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Monocyte Chemoattractant Protein-1 and 5-Lipoxygenase Products Recruit Leukocytes in Response to Platelet-Activating Factor-Like Lipids in Oxidized Low-Density Lipoprotein

Adriana R. Silva,* Edson F. de Assis,† Lara F. C. Caiado,* Gopal K. Marathe,‡§ Marcelo T. Bozza,† Thomas M. McIntyre,‡§ Guy A. Zimmerman,† Stephen M. Prescott,‡§ Patricia T. Bozza,* and Hugo C. Castro-Faria-Neto‡*

Oxidized low-density lipoprotein (LDL) contains inflammatory agents, including oxidatively fragmented phospholipids that activate the platelet-activating factor (PAF) receptor, but in vivo events caused by these pathologically generated agents are not well defined. Injection of PAF-like lipids derived from oxidized LDL, or C4-PAF that is a major PAF-like lipid in these particles, into the pleural cavity of mice resulted in rapid monocyte, neutrophil, and eosinophil accumulation. Increased numbers of intracellular lipid bodies in these cells show they were in an inflammatory environment. Leukocyte recruitment was abolished by a PAF receptor antagonist, as expected. PAF-like lipids induced 5-lipoxygenase expression in leukocytes, mRNA expression for monocyte chemoattractant protein-1 (MCP-1) and other chemokines, synthesis of MCP-1, and leukotriene B4. The 5-lipoxygenase inhibitor zileuton impaired neutrophil influx, while MCP-1 had a more global role, as determined with MCP-1−/− mice. The lack of MCP-1 abrogated leukocyte accumulation and lipid body formation both in vivo and in vitro and chemokine transcription in vivo, and reduced in vivo leukotriene B4 production. Thus, PAF-like phospholipids in oxidized LDL induce an inflammatory infiltrate through the PAF receptor, chemokine transcription, lipid body formation, and 5-lipoxygenase expression in leukocytes. MCP-1 has a key role in this inflammatory response, and 5-lipoxygenase products are essential for neutrophil recruitment into the inflamed pleural cavity. The Journal of Immunology, 2002, 168: 4112–4120.
increases atherogenesis in susceptible mice (18). Conversely, mice doubly deficient for the MCP-1 receptor (CCR2) and apolipoprotein E on a Western diet show decreased lesion formation as compared with atherosclerosis-susceptible apolipoprotein E-deficient mice (19). Activation of the PAF receptor stimulates MCP-1 synthesis by monocytes in vitro, but only when they are tethered to P-selectin and receive appropriate adhesion-dependent cosignals (20). Whether oxidized phospholipids act on PAF receptor-bearing cells and expand the acute inflammatory response through cytokine synthesis in vivo oxidative, inflammatory conditions is not known.

We investigated the mechanisms of the inflammatory effects of oxidatively fragmented phospholipids extracted from oxidized LDL on leukocyte accumulation in an animal pleurisy model. We characterized the profile of chemokine expression and assessed the involvement of MCP-1 on leukocyte influx and lipid body formation in MCP-1-deficient mice. The results showed that MCP-1 is an early response that amplifies inflammation through the production of another lipid mediator, leukotriene B4 (LTB4). These results also show that neutrophils, which are not a primary target of MCP-1 in vitro, primarily are recruited into the inflammatory environment through the induction of 5-lipooxygenases and LTB4 by MCP-1. In vivo, MCP-1 is a pleiotropic regulator with direct and secondary effects on the development of an inflammatory response even when cells possess receptors for PAF.

Materials and Methods

Materials

[α-32P]UTP was purchased from NEN Life Sciences (Boston, MA); nuc-5 in vitro hybridization kit and RNase protection kit were from BD PharMingen (San Diego, CA); anti-5-lipooxygenase rabbit IgG was from Cayman Chemicals (Ann Arbor, MI); anti-rabbit biotinylated IgG was from Vector Laboratories (Burlingame, CA); aqua Polymount was from Polyscience (Warrington, PA); BN 5201 was from Biomol (Plymouth Meeting, PA); C57-PAF, 1-O-hexadecyl-2-butanolyl-sn-glycero-3-phosphocholine was from Avanti Polar Lipids (Alabaster, AL); aminopropyl columns were from J. T. Baker (Phillipsburg, NJ); PAF, 1-O-hexadecyl-2-acetylsn-glycero-3-phosphocholine was from Sigma-Aldrich (St. Louis, MO); Pefabloc was from Pentapharm Laboratories (Basel, Switzerland); LTB4 was detected by immunoenzymatic assay with a kit from Cayman Chemicals, while the kit for MCP-1 detection was from R&D Systems (Minneapolis, MN); Ultraspec RNA isolation system was from Biotecx Laboratories (Houston, TX); zileuton was obtained from Abbott Laboratories (Abbott Park, IL). Swiss mice were maintained by the Oswaldo Cruz Foundation breeding facility. MCP-1-deficient mice or their backcrossed controls (13) were used, this was administered i.n. into naive animals as a further control. Synthetic C3-PAF when tested as an agonist was reconstituted in HBSS/BSA and injected i.t. at 1 μg per cavity. After 3, 6, 12, and 24 h, the injected animals were sacrificed in a CO2 gas chamber, and the thoracic cavity was opened and washed with 1 ml HBSS. These pleural washes were recovered, and their volume was measured. When MCP-1-deficient mice or their backcrossed controls (13) were used, this analysis was performed at the fixed time of 6 h after stimulation.

Pleurisy model

Swiss mice (20–25 g) from Oswaldo Cruz Foundation breeding unit were kept at constant temperature (25°C) with free access to diet and water in a room with a 12-h light/dark cycle. The experiments in this study received prior approval from the Oswaldo Cruz Institute’s animal welfare committee. Mice were treated with an ip dose (20 mg/kg) of the PAF antagonist BN 52013 in saline. HBSS injections were administered i.t. into naive animals. Mice were sacrificed by cervical dislocation after 30 min incubation with fura 2 (2 μM) before use. The HBSS/BSA solution alone was administered i.t. into naive animals as a further control. Synthetic C3-PAF when tested as an agonist was reconstituted in HBSS/BSA and injected i.t. at 1 μg per cavity. After 3, 6, 12, and 24 h, the injected animals were sacrificed in a CO2 gas chamber, and the thoracic cavity was opened and washed with 1 ml HBSS. These pleural washes were recovered, and their volume was measured. When MCP-1-deficient mice or their backcrossed controls (13) were used, this analysis was performed at the fixed time of 6 h after stimulation.

Effect of oxidized phospholipids on calcium mobilization in human neutrophils

Blood from healthy volunteers was drawn into a syringe containing sodium citrate (to yield a final concentration of 3.2%), and then layered over Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) and centrifuged (30 min at 500 × g) as in the neutrophil purification method described by Zimmermann et al. (23). Neutrophil pellets were suspended in 2 ml 0.2% NaCl for hemolysis, followed by the addition of 2 ml 1.6% NaCl before centrifugation (5 min at 400 × g). The neutrophils were washed with HBSS (calcium and magnesium free) and counted, and their number was adjusted to 5.5 × 106/ml before incubation with fura 2 (2 μM) for 45 min at 37°C in the dark. Ca2+ transients were determined in the fura 2-load neutrophils, as before (2).

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Purification and oxidation of human LDL

Human LDL was isolated, as described elsewhere (1). Briefly, LDL was purified from plasma of healthy volunteers after adjusting the density to 1.3 g/ml with potassium bromide. A gradient was formed from this plasma and saline overlaying it by centrifugation for 200 min at 150,000 × g. After centrifugation, the band of lipoproteins with densities between 1.019 and 1.062 was collected and dialyzed against PBS. The isolated LDL (200 μg protein/ml) was treated with a PAF acetylhydrolase inhibitor (200 μM Pefabloc) before oxidation with CuSO4 (10 μM) for 18 h at 37°C. Control LDL particles were those not subjected to oxidation.

Separation of lipids

The lipids were extracted from LDL by the method of Bligh and Dyer (21). Neutral lipids, fatty acids, and phospholipids were separated by amino-polymer chromatography using chloroform-isopropanol (2:1 v/v); acetic acid (2%) in diethyl ether; and methanol, respectively (22). The phospholipid fraction was further separated on a reversed-phase HPLC with a mobile phase of methanol (84%), acetonitrile (15%), and deionized water containing 1 mM ammonium acetate (1%), as previously described (2). The fractions were dried under a stream of N2, reconstituted with chloroform:methanol (2:1) containing BHT (10 μM), and stored at −20°C. Bioactive fractions and control fractions were suspended in HBSS/BSA (HBSS containing 0.01% BSA) and sonicated before use.

Immunocytochemistry

Cytosmear preparations from pleural washes were fixed in 3% formaldehyde, permeabilized with a solution of 0.05% saponin in HBSS, treated with a biotin blocking solution (Vector Laboratories), and then blocked with 10% normal goat serum. After washing, these cytospin preparations were incubated overnight at 4°C with the primary rabbit polyclonal antibody mouse 5-lipoxygenase Ab (diluted 1/150 in 0.05% saponin/HBSS). Pre-immune rabbit serum was used as control. After three washes of 5 min in 0.05% saponin/HBSS, the preparations were incubated with biotin-conjugated goat anti-rabbit IgG. The immunoreactive 5-lipoxygenase in cells was then identified by ABC Vectastain glucose-oxidase kit (Vector Laboratories) following the manufacturer’s instructions. The resulting glucose-oxidase immunostaining was visualized under light microscopy (25).

In vivo treatment with receptor antagonists and inhibitors

Swiss mice were treated with an ip dose (20 mg/kg) of the PAF antagonist BN 52013 in saline. When the animals were to be treated with zileuton (50 μg per cavity), this 5-lipoxygenase inhibitor was injected i.t. immediately before the injection of oxidized phospholipids or C5-PAF. BN 52013 was dissolved in saline, while zileuton was solubilized in a DMSO stock solution such that the final DMSO concentration in the injectate was 0.01%.
**In vitro lipid body formation in murine peritoneal macrophages**

The peritoneal cavities of untreated wild-type and MCP-1-deficient mice were washed with PBS to recover resident macrophages. The cell concentration in these washings was adjusted to 10^6 cells/ml before these suspended cells were cultured with PAF (1 μM), oxidized phospholipids (HPLC fractions 6–8, or 2, 10, and 11), C_4-PAF (1 μM), or vehicle (HBSS/BSA 0.1%) at 37°C. After 1 h, the cells were cytocentrifuged onto glass slides (10^4 per slide) and stained with osmium, and the number of lipid bodies in 50 consecutively scanned macrophages was determined as above.

**RNA protection assay**

RNA was purified according to the Ultraspec RNA protocol (Biotex Laboratories), and the RNase protection assays were performed according to the Riboquant protocol (BD PharMingen). In brief, leukocytes derived from the pleural cavities from each experimental group were pooled, and their total RNA was isolated. RNA (10 μg) from each pool was hybridized with the mck-5 RNase protection probe (BD PharMingen) using a previously synthesized radioactive probe. Hybridized RNA was treated with RNase, isolated and loaded on a denaturing polyacrylamide gel, and developed at 50 W. The resulting gel was adored onto a filter paper, dried, and exposed to film for quantification by autoradiography (26).

**ELISA for chemokines and LTB_4**

LTB_4 was detected by immunoenzymatic assay, according to the manufacturer’s instruction (Cayman Chemicals). The chemokine MCP-1 was quantitated in cellular supernatants by sandwich ELISA according to the MCP-1 detection kit’s (R&D Systems) accompanying protocol.

**Statistical analysis**

The data are represented as mean ± SEM and were statistically analyzed by means of ANOVA, followed by a Newman-Keuls-Student test with significance level set at p < 0.05.

**Results**

**Phospholipids purified from oxidized LDL cause the accumulation of inflammatory cells in vivo**

We previously demonstrated that phospholipids extracted from oxidized LDL could be fractioned by reversed-phase HPLC to obtain bioactive fractions, eluting at min 6–8, with proinflammatory activity both in vitro and in vivo (2). In the present study, we confirmed that the current fractions of oxidized phospholipids induced a transient rise in the concentration of free intracellular calcium in neutrophils (data not shown). This biochemical change indicated that the neutrophils had been activated, so we next performed an experiment in vivo to characterize the nature of the inflammatory response to these oxidatively generated agonists. We found that injection of these fractions into the pleural cavity of mice induced the formation of an exudate with a distinct increase in cellularity compared with animals receiving nonactive fractions (Fig. 1A). Analysis of the cellular composition of the cells recovered from animals injected with biologically inactive fractions, in this case the HPLC fractions surrounding those that contained PAF-like activity, showed that the recovered cells were mainly resident macrophages (Fig. 1B). In contrast, the exudate from animals receiving the bioactive fractions isolated from oxidized LDL revealed a significant enrichment in the neutrophil and eosinophil populations, as illustrated in Fig. 1C. The peritoneal cavities of untreated wild-type and MCP-1-deficient mice were washed with PBS to recover resident macrophages. The cell concentration in these washings was adjusted to 10^6 cells/ml before these suspended cells were cultured with PAF (1 μM), oxidized phospholipids (HPLC fractions 6–8, or 2, 10, and 11), C_4-PAF (1 μM), or vehicle (HBSS/BSA 0.1%) at 37°C. After 1 h, the cells were cytocentrifuged onto glass slides (10^4 per slide) and stained with osmium, and the number of lipid bodies in 50 consecutively scanned macrophages was determined as above.

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synthetic C₄-PAF induced an increase in the number of leukocytes, both neutrophils (Fig. 2C) and eosinophils (Fig. 2D), recovered from the pleural cavity of mice. The time relationship of this influx recapitulated that induced by the lipids extracted from oxidized LDL (compare Figs. 1 and 2).

**Oxidized phospholipids stimulate lipid body formation in leukocytes**

An enhanced burden of intracellular droplets, lipid bodies, is characteristic of activated inflammatory cells (28) and can mark an in vivo inflammatory environment. Cells isolated from the pleural cavity of animals injected with HPLC fractions from unoxidized LDL show few intracytoplasmic lipid inclusions (Fig. 3A), while cells isolated from animals receiving PAF-like lipids isolated from oxidized LDL visibly contained more lipid bodies (Fig. 3B). Quantitation of these inclusions in animals injected with active fractions derived from oxidized LDL showed a statistically significant increase in both the number of lipid bodies per cell (Fig. 3C) and a marked change in the distribution of these among cells (Fig. 3D) when compared with the number and distribution of these structures in animals injected with equivalent fractions recovered from the chromatography of unoxidized LDL or those injected simply with vehicle.

**Oxidized phospholipids induce the expression of 5-lipoxygenase, and its products are required for the inflammatory response to oxidized LDL**

Lipid bodies serve as sites for localization of esterified arachidonate and eicosanoid-forming enzymes (25, 29), and certain leukotrienes mediate some of the effects of PAF in vivo (30–32). We asked whether the cellular response to oxidized phospholipids required these eicosanoids. We first stained cells for 5-lipoxygenase, which catalyzes the committed step in leukotriene synthesis, to find that there was only a modest expression of this enzyme in cells isolated from animals injected with the inactive HPLC fractions that surround the elution position of PAF-like lipids (Fig. 4, compare A with B). In sharp contrast, cells isolated from animals injected with either HPLC fractions containing the PAF-like activity derived from oxidized LDL (Fig. 4C) or synthetic C₄-PAF (Fig. 4D) showed a distinct increase in this rate-limiting enzyme. Interestingly, a large portion of this immunostaining was punctate, potentially indicating that this 5-lipoxygenase was localized in lipid bodies, as has been observed under other conditions (25).

We assessed whether LTB₄ was one of the lipoxygenase products of activated leukocytes in the pleural cavity because it has been implicated in PAF-induced inflammation (7, 24). We found that the levels of LTB₄ were increased in the pleural wash 6 h after introduction of the active fractions derived from oxidized LDL, but that these levels were not different from control levels when the animals received the surrounding inactive HPLC fractions (Fig. 5). We investigated the role of 5-lipoxygenase metabolites on the pleurisy induced by oxidized phospholipids or C₄-PAF by treating

**FIGURE 2.** PAF-like activity induced by oxidized LDL phospholipid. The effect of BN 52021 (20 mg/kg) was analyzed on neutrophil (A) and eosinophil (B) accumulation in the pleural cavity of mice stimulated with the bioactive HPLC fractions from oxidized LDL phospholipids. Control groups were animals injected with vehicle and HPLC fractions 6–8 from normal LDL phospholipids. C and D, The kinetics of neutrophil and eosinophil accumulation, respectively, induced by the i.t. injection of C₄-PAF (1 μg per cavity) in mice. Each column or point is the mean, and vertical lines are SEM from at least six animals. *, Statistically significant differences when compared with vehicle; +, statistically significant differences when compared with oxidized LDL phospholipids.

**FIGURE 3.** Oxidized LDL phospholipids induce lipid body formation in leukocytes. A, The effect of HPLC fractions 6–8 from normal LDL phospholipids on the number of lipid bodies in leukocytes obtained from the pleural cavity. B, The presence of increased numbers of lipid bodies in leukocytes stimulated with HPLC fractions 6–8 of oxidized LDL phospholipids. C and D, The results obtained after enumeration of lipid bodies per cell in the different experimental groups. The HPLC fractions from normal phospholipids (dotted bar), oxidized phospholipids (cross-hatched bar), or vehicle (open bar) were injected i.t., and the lipid bodies were evaluated per cell recovered from pleural cavity 6 h after stimuli, considering a total of 50 cells per animal. The bars represent the mean, and vertical lines are SEM from at least four animals (C). D, A representative experiment showing the distribution of lipid bodies in leukocytes. *, Statistically significant differences when compared with vehicle.
the mice with the 5-lipoxygenase inhibitor zileuton immediately before stimulation. Injection of zileuton by itself did not induce any change in the leukocyte count in the pleural cavity of vehicle-injected animals (data not shown). However, treatment with zileuton did significantly inhibit the accumulation of neutrophils in response to the oxidized phospholipids recovered from oxidized LDL (Fig. 5). This effect was specific for neutrophils because there was no difference in the number of recruited macrophages or eosinophils between control and zileuton-treated animals (data not shown). These studies show that a product of the 5-lipoxygenase, potentially LTB₄, is required for the leukocyte response to the newly generated polar phospholipids of oxidized LDL.

<table>
<thead>
<tr>
<th>Stimuli</th>
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<tr>
<td>Vehicle</td>
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</tr>
<tr>
<td>Inactive oxidized phospholipids</td>
<td>36.5 ± 4.9</td>
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<tr>
<td>Active oxidized phospholipids</td>
<td>63.8 ± 9.6 *</td>
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**FIGURE 4.** Oxidized phospholipids induce 5-lipoxygenase expression in leukocytes. Immunohistochemistry of 5-lipoxygenase (arrows) is shown in leukocytes recovered from the pleural cavity of mice 6 h after the i.t. injection of inactive HPLC fractions (fractions 3–5) (B), bioactive HPLC fractions (fractions 6–8) (C) from oxidized LDL phospholipids, or C₄-PAF (1 μg per cavity) (D). Negative control with nonimmune serum is depicted in A.

**FIGURE 5.** 5-Lipoxygenase products are required for the inflammatory response of oxidized phospholipids. Upper panel, LTB₄ concentrations in the supernatant of the 6-h pleural wash of mice injected with bioactive HPLC fractions from oxidized LDL phospholipids (phospholipids 6–8), HPLC fractions 6–8 from unoxidized LDL phospholipids (phospholipids 6–8), or vehicle. The amount of LTB₄ was determined by ELISA in duplicate wells from at least four animals. Lower panel, The effect of the pretreatment with zileuton (i.t. at 50 μg per cavity) on the accumulation of neutrophils in the pleural cavity at the 6-h time point. The animals received i.t. injection of vehicle, inactive HPLC fractions (fractions 3–5) from oxidized LDL phospholipids, bioactive HPLC fractions (fractions 6–8), or C₄-PAF (1 μg per cavity). Each bar is the mean from at least six animals, and vertical lines are the SEM. *, +, and #, Statistically significant differences when compared with vehicle, oxidized LDL phospholipids (phospholipids 6–8), or C₄-PAF, respectively.

**Chemokine production and mRNA expression in pleural leukocytes obtained from mice exposed to oxidized phospholipids**

Our finding that leukotriene synthesis was stimulated by oxidized phospholipids suggested that the production and secretion of other inflammatory mediators might also be increased, and so we assessed the level of expression of mRNAs for chemokines by RNase protection. We analyzed mRNA from the total leukocyte population present in the pleural cavity 6 h after injection of the PAF-like lipids purified from oxidized LDL and observed that there was increased expression of mRNA for RANTES, macrophage-inflammatory protein (MIP)-1β, MIP-1α, MIP-2, IFN-γ-inducible protein-10, and MCP-1 when compared with the levels of expression of these cytokines by cells isolated from animal receiving adjacent inactive HPLC fractions (Fig. 6). This pattern of enhanced cytokine message expression was recapitulated by cells extracted from animals receiving C₄-PAF (Fig. 6).

One of the cytokines with enhanced message expression following injection of either oxidized LDL PAF-like lipids or C₄-PAF was MCP-1. We determined whether expression of MCP-1 protein also was increased in the exudate in response to the biologically active phospholipids found in oxidized LDL by quantifying this cytokine in the pleural cavity 6 h after introduction of the various phospholipid fractions. MCP-1 was secreted in vivo in response to the PAF-like lipids, as we detected an increased amount of it in the pleural wash of animals injected with active oxidized phospholipids or C₄-PAF, but not with control phospholipids (Fig. 6).

**Oxidized phospholipids induced inflammatory response is reduced in MCP-1-deficient mice**

MCP-1 is implicated in inflammatory diseases (9, 16), and, as described above, MCP-1 mRNA expression and protein production...
were increased in mice injected with the inflammatory phospholipids isolated from oxidized LDL. We investigated the involvement of MCP-1 in the inflammation triggered by oxidized phospholipids using mice that had been genetically engineered to be deficient in MCP-1 (MCP-1\(^{-/-}\)). PAF-like lipids purified from oxidized LDL, but not the surrounding inactive fractions (data not shown), induced mononuclear cell (data not shown), neutrophil, and eosinophil accumulation in thoracic cavity 6 h after stimulation of wild-type animals (Fig. 7). In contrast, in strain-matched MCP-1\(^{-/-}\) mice there was a marked deficit in the number of neutrophils (Fig. 7A) and eosinophils (Fig. 7B) recruited into the pleural cavity in these mice in response to the inflammatory phospholipids of oxidized LDL.

Ablation of MCP-1 synthesis also abolished the inflammatory environment in the pleural cavity as detected by in vivo lipid body accumulation. There was an increase in lipid bodies in leukocytes recovered from the pleural cavities of wild-type mice, but this increase was not observed in pleural leukocytes derived from MCP-1\(^{-/-}\) mice exposed to the same stimulus (Fig. 8A). MCP-1 acted on the exudate cells to promote lipid body accumulation as shown in experiments with cultured peritoneal macrophages from wild-type and MCP-1\(^{-/-}\) animals. In vitro exposure to active oxidized phospholipids failed to induce lipid body formation in cells from MCP-1\(^{-/-}\) mice under conditions in which significant stimulation was observed in cells from wild-type mice (Fig. 8B).

Thus, MCP-1 is required for a complete inflammatory response to oxidized phospholipids.

We questioned whether the requirement for MCP-1 to mount an inflammatory response to oxidized LDL lipids was upstream or downstream of the synthesis of LT\(_B_4\). To order these events, we exposed wild-type or MCP-1\(^{-/-}\) animals to oxidized phospholipids and measured the accumulation of LT\(_B_4\) in the pleural fluid. We found that the MCP-1\(^{-/-}\) mice expressed significantly less LT\(_B_4\) than the wild-type animals (Fig. 7C). The lack of cellular response to oxidized phospholipids in MCP-1\(^{-/-}\) mice was also reflected in the profile of mRNA expression. Although cells from wild-type mice had similar profiles of mRNA expression compared with Swiss mice leukocytes, cytokine mRNA expression in leukocytes from MCP-1\(^{-/-}\) mice differed dramatically. As shown in Fig. 9, mRNA expression was significantly decreased for all the chemokines probed, especially RANTES, in which the signal practically disappeared in MCP-1\(^{-/-}\) mice. Taken together, these data demonstrate that MCP-1 has a central, early role in the pleural inflammation induced by oxidized, PAF-like phospholipids.

**Discussion**

Oxidatively modified LDL has a well-recognized role in inflammation through the generation of several classes of biologically active mediators. In this study, we characterized the inflammatory activity of phospholipids extracted from oxidized LDL using a model of murine pleurisy. Our analysis of leukocyte accumulation, leukocyte lipid body formation, and the production of inflammatory mediators shows that the PAF-like lipid fraction alone induces a rapid and mixed cellular infiltrate. These same responses were induced by synthetic C\(_4\)-PAF, a prominent component of this mixture of bioactive phospholipid oxidation products of LDL. We find that PAF-like lipids in oxidized LDL act through MCP-1, and an...
essential subsequent step for neutrophil recruitment was the induction of 5-lipoxygenase and its products. Thus, even though white blood cells may have PAF receptors and respond to PAF and its mimetics, the inflammatory reaction in vivo is extended through the PAF receptor.

Oxidation of LDL particles generates phospholipids with PAF-like biologic activity (1, 2) that can induce MCP-1 production. Inflammatory reaction in vivo is extended through the PAF receptor.

The oxidation of LDL generates a number of phospholipid oxidation products that are still phospholipids. These include the PAF-like lipid oxidation products in which the minor class of alkyl phosphatidylcholines is fragmented to a point in which the highly selective PAF receptor recognizes and responds to these products of a radical chemical reaction. Oxidation of this class of phospholipids also generates products with slightly longer residues with an α-carboxy function that activates gene transcription through PPARγ (4). Oxidatively fragmented phospholipids themselves are precursors for the vasoactive lipid lysophosphatidylcholine because they are substrates for the LDL-associated enzyme PAF acetylhydrolase (7). Other biologically active phospholipids generated during the oxidation of LDL include lysophosphatidic acid (3), isoprostane-containing phosphatidylcholine (6), and fragmented diacyl phosphatidylcholines (5). In this study, we find that a synthetic PAF-like lipid induced all of the in vivo effects of the inflammatory phospholipids isolated from LDL, leading us to conclude that these PAF-like lipids underlie much of the inflammatory activity of oxidatively modified LDL particles.

We analyzed the pattern of chemokine mRNA expression in the pleurisy triggered by oxidized phospholipids and C4-PAF to better understand the mechanisms implicated in the inflammatory reaction to oxidized phospholipids. Both stimuli induced an increase in mRNA for RANTES, MIP-1β, MIP-1α, MIP-2, IFN-γ-inducible protein-10, and MCP-1 in cells obtained from pleural cavity 6 h poststimulation. Among these chemokines, RANTES, MIP-1α, and MIP-1β have been implicated in the formation of atherosclerotic lesions (36). The role of the C-C chemokine MCP-1 in the pathophysiology of atherosclerosis, now recognized as a chronic inflammatory condition, has been demonstrated in humans (16) and animal models of this disease (18, 37–40). MCP-1 participates in the development of allergic inflammation (12), and is positioned for a similar role in pleurisy.

In the latter model, the anti-MCP-1 Ab reduced peritoneal LTβ4 levels and an LTβ4 antagonist reduced both monocyte and neutrophil influx.

**FIGURE 8.** Oxidized LDL phospholipid-induced lipid body formation in leukocytes is abolished in MCP-1-deficient mice. A, In vivo results. Wild-type or MCP-1-deficient mice received i.t. injection of inactive HPLC fractions (fractions 2, 10, and 11) from oxidized LDL phospholipids or bioactive HPLC fractions (fractions 6–8). Pleural washes were recovered 6 h after stimulation. B, In vitro results. Peritoneal macrophages from wild-type or MCP-1-deficient mice were exposed to oxidized phospholipid fractions for 1 h before the number of cellular lipid bodies were evaluated. The bars are the mean of lipid body counts (using a total of 50 cells per condition), and vertical lines are SEM from at least four animals. *p < 0.5 statistically significant differences when compared with oxidized LDL phospholipids 2, 10, and 11.

**FIGURE 9.** MCP-1 deficiency reduces oxidized LDL phospholipid-induced C-C chemokine transcription. RNase protection assays were performed with RNA extracted from leukocytes recovered from the pleural cavity of wild-type or deficient mice 6 h after stimulation with bioactive HPLC fractions (fractions 6–8) from oxidized LDL phospholipids.
We find a marked dependence of monocytes, eosinophils, and neutrophils trafficking on the ability to increase MCP-1 levels in a closed compartment in response to a relevant inflammatory insult. The CCR2 receptor for MCP-1 normally is not expressed by neutrophils (41), and disruption of its gene does not block neutrophil influx into the peritoneum (42), so it is not readily apparent that loss of MCP-1 would impact neutrophil migration into the pleural cavity. However, anti-MCP-1 Abs reduced the neutrophilic influx into the lungs of mice infected with Cryptococcus neoformans (43) and briefly attenuated neutrophil influx in the more complex model of cecal ligation and puncture (44). We do not believe that this is a primary reaction to the disruption of the MCP-1 gene, but rather that the neutrophil accumulation induced by inflammatory oxidized phospholipids is secondary to the generation of 5-lipoxygenase products, most likely LTB4. We found enhanced levels of LTB4 in the pleural exudate, similar to the increased levels of LTB4 in a model of peritonitis in which MCP-1 induction of this mediator has a major role in leukocyte recruitment (44). LTB4 is a potent chemoattractant for neutrophils (45), and disruption of the gene for 5-lipoxygenase interferes with PAF-induced neutrophil recruitment (46). Inhibition of 5-lipoxygenase activity with zileuton shows that its products are essential for the recruitment of neutrophils, and not eosinophils or monocytes (data not shown), into the pleural cavity after introduction of PAF-like oxidized phospholipids.

We detected increased levels of LTB4 in the pleural fluid of animals injected with the active fractions isolated from oxidized LDL, and increased 5-lipoxygenase expression in the accompanying cells. MCP-1 stimulates monocyte LTB4 synthesis (44), suggesting that these cells were the source of this agonist. However, the influx of neutrophils markedly preceded the increase in infiltrating monocytes, a result that indicates that the resident macrophage population, which does not require MCP-1 for its establishment (13), is a more probable source of this eicosanoid.

PAF-like lipids isolated from oxidized LDL or pure C4-PAF induced lipid body accumulation in leukocytes recovered from the pleural cavity. Lipid body formation in response to PAF activation of the PAF receptor and a downstream G protein has been previously established (25). Lipid bodies are membraneless cytoplasmic inclusions whose numbers are increased in endothelial cells, eosinophils, neutrophils, and macrophages upon appropriate activation. In this study, we use this property to mark the development of an inflammatory milieu.

Lipid bodies may have a functional role in this model, as they localize esterified arachidonate and eicosanoid-forming enzymes to facilitate the production of eicosanoids by activated cells (24). The presence of increased numbers of lipid bodies in leukocytes stimulated by the oxidized phospholipids correlated with the increase in LTB4 levels found in the pleural exudate of stimulated animals, and we find that incubation of pleural leukocytes with oxidized phospholipids in vitro primes those cells for enhanced calcium ionophore-induced LTB4 production (E. F. de Assis et al., in preparation). We note that most of the 5-lipoxygenase detected by immunohistochemistry displays a granular cytoplasmic localization in cells isolated from animals injected with phospholipids isolated from oxidized LDL, as previously detected by Bozza et al. (24), in response to PAF, and so may be positioned to act in this way. Recently, a direct proof of the involvement of lipid bodies as sites of leukotriene production was provided by the demonstration of intracellular immunofluorescent localization of newly formed LTC4 within lipid bodies in chemokine-stimulated human eosinophils (47).

Pleurisy offers a quantitative way to examine the early events of acute inflammation. This model provides an easy way to detect cell influx and the release of chemical mediators in the inflammatory milieu. In this study, we demonstrated that PAF-like lipid oxidation products induce a rapid influx of neutrophils that progresses to a late phase marked by monocyte and eosinophil influx. Accumulation of each of these cells is characteristic of pleurisy evoked by distinct types of insults (48). Carrageenan-induced pleurisy, for example, creates an oxidative stress in which the neutrophilic infiltration and damage are attenuated by antioxidant treatment (49). PAF biologic activity is recoverable from pleural washes from patients with select etiologies that correlate with neutrophil and eosinophil numbers (50). There may be an oxidative component to pleurisy in which the events we define in this work come into play, but it is the quantitative aspects of the model that are particularly relevant. Oxidized LDL (51), oxidatively fragmented phospholipids (5), MCP-1 (16), and monocytes with MCP-1 receptors (17) are all found in atherosclerotic lesions, but individually assessing the role of each early in this prolonged inflammatory event is difficult. A quantifiable model of acute inflammation has connected oxidatively modified phospholipids with PAF-like activity derived from oxidized LDL to the accumulation of several classes of white blood cells through enhanced synthesis of MCP-1. The sequence of events following the stimulus by oxidized phospholipids and the generation of MCP-1 and other chemokines may be of fundamental importance in the early stages of atherosclerosis and other inflammatory disorders.

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References


