation is eosinophil (44), monocyte (45), and neutrophil (46) chemotaxis \textit{in vivo}. The neutrophilic, monocytic, and eosinophilic influx was abolished and the edema sharply curtailed by \textit{in vivo} treatment with a specific PAF receptor antagonist or by treatment of the oxidized lipids with PAF acetylhydrolase prior to injection. Thus, PAF-like oxidation products are inflammatory in an \textit{in vivo} model, and they account for all of the inflammatory properties over the first few hours of the response of the lipids extracted from oxidized LDL.

The acyl analog of PAF is several hundredfold less potent as a PAF receptor agonist than PAF, and we found a similar requirement for the \( sn-1 \) ether bond when we compared oxidized phospholipids. The activity ratio of oxidized 1-O-hexadecyl-2-arachidonoyl-sn-glycero-3-phosphocholine to its \( sn-1 \) acyl homolog in stimulating \( Ca^{2+} \) flux in PMN cells was approximately 800, suggesting that highly potent diacyl oxidation products are not present in abundance. Lipoproteins transport small amounts of alkyl acyl phosphatidylcholines (21), which varies with LDL subtype (22). The nature of the \( sn-1 \) bond (with the exception of 1’ alkenyl phospholipids; Ref. 47) does
not affect the types of oxidative reactions that fragment unsaturated acyl residues, so oxidation of diacyl and alkylacyl phosphatidylcholines produce homologous species (20). Thus we expect that LDL oxidation should produce homologous fragmented diacyl and alkyl acyl phosphatidylcholine products in relation to the abundance of their precursors, which is approximately 150 to 1. Accordingly, we found that treatment of phosphatidylcholines from oxidized LDL by phospholipase A1 hydrolyzed the great majority of the phospholipid mass. This treatment did not affect the amount of PAF-like activity in these fractions, suggesting they were mostly derived from the oxidation of the rare alkyl phosphatidylcholines in LDL.

We (16) and others (12, 17, 39) have shown that oxidation of diacyl phosphatidylcholines, like that shown here after oxidation of alkyl acyl phosphatidylcholines, generates PAF-like lipids. Here we find that, just as with PAF and its acyl homolog (e.g. Fig. 4), that oxidation of alkyl acyl phosphatidylcholine generates PAF-like compounds that are around 800-fold more active than those generated by oxidation of a diacyl homolog (Fig. 3).\(^2\) Consistent with this, we find that phospholipase A\(_1\) digestion of the 150-fold excess diacyl phosphatidylcholine in LDL did not significantly reduce the PAF-like bioactivity in the crude lipid extract. Thus, the contribution of diacyl compounds to the total PAF-like activity may just be undetectable in the presence of their more active alkyl homologs.

We identified oxidatively fragmented alkyl phosphatidylcholines in oxidized LDL by tandem mass spectrometry, and correlated the elution of these with biologic activity. Abundant ions that correlated with PAF receptor activation were identified as a butenoyl analog of PAF and its saturate butanoyl homolog. These two fragmented phospholipids were also abundant products when synthetic hexadecyl arachidonoyl phosphatidylcholine was oxidized, suggesting this may have been the precursor in LDL. Butanoyl-PAF has been shown to be 10-fold less potent than PAF, and we show here that introduc-

\(^2\) S. S. Davies, G. K. Marathe, K. A. Harrison, R. C. Murphy, S. M. Prescott, and T. M. McIntyre, unpublished data.
tion of a double bond at the 2-position (rather than the 3-position most likely found in LDL-derived material) did not significantly alter this receptor stimulation.

We additionally found small amounts of PAF in oxidized LDL, and the origin of both the C₄₋ and C₅₋PAF-like lipids could result from the decomposition of arachidonoyl hydroperoxy radical species (Fig. 9). Hydrogen atom rearrangement of the 5-hydroperoxy radical intermediate of 1-α-alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine and PAF. A possible mechanism for the decomposition of the alkoxyl radical to this species of C₄₋-PAF (Fig. 9) involves the formation of a stable aldehyd and an intermediate carbon centered radical at the 2-position. This intermediate could also undergo loss of ethylene (48) followed by hydrogen atom extraction to yield PAF. All these products were found after the oxidation of synthetic 1-α-hexadecyl-2-butanoyl-sn-glycero-3-phosphocholine and PAF. A possible mechanism for the postulated loss of the ethylene group appears to be a low probability event compared with hydrogen ion extraction. The probability of PAF compared with these C₄ homologs increases the contribution of PAF to the total activity of oxidized LDL, but this still can account for only about 10% of the biologic activity (Figs. 5 and 6). Thus, the postulated loss of the ethylene group appears to be a low probability event compared with hydrogen ion extraction. The 10-fold greater potency of PAF compared with these C₄ homologs increases the contribution of PAF to the total activity of oxidized LDL, but this still can account for only about 10% of the total PAF-like bioactivity. Other phospholipid oxidation products may contribute to the proatherogenic activity of oxidized LDL, but two of the major PAF-like lipids that accumulate in oxidized LDL are formed during fragmentation of an arachidonoyl residue.

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