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

This information is current as of April 18, 2020.

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

---

References   This article **cites 51 articles**, 23 of which you can access for free at:
http://www.jimmunol.org/content/168/8/4112.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision

- **No Triage!** Every submission reviewed by practicing scientists

- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription   Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions   Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts   Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

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

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[2]*

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 C_4-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 B_4. 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 B_4 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.

Oxidative attack on low-density lipoprotein (LDL)[3] particles generates a number of biologically active lipids that result from the oxidation and fragmentation of the phospholipid shell that surrounds the hydrophobic lipid core. These newly formed agents include fragmented phospholipids that activate the platelet-activating factor (PAF) receptor expressed on inflammatory cells (1, 2), the edg receptor agonist lysophosphatidic acid (3), and those that bind and activate the nuclear hormone receptor peroxisome proliferator-activated receptor-γ (PPARγ) (4). Other oxidized phospholipids are generated by the oxidative attack on LDL with less defined mechanisms of action (5, 6). Formation of all of these types of biologically active phospholipid oxidation products results from a series of radical catalyzed chemical reactions, and so is unregulated.

Oxidized LDL and phospholipids isolated from oxidized LDL particle induce neutrophil adhesion and smooth muscle cell proliferation in vitro through activation of the PAF receptor (1). These also stimulate the PAF receptor in vivo, and much of this effect results from C_4 analogs of PAF derived from the oxidation of arachidonoyl-containing phosphatidylcholines (2). PAF is an inflammatory phospholipid in which the *sn*-1 ether bond, the short *sn*-2 acetyl residue, and the phosphocholine head group are recognized by a single G protein-coupled PAF receptor at picomolar levels (7). Oxidatively generated PAF analogs differ from PAF by the presence of longer and sometimes derivatized residues at the *sn*-2 position of the glycerol backbone and, although less potent than PAF, are considerably more abundant than PAF after oxidation of polyunsaturated phosphatidylcholines.

Monocyte chemoattractant protein-1 (MCP-1) is a CC chemokine whose role in the pathogenesis of inflammatory diseases is extensive (8). By example, MCP-1 has a key role in conditions ranging from acute respiratory distress syndrome to rheumatoid arthritis (9). MCP-1 is detected in the bronchoalveolar lavage fluid of allergic asthmatic patients (10) and in bronchoalveolar lavage fluid and plasma of patients with interstitial lung diseases (11), suggesting that it may also play a fundamental role in development of allergic responses as well (12). MCP-1 is essential for monocyte recruitment in in vivo models of inflammation, and impaired monocyte accumulation has been noted in MCP-1-deficient mice stimulated by thioglycolate (13).

Oxidized LDL particles stimulate MCP-1 synthesis by vascular cells (14, 15), and MCP-1 is expressed in inflamed macrophage-rich areas of atherosclerotic lesions (16). Extravasated macrophages in these lesions express MCP-1 receptors (17), and its overexpression

*Laboratório de Imunofarmacologia, Departamento de Fisiologia e Farmacodinâmica, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, and [†]Laboratório Inflamação e Imunidade, Departamento de Imunologia, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; and Departments of [‡]Internal Medicine and [§]Pathology, and [¶]Huntsman Cancer Institute, University of Utah, Salt Lake City, UT 84112

[2] Address correspondence and reprint requests to Dr. Hugo C. Castro-Faria-Neto, Laboratório de Imunofarmacologia, Departamento de Fisiologia e Farmacodinâmica, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Av. Brasil 4365, Manguinhos, Rio de Janeiro, RJ, CEP 21045-900, Brazil. E-mail address: hcastro@gene.dbbm.fiocruz.br

[3] Abbreviations used in this paper: LDL, low-density lipoprotein; i.t., intrathoracic; LTB$_4$, leukotriene B$_4$; MCP-1, monocyte chemoattractant protein 1; MIP, macrophage-inflammatory protein; PAF, platelet-activating factor; PPARγ, peroxisome proliferator-activated receptor-γ.

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 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 B_4 (LTB_4). 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-lipoxygenases and LTB_4 by MCP-1. In vivo, MCP-1 is a pleiotrophic 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-^{32}P]UTP was purchased from NEN Life Sciences (Boston, MA); *mck*-5 in vitro hybridization kit and RNase protection kit were from BD Phar-Mingen (San Diego, CA); anti-5-lipoxygenase 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 52021 was from Biomol (Plymouth Meeting, PA); C_4-PAF, 1-*O*-hexadecyl-2-butanoyl-*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-acetyl-*sn*-glycero-3-phosphocholine was from Sigma-Aldrich (St. Louis, MO); Pefabloc was from Pentapharm Laboratories (Basel, Switzerland); LTB_4 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$^{-/-}$ mice and their backcrossed have been previously described (13) and were kindly provided by C. Gerard (Pelmutter Laboratory, Children's Hospital, Harvard Medical School, Boston, MA).

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 with potassium bromide. A gradient was formed from this plasma and saline overlaying it by centrifugation for 200 min at $150,000 \times g$. 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 $CuSO_4$ (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 aminopropyl 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 N_2, 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.

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 $500 \times g$) as in the neutrophil purification method described by Zimmerman 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 $400 \times g$). The neutrophils were washed with HBSS (calcium and magnesium free) and counted, and their number was adjusted to 5.5×10^6/ml before incubation with fura 2 (2 μM) for 45 min at 37°C in the dark. Ca^{2+} transients were determined in the fura 2-loaded neutrophils, as before (2).

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 received an intrathoracic (i.t.) injection of pooled HPLC fractions 6–8 containing material derived from oxidized LDL, which activated a Ca^{2+} flux in polymorphonuclear cells, or with the surrounding inactive fractions (fractions 2–4, 10, and 11) from the same chromatographic separation of oxidized LDL. We also used the corresponding fractions 6–8 from the chromatographic separation of polar lipids extracted from unoxidized LDL as a control. The stored HPLC fractions were dried under nitrogen and resuspended in sterile HBSS containing 0.01% BSA just before use. The HBSS/BSA solution alone was administered i.t. into naive animals as a further control. Synthetic C_4-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 CO_2 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 and their backcrossed controls (13) were used, this analysis was performed at the fixed time of 6 h after stimulation.

Pleural wash samples were diluted in Turk fluid (2% acetic acid) for total leukocyte counts using Neubauer chambers. Differential analysis was performed on cytosmears treated with May Grunwald-Giemsa stain. Lipid body formation in leukocytes was evaluated on cytosmears stained with osmium (24). Briefly, cells on cytosmears were fixed with 3% formalin and stained with cacodilic acid and 1.5% osmium. After 30 min, the cytosmears were washed with water and incubated with 1% thiocarbohydrazide for 3 min, and then with cacodilic acid and osmium for 5 min. Intracellular lipid body content in these cells was then evaluated by counting them in 50 cells magnified under an optical microscope. Pleural wash fluid samples were centrifuged to pellet the cells, and the supernatant from this procedure was stored at −70°C for ELISA determinations. The pelleted cells were resuspended in Ultraspec RNA isolation reagent (Biotecx Laboratories).

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 anti-mouse 5-lipoxygenase Ab (diluted 1/150 in 0.05% saponin/HBSS). Preimmune 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 i.p. dose (20 mg/kg) of the PAF antagonist BN 52021 30 min in advance of the i.t. injection of oxidized phospholipids. 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 C_4-PAF. BN 52021 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 stimulated 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 (10^5 per slide) and stained with osmium, and the number of lipid bodies in 50 consecutively scanned macrophages was determined as above.

RNase protection assay

RNA was purified according to the Ultraspec RNA protocol (Biotecx 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 adsorbed 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 time relationship of inflammation showed the influx of neutrophils was rapid, with a significant increase by 3 h postinjection that remained elevated for another 9 h before this accumulation resolved (Fig. 1D). This influx was accompanied by mononuclear cell infiltration 6 h after injection ($0.83 \pm 0.21 \times 10^6$ to $4.1 \pm 1.1 \times 10^6$) and by eosinophil accumulation from 6 to 24 h postinjection (Fig. 1E). Vehicle-injected animals did not show a significant influx of either of these types of leukocytes, nor was there an inflammatory response when the animals were injected with inactive fractions surrounding those that contained this PAF-like ac-
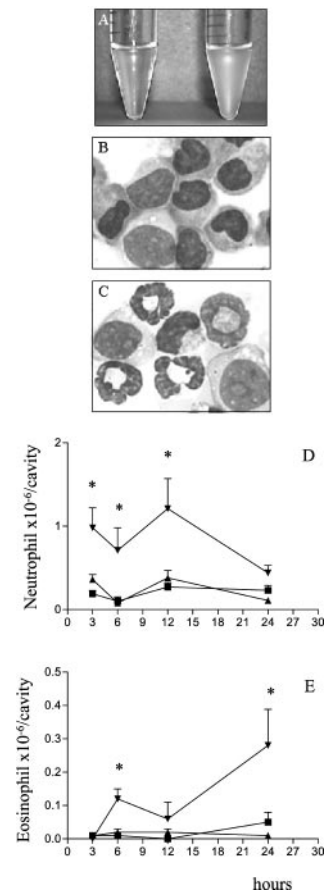

FIGURE 1. Phospholipids from oxidized LDL stimulate neutrophil and eosinophil accumulation into mice pleural cavity. Animals received i.t. injection of vehicle (■), bioactive (fractions 6–8) (▼), or inactive (fractions 2, 3, and 10) (▲) HPLC fractions of oxidized phospholipids. The cell analysis of the exudate revealed a significant cell accumulation (*A*) with enrichment in the neutrophil and eosinophil populations, as illustrated in *C*, whereas *B* shows only mononuclear cells obtained from inactive oxidized phospholipids-injected group. Neutrophil (*D*) and eosinophil (*E*) counts were performed in samples obtained from a cytospin of the pleural wash. Each point is the mean, with SEM represented by vertical lines, from at least eight animals. ∗, Statistically significant differences as compared with animals injected with vehicle.

tivity. We analyzed the effect of the bioactive fractions on the accumulation of T cell subtypes by flow cytometry to find that the bioactive fractions did not increase the number of $CD3^+$, $CD4^+$, $CD8^+$, or $\gamma\delta^+$ T lymphocytes in pleural cavity of mice at 24 h poststimulus (data not shown).

PAF antagonists block the effects of oxidized phospholipids on leukocytes: potential involvement of C_4-PAF

The polar phospholipids generated by LDL oxidation include PAF-like lipids (1), PPARγ agonists (4), lysophosphatidic acid (27), as well as other products (5, 6). We tested whether the inflammatory actions that we had observed arose from oxidized phospholipids acting on the PAF receptor on target leukocytes. Fig. 2 shows that pretreatment of the animals with the PAF receptor antagonist BN 52021 inhibited the influx of both neutrophils (Fig. 2A) and eosinophils (Fig. 2B) into the thoracic cavity at 6 h after injection of the biologically active fractions derived from oxidized LDL. Previous studies using mass spectrometry identified C_4-PAF as a major component of the bioactive fractions responsible for the PAF-like activity of oxidized LDL (2). We found that
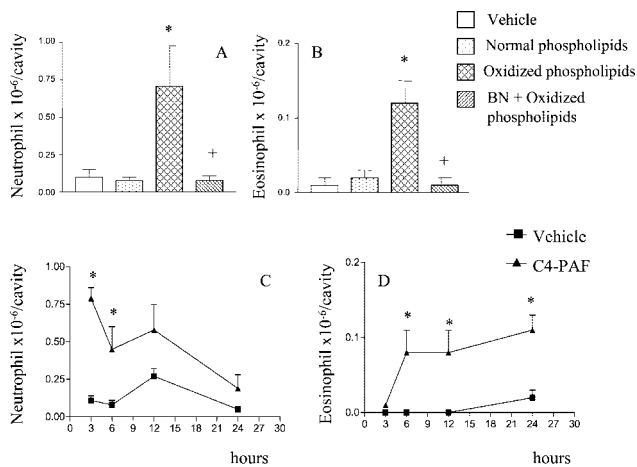
FIGURE 2. PAF-like activity induced by oxidized LDL phospholipid. The effect of BN 52021 (20 mg/kg) was analyzed on neutrophil (*A*) and eosinophil (*B*) accumulation in the pleural cavity of mice stimulated with the bioactive HPLC fractions from oxidized LDL phospholipids. Control groups were animals injected with vehicle and HLPC 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 C_4-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.

synthetic C_4-PAF induced an increase in the number of leukocytes, both neutrophils (Fig. 2*C*) and eosinophils (Fig. 2*D*), 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. 3*A*), while cells isolated from animals receiving PAF-like lipids isolated from oxidized LDL visibly contained more lipid bodies (Fig. 3*B*). 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. 3*C*) and a marked change in the distribution of these among cells (Fig. 3*D*) 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
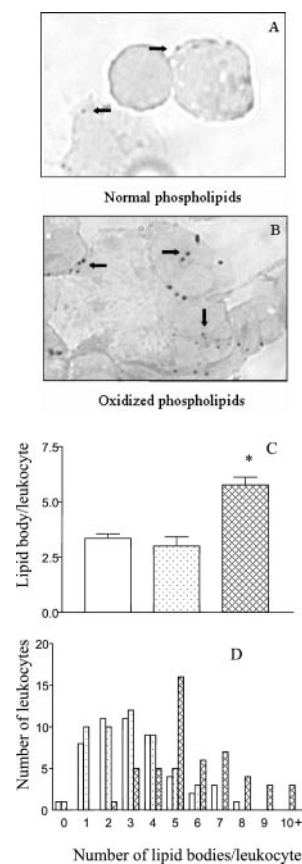

FIGURE 3. Oxidized LDL phospholipids induce lipid body formation in leukocytes. *A*, The effect of HPLC fractions 6–8 from normal LDL phospholipids on the number of lipid bodies in leukocytes obtained from the pleural cavity. *B*, The presence of increased numbers of lipid bodies in leukocytes stimulated 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 leukokocytes. ∗, Statistically significant differences when compared with vehicle.

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. 4*C*) or synthetic C_4-PAF (Fig. 4*D*) 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 LTB_4 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 LTB_4 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 C_4-PAF by treating
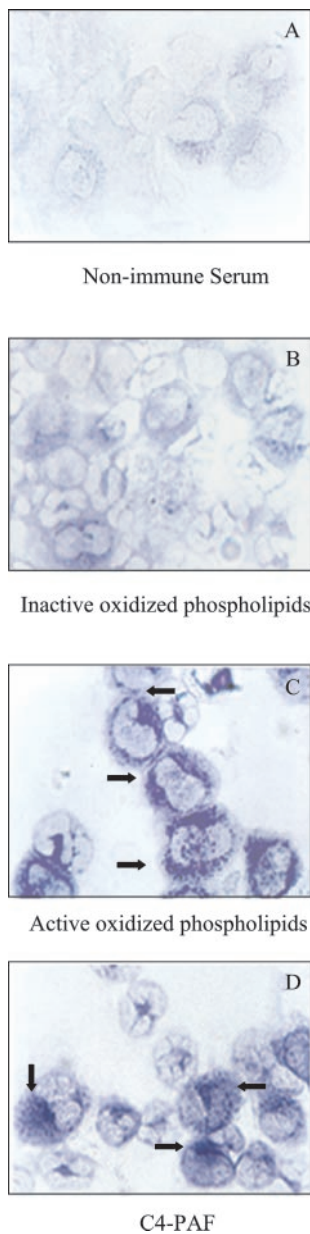
FIGURE 4. Oxidized phospholipids induce 5-lipoxygenase expression in leukocytes. Immunohistochemistry of 5-lipoxygenase (arrows) is showed in leukocytes recovered from the pleural cavity of mice 6 h after the i.t. injection of inactive HPLC fractions (fractions 3–5) (*B*), bioactive HPLC fractions (fractions 6–8) (*C*) from oxidized LDL phospholipids, or C$_4$-PAF (1 μg per cavity) (*D*). Negative control with nonimmune serum is depicted in *A*.

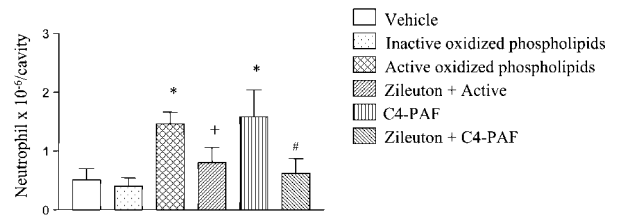| Stimuli	Leukotriene B$_4$ (pg/cavity)
Vehicle	42.0 ± 4.0
Inactive oxidized phospholipids	36.5 ± 4.9
Active oxidized phospholipids	63.8 ± 9.6 *

FIGURE 5. 5-Lipoxygenase products are required for the inflammatory response of oxidized phospholipids. *Upper panel*, LTB$_4$ concentrations in the supernatant of the 6-h pleural wash of mice injected with bioactive HPLC fractions from oxidized LDL phospholipids (phospholipids 6–8), HLPC fractions 6–8 from unoxidized LDL phospholipids (phospholipids 6–8), or vehicle. The amount of LTB$_4$ 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 mice 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 C$_4$-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 C$_4$-PAF, respectively.

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 LTB$_4$, is required for the leukocyte response to the newly generated polar phospholipids of oxidized LDL.

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 C$_4$-PAF (Fig. 6).

One of the cytokines with enhanced message expression following injection of either oxidized LDL PAF-like lipids or C$_4$-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 C$_4$-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
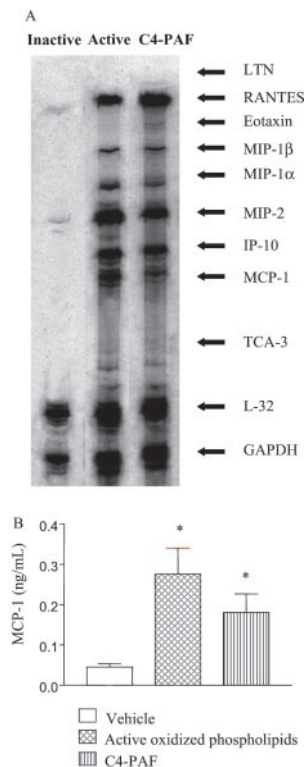
FIGURE 6. Oxidized phospholipids stimulate C-C chemokine synthesis. *A*, RNase protection assays were performed with the RNA extracted from leukocytes recovered at 6 h from the pleural cavity of mice stimulated with inactive HPLC fractions (fractions 3–5) from oxidized LDL phospholipids, bioactive HPLC fractions (fractions 6–8), or C_4-PAF (1 μg per cavity). IP-10, IFN-γ-inducible protein-10. L32 and GAPDH are loading controls. *B*, Detection of MCP-1 by ELISA in the supernatant of the 6-h pleural wash of mice stimulated with bioactive HPLC fractions (fractions 6–8) from oxidized LDL phospholipids, C_4-PAF (1 μg per cavity), or vehicle. Each column is the mean from at least six animals, and vertical lines are the SEM. *, Statistically significant differences when compared with vehicle.

FIGURE 7. Leukocyte infiltration and LTB_4 production stimulated by oxidized LDL phospholipids are abolished in MCP-1-deficient mice. Neutrophil (*A*) and eosinophil (*B*) accumulation and LTB_4 production (*C*) in the pleural cavity of wild-type and MCP-1-deficient mice 6 h after i.t. injection of inactive HPLC fractions (fractions 2, 10, and 11) from oxidized LDL phospholipids or bioactive HPLC fractions (fractions 6–8). Each bar is the mean from six animals, and vertical lines are the SEM. * and +, Statistically significant differences when compared with wild-type or MCP-1-deficient mice injected with oxidized LDL phospholipids 2, 10, and 11, respectively; #, statistically significant differences when compared with wild-type animals stimulated with oxidized LDL phospholipids 6–8.

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. 7*A*) and eosinophils (Fig. 7*B*) 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. 8*A*). 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. 8*B*). Thus,
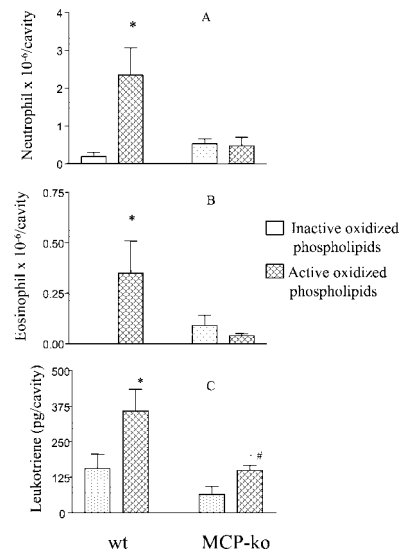
MCP-1 is required for a complete inflammatory response to oxidized phospholipids.

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 LTB_4. To order these events, we exposed wild-type or MCP-$1^{-/-}$ animals to oxidized phospholipids and measured the accumulation of LTB_4 in the pleural fluid. We found that the MCP-$1^{-/-}$ mice expressed significantly less LTB_4 than the wild-type animals (Fig. 7*C*). The lack of cellular response to oxidized phospholipids in MCP-$1^{-/-}$ mice also was 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
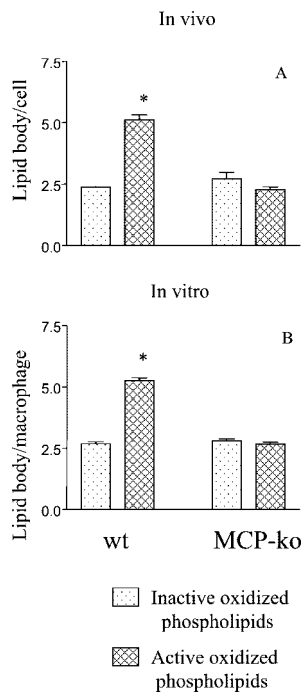
FIGURE 8. Oxidized LDL phospholipid-induced lipid body formation in leukocytes is abolished in MCP-1-deficient mice. *A*, In vivo results. Wild-type or MCP-1-deficient mice received i.t. injection of inactive HPLC fractions (fractions 2, 10, and 11) from oxidized LDL phospholipids or bioactive HPLC fractions (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. ∗, Statistically significant differences when compared with oxidized LDL phospholipids 2, 10, and 11.
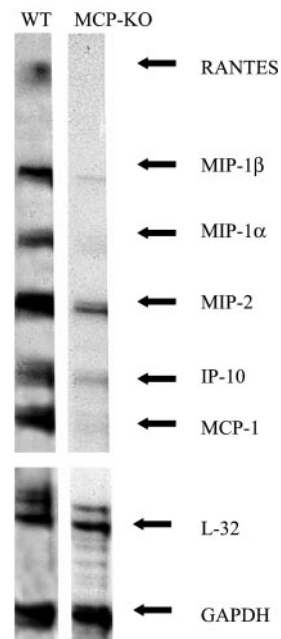

FIGURE 9. MCP-1 deficiency reduces oxidized LDL phospholipid-induced C-C chemokine transcription. RNase protection assays were performed with RNA extracted from leukocytes recovered from the pleural cavity of wild-type or deficient mice 6 h after stimulation with bioactive HPLC fractions (fractions 6–8) from oxidized LDL phospholipids.

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 MCP-1 production.

Oxidation of LDL particles generates phospholipids with PAF-like biologic activity (1, 2) that can induce inflammation in a rat model of pleurisy (2). We sought to define the molecular mechanisms responsible for this response and so established a similar model in mice. The features of the murine model were similar to that of rats, in which there was a rapid influx of neutrophils that was maximal by 3 h and had resolved by 24 h. The influx of eosinophils was slower, but then this increased cellularity remained elevated for up to 24 h after exposure to the inciting oxidized phospholipids. This response was expected because PAF, as a biosynthetic product of activated inflammatory cells, is an important component of the inflammatory response in mice (33) that elicits an inflammatory exudate and edema (7).

In addition to neutrophil accumulation, we find that the PAF-like lipids from oxidized LDL as well as C$_4$-PAF induced monocyte and eosinophil accumulation in the thoracic cavity of mice. PAF is a potent and direct chemoattractant for eosinophils in vitro (34), and PAF induces eosinophil accumulation in the pleural cavity of rats, an effect secondary to the generation of a protein with specific eosinophil chemotactic activity (35). These observations support the conclusion that bioactive fractions of oxidized LDL phospholipids, C$_4$-PAF, and PAF all trigger inflammatory response 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 *sn*-2 residue of 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 ω-carboxy function that actives 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 C$_4$-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 those 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 LTB$_4$ levels and an LTB$_4$ antagonist reduced both monocyte and neutrophil influx.

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 LTB_4. We found enhanced levels of LTB_4 in the pleural exudate, similar to the increased levels of LTB_4 in a model of peritonitis in which MCP-1 induction of this mediator has a major role in leukocyte recruitment (44). LTB_4 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 LTB_4 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 LTB_4 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 C_4-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 LTB_4 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 LTB_4 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 LTC_4 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 monocytic cells 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.

Acknowledgments

References

1. Heery, J. M., M. Kozak, D. M. Stafforini, D. A. Jones, G. A. Zimmerman, T. M. McIntyre, and S. M. Prescott. 1995. Oxidatively modified LDL contains phospholipids with PAF-like activity and stimulates the growth of smooth muscle cells. *J. Clin. Invest. 96:2322.*

2. Marathe, G. K., S. S. Davies, K. A. Harrison, A. R. Silva, R. C. Murphy, H. C. Castro-Faria Neto, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1999. Inflammatory PAF-like lipids in oxidized low density lipoproteins are fragmented alkyl phosphatidylcholines. *J. Biol. Chem. 274:28395.*

3. Siess, W., K. J. Zangl, M. Essler, M. Bauer, R. Brandl, C. Corrinth, R. Bittman, G. Tigyi, and M. Aepfelbacher. 1999. Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA 96:6931.*

4. Davies, S. S., A. V. Pontsler, G. K. Marathe, K. A. Harrison, R. C. Murphy, J. C. Hinshaw, G. D. Prestwich, A. St. Hilaire, S. M. Prescott, G. A. Zimmerman, et al. 2001. Oxidized phospholipids are specific, high affinity PPARγ ligands and agonists. *J. Biol. Chem. 276:16015.*

5. Watson, A. D., N. Leitinger, M. Navab, K. F. Faull, S. Horkko, J. L. Witztum, W. Palinski, D. Schwenke, R. G. Salomon, W. Sha, et al. 1997. Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. *J. Biol. Chem. 272:13597.*

6. Watson, A. D., G. Subbanagounder, D. S. Welsbie, K. F. Faull, M. Navab, M. E. Jung, A. M. Fogelman, and J. A. Berliner. 1999. Structural identification of a novel pro-inflammatory epoxyisoprostane phospholipid in mildly oxidized low density lipoprotein. *J. Biol. Chem. 274:24787.*

7. Prescott, S. M., G. A. Zimmerman, D. M. Stafforini, and T. M. McIntyre. 2000. Platelet-activating factor and related lipid mediators. *Annu. Rev. Biochem. 69:419.*

8. Gu, L., S. C. Tseng, and B. J. Rollins. 1999. Monocyte chemoattractant protein-1. *Chem. Immunol. 72:7.*

9. Luster, A. D. 1998. Chemokines: chemotactic cytokines that mediate inflammation. *N. Engl. J. Med. 338:436.*

10. Alam, R., J. York, M. Boyars, S. Stafford, J. A. Grant, J. Lee, P. Forsythe, T. Sim, and N. Ida. 1996. Increased MCP-1, RANTES, and MIP-1α in bronchoalveolar lavage fluid of allergic asthmatic patients. *Am. J. Respir. Crit. Care Med. 153: 1398.*

11. Suga, M., K. Iyonaga, H. Ichiyasu, N. Saita, H. Yamasaki, and M. Ando. 1999. Clinical significance of MCP-1 levels in BALF and serum in patients with interstitial lung diseases. *Eur. Respir. J. 14:376.*

12. Luster, A. D., and M. E. Rothenberg. 1997. Role of the monocyte chemoattractant protein and eotaxin subfamily of chemokines in allergic inflammation. *J. Leukocyte Biol. 62:620.*

13. Lu, B., B. J. Rutledge, L. Gu, J. Fiorillo, N. W. Lukacs, S. L. Kunkel, R. North, C. Gerard, and B. J. Rollins. 1998. Abnormalities in monocyte recruitment and

cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J. Exp. Med.* 187:601.

14. Cushing, S. D., J. A. Berliner, A. J. Valente, M. C. Territo, M. Navab, F. Parhami, R. Gerrity, C. J. Schwartz, and A. M. Fogelman. 1990. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc. Natl. Acad. Sci. USA* 87:5134.

15. Navab, M., S. S. Imes, S. Y. Hama, G. P. Hough, L. A. Ross, R. W. Bork, A. J. Valente, J. A. Berliner, D. C. Drinkwater, H. Laks, et al. 1991. Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein 1 synthesis and is abolished by high density lipoprotein. *J. Clin. Invest.* 88:2039.

16. Ylä-Herttuala, S., B. A. Lipton, M. E. Rosenfeld, T. Sarkioja, T. Yoshimura, E. J. Leonard, J. L. Witztum, and D. Steinberg. 1991. Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA* 88:5252.

17. Ohtsuki, K., M. Hayase, K. Akashi, S. Kopiwoda, and H. W. Strauss. 2001. Detection of monocyte chemoattractant protein-1 receptor expression in experimental atherosclerotic lesions: an autoradiographic study. *Circulation* 104:203.

18. Aiello, R. J., P. A. Bourassa, S. Lindsey, W. Weng, E. Natoli, B. J. Rollins, and P. M. Milos. 1999. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 19:1518.

19. Boring, L., J. Gosling, M. Cleary, and I. F. Charo. 1998. Decreased lesion formation in CCR2$^{-/-}$ mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* 394:894.

20. Weyrich, A. S., T. M. McIntyre, R. P. McEver, S. M. Prescott, and G. A. Zimmerman. 1995. Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor-α secretion: signal integration and NF-κB translocation. *J. Clin. Invest.* 95:2297.

21. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911.

22. Kaluzny, M. A., L. A. Duncan, M. V. Merritt, and D. E. Epps. 1985. Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J. Lipid Res.* 26:135.

23. Zimmerman, G. A., T. M. McIntyre, and S. M. Prescott. 1985. Thrombin stimulates the adherence of neutrophils to human endothelial cells in vitro. *J. Clin. Invest.* 76:2235.

24. Bozza, P. T., J. L. Payne, J. L. Goulet, and P. F. Weller. 1996. Mechanisms of platelet-activating factor-induced lipid body formation: requisite roles for 5-lipoxygenase and de novo protein synthesis in the compartmentalization of neutrophil lipids. *J. Exp. Med.* 183:1515.

25. Bozza, P. T., W. Yu, J. F. Penrose, E. S. Morgan, A. M. Dvorak, and P. F. Weller. 1997. Eosinophil lipid bodies: specific, inducible intracellular sites for enhanced eicosanoid formation. *J. Exp. Med.* 186:909.

26. Gilman, M. 1993. Ribonuclease protection assay. In *Current Protocols in Molecular Biology*, Vol. 1. F. M. Ausube, R. Brent, R. E. Kingston, D. D. Morre, J. G. Seidman, J. A. Smith, and K. Stuhl, eds. Wiley, New York, p. 4.7.1.

27. Rizza, C., N. Leitinger, J. Yue, D. J. Fischer, D. A. Wang, P. T. Shih, H. Lee, G. Tigyi, and J. A. Berliner. 1999. Lysophosphatidic acid as a regulator of endothelial/leukocyte interaction. *Lab. Invest.* 79:1227.

28. Weller, P. F., and A. M. Dvorak. 1994. Lipid bodies: intracellular sites for eicosanoid formation. *J. Allergy Clin. Immunol.* 94:1151.

29. Dvorak, A. M., P. F. Weller, V. S. Harvey, E. S. Morgan, and H. F. Dvorak. 1993. Ultrastructural localization of prostaglandin endoperoxide synthase (cyclooxygenase) to isolated, purified fractions of guinea pig peritoneal macrophage and line 10 hepatocarcinoma cell lipid bodies. *Int. Arch. Allergy Immunol.* 101:136.

30. Bonnet, J., D. Thibaudeau, and P. Bessin. 1983. Dependency of the Paf-acether induced bronchospasm on the lipoxygenase pathway in the guinea-pig. *Prostaglandins* 26:457.

31. Young, J. M., P. J. Maloney, S. N. Jubb, and J. S. Clark. 1985. Pharmacological investigation of the mechanisms of platelet-activating factor induced mortality in the mouse. *Prostaglandins* 30:545.

32. Nagase, T., S. Ishii, H. Katayama, Y. Fukuchi, Y. Ouchi, and T. Shimizu. 1997. Airway responsiveness in transgenic mice overexpressing platelet-activating fac-tor receptor: roles of thromboxanes and leukotrienes. *Am. J. Respir. Crit. Care Med.* 156:1621.

33. Ishii, S., T. Nagase, F. Tashiro, K. Ikuta, S. Sato, I. Waga, K. Kume, J. Miyazaki, and T. Shimizu. 1997. Bronchial hyperreactivity, increased endotoxin lethality and melanocytic tumorigenesis in transgenic mice overexpressing platelet-activating factor receptor. *EMBO J.* 16:133.

34. Wardlaw, A. J., R. Moqbel, O. Cromwell, and A. B. Kay. 1986. Platelet-activating factor: a potent chemotactic and chemokinetic factor for human eosinophils. *J. Clin. Invest.* 78:1701.

35. Silva, P. M., M. A. Martins, H. C. Castro-Faria-Neto, R. S. B. Cordeiro, and B. B. Vargaftig. 1991. Generation of an eosinophilotactic activity in the pleural cavity of platelet-activating factor-injected rats. *J. Pharmacol. Exp. Ther.* 257:1039.

36. Reape, T. J., and P. H. Groot. 1999. Chemokines and atherosclerosis. *Atherosclerosis* 147:213.

37. Wang, N., I. Tabas, R. Winchester, S. Ravalli, L. E. Rabbani, and A. Tall. 1996. Interleukin 8 is induced by cholesterol loading of macrophages and expressed by macrophage foam cells in human atheroma. *J. Biol. Chem.* 271:8837.

38. Gu, L., Y. Okada, S. K. Clinton, C. Gerard, G. K. Sukhova, P. Libby, and B. J. Rollins. 1998. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol. Cell* 2:275.

39. Gosling, J., S. Slaymaker, L. Gu, S. Tseng, C. H. Zlot, S. G. Young, B. J. Rollins, and I. F. Charo. 1999. MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. *J. Clin. Invest.* 103:773.

40. Rayner, K., S. Van Eersel, P. H. Groot, and T. J. Reape. 2000. Localization of mRNA for JE/MCP-1 and its receptor CCR2 in atherosclerotic lesions of the ApoE knockout mouse. *J. Vasc. Res.* 37:93.

41. Johnston, B., A. R. Burns, M. Suematsu, T. B. Issekutz, R. C. Woodman, and P. Kubes. 1999. Chronic inflammation up-regulates chemokine receptors and induces neutrophil migration to monocyte chemoattractant protein-1. *J. Clin. Invest.* 103:1269.

42. Kuziel, W. A., S. J. Morgan, T. C. Dawson, S. Griffin, O. Smithies, K. Ley, and N. Maeda. 1997. Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proc. Natl. Acad. Sci. USA* 94:12053.

43. Huffnagle, G. B., R. M. Strieter, T. J. Standiford, R. A. McDonald, M. D. Burdick, S. L. Kunkel, and G. B. Toews. 1995. The role of monocyte chemotactic protein-1 (MCP-1) in the recruitment of monocytes and CD4$^+$ T cells during a pulmonary *Cryptococcus neoformans* infection. *J. Immunol.* 155:4790.

44. Matsukawa, A., C. M. Hogaboam, N. W. Lukacs, P. M. Lincoln, R. M. Strieter, and S. L. Kunkel. 1999. Endogenous monocyte chemoattractant protein-1 (MCP-1) protects mice in a model of acute septic peritonitis: cross-talk between MCP-1 and leukotriene B$_4$. *J. Immunol.* 163:6148.

45. Samuelsson, B., S.-E. Dahlen, J.-A. Lindgren, C. A. Rouzer, and C. N. Serhan. 1987. Leukotrines and lipoxins: structures, biosynthesis, and biological effects. *Science* 237:1171.

46. Chen, X.-S., J. R. Sheller, E. N. Johnson, and C. D. Funk. 1994. Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. *Nature* 372:179.

47. Bandeira-Melo, C., M. Phoofolo, and P. F. Weller. 2001. Extranuclear lipid bodies, elicited by CCR3-mediated signaling pathways, are sites of chemokine-enhanced leukotriene C$_4$ production in eosinophils and basophils. *J. Biol. Chem.* 276:22779.

48. Kroegel, C., and V. B. Antony. 1997. Immunobiology of pleural inflammation: potential implications for pathogenesis, diagnosis and therapy. *Eur. Respir. J.* 10:2411.

49. Cuzzocrea, S., E. Mazzon, L. Dugo, I. Serraino, A. Ciccolo, T. Centorrino, A. De Sarro, and A. P. Caputi. 2001. Protective effects of *n*-acetylcysteine on lung injury and red blood cell modification induced by carrageenan in the rat. *FASEB J.* 15:1187.

50. Oda, M., K. Satouchi, K. Ikeda, M. Sakakura, K. Yasunaga, and K. Saito. 1990. The presence of platelet-activating factor associated with eosinophil and/or neutrophil accumulations in the pleural fluids. *Am. Rev. Respir. Dis.* 141:1469.

51. Boren, J., M. Gustafsson, K. Skalen, C. Flood, and T. L. Innerarity. 2000. Role of extracellular retention of low density lipoproteins in atherosclerosis. *Curr. Opin. Lipidol.* 11:451.