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Synergism Between Platelet-Activating Factor-Like Phospholipids and Peroxisome Proliferator-Activated Receptor γ Agonists Generated During Low Density Lipoprotein Oxidation That Induces Lipid Body Formation in Leukocytes[1]

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Oxidized low density lipoprotein (LDL) has an important proinflammatory role in atherogenesis. In this study, we investigated the ability of oxidized LDL (oxLDL) and its phospholipid components to induce lipid body formation in leukocytes. Incubation of mouse peritoneal macrophages with oxidized, but not with native LDL led to lipid body formation within 1 h. This was blocked by platelet-activating factor (PAF) receptor antagonists or by preincubation of oxLDL with rPAF acetylhydrolase. HPLC fractions of phospholipids purified from oxLDL induced calcium flux in neutrophils as well as lipid body formation in macrophages. Injection of the bioactive phospholipid fractions or butanoyl and butenoyl PAF, a phospholipid previously shown to be present in oxLDL, into the pleural cavity of mice induced lipid body formation in leukocytes recovered after 3 h. The 5-lipoxygenase and cyclooxygenase-2 colocalized within lipid bodies formed after stimulation with oxLDL, bioactive phospholipid fractions, or butanoyl and butenoyl PAF. Lipid body formation was inhibited by 5-lipoxygenase antagonists, but not by cyclooxygenase-2 inhibitors. Azelaoyl-phosphatidylcholine, a peroxisome proliferator-activated receptor-γ agonist in oxLDL phospholipid fractions, induced formation of lipid bodies at late time points (6 h) and synergized with suboptimal concentrations of oxLDL. We conclude that lipid body formation is an important proinflammatory effect of oxLDL and that PAF-like phospholipids and peroxisome proliferator-activated receptor-γ agonists generated during LDL oxidation are important mediators in this phenomenon. ***The Journal of Immunology*****, 2003, 171: 2090–2098.**

Atherosclerosis is a chronic inflammatory disease, in which the earliest recognizable event is the accumulation of foam cells in arterial intima with fatty streak formation (1, 2). Foam cell formation is the result of unregulated uptake of oxidized low density lipoprotein (LDL)[3] by macrophages through scavenger receptors (3, 4). LDL oxidation contributes to the initiation and development of atherosclerosis not only by foam cell and fatty streak formation, but also as a source of proinflammatory lipids (5). LDL oxidation generates phospholipids with biological activity that accounts for many of the biological effects attributed to oxidized LDL (oxLDL), including induction of chemokine synthesis and leukocyte adhesion (6–8). Moreover, the uptake of oxLDL seems also to be influenced by the presence of oxidized phospholipids because Boullier et al. (9) showed that oxLDL phospholipids are recognized by the scavenger receptor CD36, which may partially mediate uptake of oxLDL by macrophages (10, 11).

Heery et al. (12) demonstrated the presence of platelet-activating factor (PAF)-like phospholipids in oxLDL using models of smooth muscle cell proliferation and neutrophil adhesion. We expanded these observations by showing that HPLC-purified fractions of oxLDL phospholipids activate the PAF receptor both in vitro and in vivo, and that their effects are blocked by treatment with PAF acetylhydrolase. The main PAF-like phospholipids found in those fractions were 1-*O*-hexadecyl-2-butanoyl-*sn*-glycero-3-phosphocoline and 1-*O*-hexadecyl-2-butenoyl-*sn*-glycero-3-phosphocoline (C4-PAF) (13, 14).

PAF-like phospholipids are involved in many human diseases. Those substances are generated in the blood of patients with sepsis (15, 16), active systemic lupus erythematosus (17), and in plasma lipoproteins of cigarette smokers (18). PAF-like lipids are potent proinflammatory mediators and seem to be involved on earliest events of atherosclerosis in mice because the PAF receptor antagonist, WEB 2086, decreased monocyte-endothelial interactions and fatty streak formation in LDL receptor-deficient animals fed a lipid-rich diet (19). It is important to note that authentic PAF induces lipid body formation in leukocytes such as neutrophils (20) and eosinophils (21, 22). Those lipid bodies are membraneless lipid-rich cytoplasmic inclusions localized in leukocytes and in

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[3] Abbreviations used in this paper: LDL, low density lipoprotein; 5-LO, 5-lipoxygenase; azPC, azelaoyl phosphatidylcholine; C4-PAF, butanoyl and butenoyl-PAF; COX-2, cyclooxygenase-2; i.t., intrathoracic; LT, leukotriene; MCP-1, monocyte chemoattractant protein 1; oxLDL, oxidized LDL; PAF, platelet-activating factor; PPARγ, peroxisome proliferator-activated receptor-γ; rhPAF-AH, human rPAF acetylhydrolase.

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other inflammation-associated cell types. They are constitutive in many leukocytes, increase in size and number after stimulation (23, 24), and are sites for localization of enzymes involved in arachidonate mobilization and metabolism (21, 25, 26).

In addition to PAF-like phospholipids, Davies et al. (27) have identified another oxidatively modified alkyl phospholipid in bioactive fractions of oxLDL, hexadecyl azelaoyl phosphatidylcholine (azPC), as a high affinity ligand and agonist for peroxisome proliferator-activated receptor-γ (PPARγ). This phospholipid induced expression of CD36 in primary human monocytes. Interestingly, Nagy et al. (28) showed that oxLDL binds to CD36 and induces PPARγ expression, which regulates the transcription process of several genes related to lipid metabolism. Moreover, ligand-induced activation of PPARγ led to CD36 synthesis and expression on macrophage membranes, and enhanced the uptake of oxLDL (28, 29). In addition, Tontonoz et al. (29) have demonstrated that PPARγ ligands induce morphological changes, and a tendency to adhere to the culture dish and lipid droplet accumulation in the cytoplasm of a monocytic cell line.

In this context, we considered that PAF-like phospholipids and PPARγ agonists present in the phospholipid fractions of oxLDL might induce leukocyte activation and lipid body formation with consequences to arachidonate metabolism. Therefore, our aim was to analyze whether oxLDL and its phospholipid fractions are able to induce lipid body formation in leukocytes and study the mechanisms underlying this response.

Materials and Methods

Materials

Anti-5-lipoxygenase (5-LO) rabbit IgG was from Cayman Chemicals (Ann Arbor, MI); anti-rabbit biotinylated IgG was from Vector (Burlingame, CA); aqua Polymount was from Polyscience (Warrington, PA); BN 52021 [3-(1,1-dimethylethyl) hexahydro-1,4,7b-trihidroxy-8-methyl-9H-1,7α (epoxy-methano)-1H,6αH-cyclopenta (c) furo (2,3-b) furo (3′,2′:3,4) cyclopenta (1,2-d) furan-5,9,12 (4H)-trione] was from Biomol (Plymouth Meeting, PA); SR 27417 (*N*-(2-dimethylamino ethyl)-*N*-(3-pyridinyl methyl) [4-(2,4,6-triisopropylphenyl) thiazol-2-yl] amine) was a kind gift from J. Herbert from Sanofi Rechecher (Toulouse, France); WEB 2086 [3-(4-(2-chlorophenyl)-9-methyl-6H-thieno-2,2-f-(1,2,4) triazolo (4,3-a) (1,4)-diazepin-2-yl-1-(4-morpholinyl)-1-propanone] was a gift from H. Heuer from Boehringer Ingelheim (Ingelheim am Rhein, Germany); C4-PAF was from Avanti Polar Lipids (Alabaster, AL); celecoxib was from Searle (St. Louis, MO); aminopropyl columns were from J. Baker (Phillipsburg, NJ); PAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocoline, was from Sigma-Aldrich (St. Louis, MO); Pefablock [4-(2-aminoethyl) benzenesulfonylfluoride] was from Pentapharm A.G. Laboratories (Basel, Switzerland); leukotriene B_4 (LTB_4) was detected by immunoenzymatic assay from Cayman Chemicals; zileuton was obtained from Abbott Laboratories (Abbott Park, IL); and human rPAF acetylhydrolase (rhPAF-AH) was from ICOS (Bothell, WA).

Purification and oxidation of human LDL

Human LDL was isolated from plasma after density adjustment to 1.3 g/ml with KBr. A gradient of plasma and saline solution (NaCl 0.9%) was centrifuged for 2 h 40 min/150,000 $\times$ *g*. After centrifugation, the band of lipoproteins with density between 1.019 and 1.062 was collected and dialyzed against PBS (1$\times$) overnight. The LDL isolated (200 μg of protein/ml) was treated with a PAF acetylhydrolase inhibitor ([4-(2-aminoethyl) benzenesulfonylfluoride], 200 μM) before oxidation with $CuSO_4$ (10 μM) for 18 h/37°C (12). Control LDL was not subjected to oxidation, and EDTA (40 μM) was added to this material.

Separation of lipids

The lipids were extracted from LDL according to the method described by Bligh and Dyer (30). Neutral lipids, fatty acids, and phospholipids were separated by aminopropyl cromathography using chloroform:isopropanol (2:1 v/v), acetic acid (2%) in diethyl ether, and methanol, respectively (31). 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%) (13). Fractions were collected every minute, dried under a stream of N_2, reconstituted with methanol containing BHT (10 μM), and stored at -20°C. Before the experiments, the fractions were dried under N_2, suspended in HBSS/BSA, and sonicated for 30 s before use. The bioactivity of the fractions was determined in a calcium flux assay, as previously described (13).

Induction of lipid body formation in vitro

The peritoneal spaces of mice were washed with PBS, and the resident macrophages from this peritoneal lavage (10^6cells/ml) were stimulated with PAF (1 μM), unoxidized LDL, oxLDL (50, 100, or 200 μg lipoprotein/ml), oxidized phospholipids (HPLC fractions 2–11), C4-PAF (1 μM), PPARγ agonists BRL 49653 or azPC (1 and 5 μM) alone or in association with oxLDL (50 and 200 μg/ml), or C4-PAF (0.1 nM) or arachidonic acid (1 and 10 μM) or vehicle (HBSS/BSA 0.1%) at 37°C for 1 h. In other experiments, peritoneal macrophages were stimulated with unoxidized LDL, oxLDL (200 μg lipoprotein/ml), or azPC (1 μM) in albumin, HEPES, and glucose-supplemented PBS at 37°C for 6 h. The macrophages were pretreated with PAF antagonists (BN 52021, WEB 2086, or SR 274117A) at 10 μM for 15 min before the incubation with PAF (1 μM), oxidized phospholipids (HPLC fractions 6, 7, and 8), or C4-PAF (1 μM) at 37°C. Some oxidized phospholipid fractions were incubated with rh-PAF-AH (4 μg) for 1 h at 37°C. The cells were centrifuged in a cytocentrifuge (Shandon cytospin III) (550 rpm/5 min) onto glass slides (10^5 cells/slide) and stained with osmium tetroxide, as described (20). Briefly, while still moist, leukocytes on cytospin slides were fixed in 3.7% formaldehyde in Ca^{2+}/Mg^{2+}-free HBSS, pH 7.4, rinsed in 0.1 M cacodylate buffer 1.5% OsO_4 (30 min), rinsed in dH_2O, immersed in 1.0% thiocarbohydazide (5 min), rinsed in 0.1 M cacodylate buffer, restained in 1.5% OsO_4 (3 min), rinsed in dH_2O, and then dried and mounted. The morphology of fixed cells was observed, and lipid bodies were enumerated by light microscopy with a $\times$100 objective lens in 50 consecutively scanned leukocytes.

Pleurisy model and lipid body formation

Male or female Swiss mice (20–25 g) of the 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 animals received an intrathoracic (i.t.) injection of pooled bioactive (HPLC fractions 6, 7, and 8) or inactive phospholipid fractions (HPLC fractions 2, 3, 4, 10, and 11) or C4-PAF (1 μg/cavity). A negative control was also performed using phospholipid fractions obtained from unoxidized LDL. The HPLC fractions and C4-PAF were dried under nitrogen and resuspended in sterile HBSS containing 0.01% of BSA. The HBSS/BSA solution was administered i.t. into naive animals, constituting the control group. After 3, 6, 12, and 24 h, the animals were sacrificed in a CO_2 gas chamber, and the thoracic cavity was open and washed with 1 ml of HBSS. The pleural wash was recovered, and the volume was measured on a graduated syringe. Lipid body formation in leukocytes was evaluated on cytosmears stained with osmium, as described earlier.

In vivo treatments

Animals were treated with the PAF antagonist BN 52021 at the dose of 20 mg/kg i.p. 30 min before stimulation. In a different experimental protocol, the animals were treated with the cyclooxygenase-2 (COX-2) inhibitor, celecoxib (5 mg/kg, i.p.), or the 5-LO inhibitor, zileuton (50 μg/cavity, in situ), before being stimulated with oxidized phospholipids or C4-PAF. BN 52021 was dissolved in 0.9% saline, and celecoxib and zileuton were dissolved in DMSO to obtain a stock solution and further diluted in 0.9% saline. The final DMSO concentration was 0.01%.

Immunocytochemistry

Pleural leukocytes in suspension were incubated for 1 h with 1 μM fluorescent fatty acid-containing diglyceride, 1-acyl-2-(7-octyl-BODIPY-1-pentanoyl)-sn-glycerol (Molecular Probes, Eugene, OR), to fluorescently label lipid bodies. After the incubation, cells were washed twice in Ca^{2+}/Mg^{2+}-free HBSS. Cytosmears were prepared and fixed in 3% formaldehyde, permeabilized with 0.05% saponin/HBSS solution, treated with biotin-blocking solution, and blocked with 10% normal goat serum. After washing, cytospin preparations were incubated overnight at 4°C with rabbit polyclonal anti-mouse PGHS-2 (anti-COX-2) or rabbit polyclonal anti-mouse 5-LO Ab (1:150) diluted in 0.05% saponin/HBSS solution. Preimmune rabbit serum was used as control. After three washes of 5 min in 0.05% saponin/HBSS solution, the preparations were incubated with biotin-conjugated goat anti-rabbit IgG. The immunoreactive COX-2 and 5-LO in cells were then identified by ABC Vectastin glucose-oxidase kit

following the manufacturer's instruction (Vector, CA). The glucose-oxidase immunostaining was visualized under light microscopy, and fluorescent lipid bodies were identified under FITC filter (21).

Priming for LTB_4 generation

Macrophages incubated with phospholipids extracted from oxLDL or C4-PAF for 1 h were washed in calcium-free HBSS and resuspended in HBSS. Macrophages were then stimulated with calcium ionophore (0.5 μM) for 15 min, and LTB_4 released in the supernatant was detected by immunoenzymatic assay, according to manufacturer's instruction (Cayman Chemicals).

Statistical analysis

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

Results

oxLDL and oxLDL phospholipids induce lipid body formation in peritoneal macrophages

Lipid bodies are arachidonic acid-rich cytoplasmic inclusions found in increased numbers in inflammatory cells (23, 32). Fig. 1*A* illustrates the presence of lipid bodies in macrophages exposed to 200 μg/ml of native LDL or oxLDL, respectively. There was an increase in lipid body numbers at concentrations of 100 and 200 μg/ml of oxLDL, but there was no lipid body formation with similar concentrations of native LDL. These results demonstrate that the oxidation of LDL particle generates bioactive components with ability to induce lipid body formation.

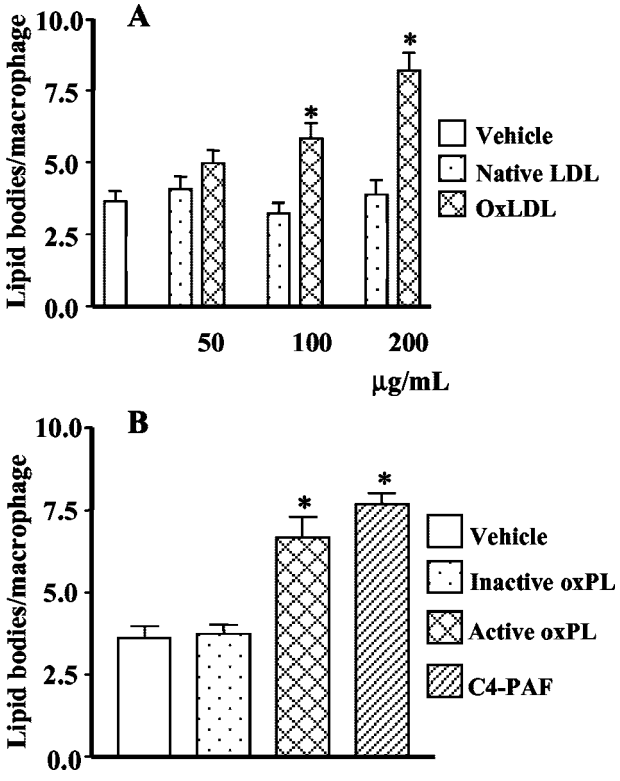

FIGURE 1. Concentration-dependent effect of oxLDL (50, 100, 200 μg/ml) in lipid body formation in macrophages stimulated for 1 h (*A*). The effect of HPLC fractions 2, 3, 10 (inactive), and 6, 7, 8 (active) of oxidized phospholipids (oxPL) or C4-PAF (1 μM) (*B*) on lipid body formation in macrophages. Mice peritoneal macrophages were harvested with PBS and stimulated for 1 h with the different stimuli. The lipid body count was performed in 50 cells. Each column is the mean and vertical lines are SEM from at least three animals. ∗, Indicates statistically significant differences when compared with HBSS/BSA (vehicle).

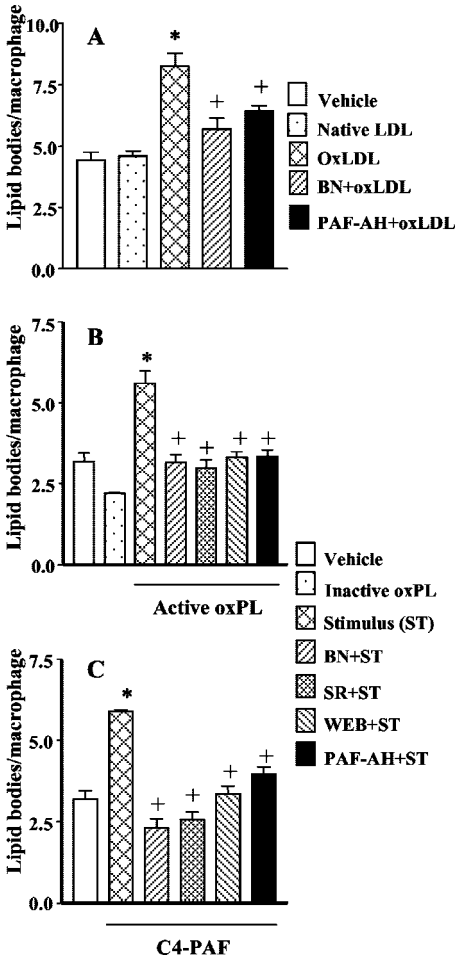

FIGURE 2. PAF antagonists and rhPAF-AH inhibit lipid body formation by oxLDL and its bioactive phospholipids. *A*, Shows the effect of BN52021 (BN) (10 μM) and rhPAF-AH (4 μg) on lipid body formation in mice peritoneal macrophages stimulated with oxLDL. The effect of three structurally unrelated PAF antagonists BN52021 (BN) (10 μM), WEB, 2086 (WEB) (10 μM), and SR 27417A (SR) (10 μM), or of rhPAF-AH (4 μg) on lipid body formation in mice peritoneal macrophages stimulated with bioactive HPLC fractions obtained from oxLDL phospholipids (oxPL) (*B*) or synthetic C4-PAF (1 μM) (*C*) is also shown. Control groups were macrophages incubated with HBSS/BSA (vehicle), LDL (*A*), or inactive HPLC fractions (*B*). The lipid body count was performed in 50 cells. Each column is the mean and vertical lines are SEM from at least three animals. ∗, Indicates statistically significant differences when compared with HBSS/BSA, and +, when compared with oxLDL (*A*), bioactive fractions (*B*), or C4-PAF alone (*C*).

In a previous study, we purified the polar lipids from oxLDL and fractionated them on a reversed-phase HPLC. Only phospholipid fractions eluting at 6, 7, and 8 min induced calcium mobilization in neutrophils in vitro and inflammation in vivo through the activation of the PAF receptor. We also found that the main bioactive component of those fractions was C4-PAF (13). As shown in Fig. 1*B*, phospholipids extracted from oxLDL induced lipid body formation, which was induced by the same fractions (HPLC fractions 6, 7, and 8) or C4-PAF that were active in other assays. Fractions previously shown to be inactive in a calcium mobilization assay or in an inflammatory model in vivo (fractions 2, 3, 4, 10, and 11) (13, 33) did not stimulate lipid body formation. These results suggest that the effect of oxLDL may be attributed to the oxidatively modified phospholipids present on oxLDL, in particular to C4-PAF.

We further analyzed the mechanisms underlying lipid body formation induced by oxLDL or oxLDL phospholipids by preincubating macrophages with a PAF receptor antagonist (BN 52021) before the incubation with oxLDL. Alternatively, we treated oxLDL with the selective degradative enzyme rhPAF-AH before addition to the macrophages. Both procedures blocked lipid body formation (Fig. 2*A*). Also, the effect of the bioactive oxidized phospholipids or C4-PAF was inhibited by the treatment with three structurally unrelated PAF antagonists (BN 52021, WEB 2086, and SR 27417A) and rhPAF-AH (Fig. 2, *B* and *C*). These results suggest that oxLDL and oxLDL phospholipids induce lipid body formation in vitro through activation of the PAF receptor.

Lipid bodies are induced in leukocytes recovered from the pleural cavity of mice stimulated with oxidized phospholipids and C4-PAF

We performed in vivo experiments to further study lipid body formation. Pleural leukocytes were recovered at different time intervals after the i.t. injection of oxidized phospholipids or C4-PAF. The injection of bioactive oxidized phospholipid fractions or C4-PAF into pleural space of mice induced lipid body formation in leukocytes recovered after 3 and 6 h. At 12 h, lipid body numbers returned to basal levels (Fig. 3*A*). Neutrophils were the main leukocyte subtype infiltrating the pleural cavity at the times when lipid body numbers were augmented (data not shown). Treatment with the PAF antagonist, BN 52021, blocked both leukocyte accumulation (data not shown) and lipid body formation at 6 h (Fig. 3*B*), showing that oxidized phospholipids induced lipid body formation in vivo through activation of the PAF receptor. These data provide strong evidence for C4-PAF as the main PAF-like component of oxLDL that leads to lipid body formation in leukocytes, not only in vitro, but also in vivo.

COX-2 and 5-LO localize in lipid bodies formed after oxidized phospholipid stimulation

Lipid bodies are sites of localization for arachidonate and for eicosanoid-forming enzymes in leukocytes (21, 25, 26, 34). In Fig. 4, we show that the incubation of macrophages with oxLDL, but not with native LDL, for 6 h induced expression of both COX-2 (*B* and *E*) and 5-LO (*C* and *F*) with a clear accumulation of those enzymes in punctated structures in the cytoplasm (arrows). We evaluated whether enzymes involved in arachidonic acid metabolism were localized in lipid bodies induced after i.t. injection of C4-PAF. As shown in Fig. 5*B*, 6 h after the injection of C4-PAF COX-2 was expressed in punctated cytoplasmic structures, although a faint perinuclear staining could also be noted. In contrast, at 24 h, COX-2 immunostaining lost its granular aspect and showed a much more diffuse pattern in the cytoplasm, although a perinuclear staining could still be seen (Fig. 5*D*). Of importance, lipid body numbers were back to basal 24 h after stimulation (Fig. 3*A*). This suggests that the granular staining pattern of COX-2 expression may be related to COX-2 localization on lipid bodies. As was demonstrated in our previous work (33), 5-LO also showed a granular pattern of expression, consistent with its localization in lipid bodies, in leukocytes recovered from the pleural cavity 6 h after the i.t. injection of bioactive oxidized phospholipid fractions or C4-PAF (Fig. 6*C*).

To reinforce these findings, we performed studies using cells recovered from the pleural cavity of C4-PAF-stimulated animals, associating the specific immunostaining for the enzymes with fluorescent fatty acids incorporated into the lipid bodies. The results

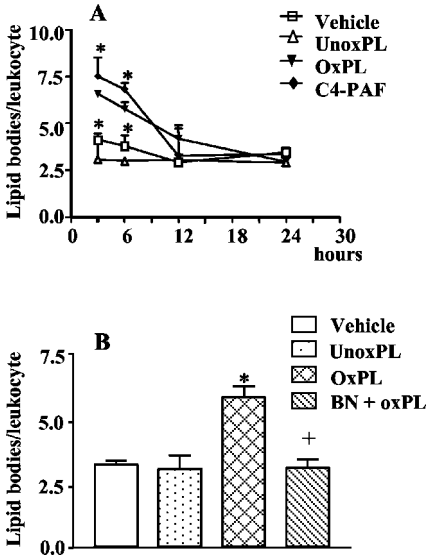

FIGURE 3. Kinetics of lipid body formation in leukocytes after i.t. injection of vehicle, inactive (2, 3, 10), bioactive (6, 7, 8) HPLC fractions obtained from oxLDL phospholipids (oxPL) or C4-PAF (1 μg/cavity) in the pleural cavities of mice (*A*). In vivo administration of BN52021 (BN) (20 mg/kg) blocks lipid body formation in leukocytes recovered from the pleural cavity of mice stimulated with bioactive fractions obtained from oxPL. Control groups were animals injected with HBSS/BSA (vehicle) and the same HPLC fractions (6, 7, 8) obtained from native LDL phospholipids (unoxidized phospholipids, unoxPL) (*B*). The lipid body count was performed in 50 cells. ∗, Indicates statistically significant differences when compared with HBSS/BSA, and +, when compared with bioactive fractions. Each point (*A*) or column (*B*) is the mean from at least six animals, and vertical lines are the SEM.

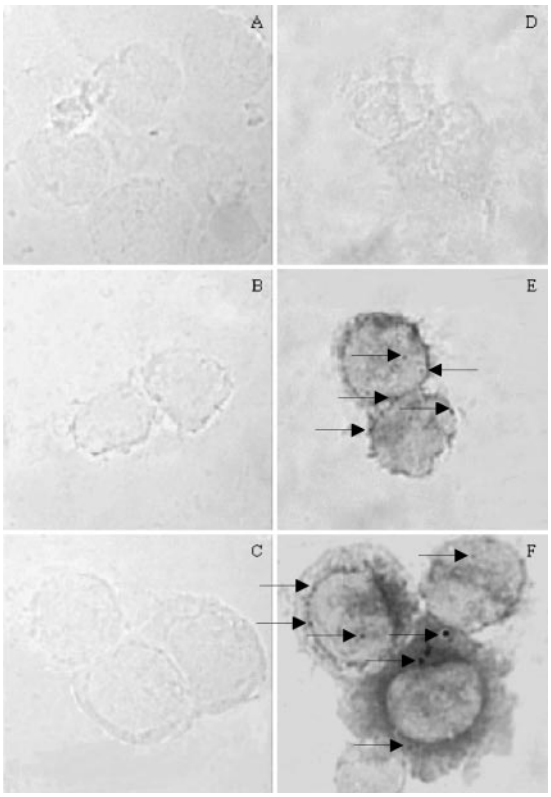

FIGURE 4. Immunolocalization of COX-2 (*B* and *E*) and 5-LO (*C* and *F*) (arrows) in mice peritoneal macrophages stimulated for 6 h with oxidized (oxLDL) (*E*, *F*) or native LDL (*B*, *C*). Negative control with nonimmune serum is depicted on *A* and *D*.

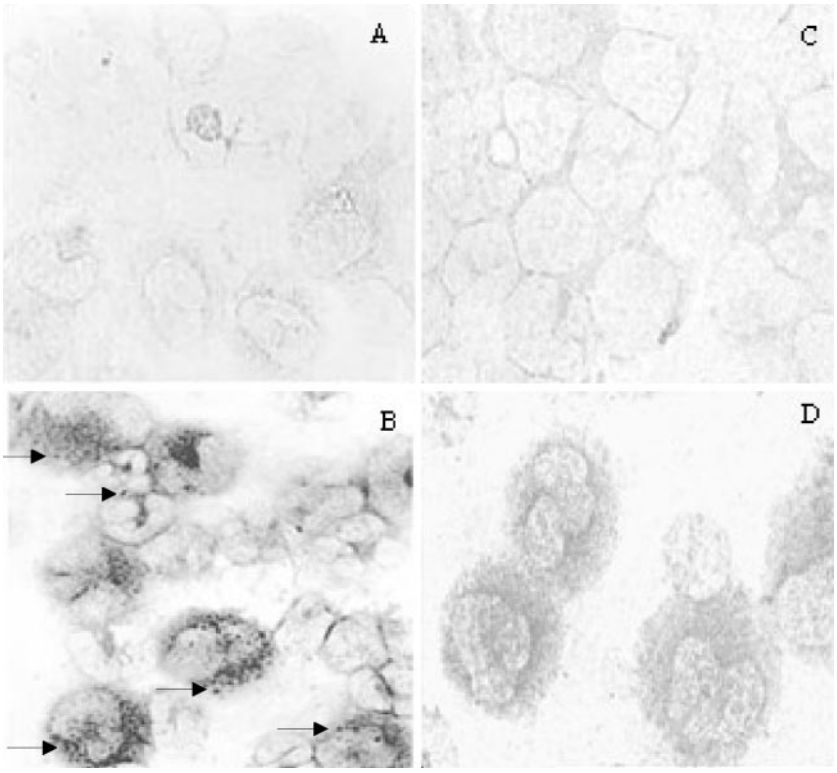

FIGURE 5. Immunolocalization of COX-2 (arrows) in leukocytes recovered from the pleural cavity of mice 6 (*B*) and 24 h (*D*) after the i.t. injection of C4-PAF (1 μg/cavity). Negative control with nonimmune serum is depicted on *A* and *C*.

obtained are depicted on Fig. 6 and clearly demonstrate that immunostaining for both COX-2 and 5-LO have the same cellular localization of fluorescent lipid bodies. Similar results were previously reported in eosinophils exposed to authentic PAF (21). Thus, lipid body formation in leukocytes was enhanced after in vivo exposure to C4-PAF, and these lipid bodies are sites of localization for both COX-2 and 5-LO.

The 5-LO metabolites are important mediators for lipid body formation induced by oxidized phospholipids

In light of the information that both COX-2 and 5-LO localize on lipid bodies after stimulation with bioactive oxidized phospholipid fractions or C4-PAF, we addressed the question of whether enzymatic activity is needed for lipid body formation. We treated the animals with celecoxib, a selective inhibitor of COX-2, or zileuton, a selective inhibitor of 5-LO. Celecoxib treatment showed no effect on lipid body formation induced by C4-PAF (Fig. 7*B*). This COX-2 inhibitor blocked zymosan-induced protein extravasation into mice pleural cavity (data not shown). In contrast, zileuton blocked the lipid body formation in leukocytes stimulated by bioactive oxidized phospholipids or C4-PAF (Fig. 7, *A* and *B*). This implicates 5-LO, but not COX metabolites, in the formation of lipid bodies after in vivo stimulation with bioactive oxidized phospholipids or C4-PAF. We also observed that the active oxidized phospholipids or C4-PAF primed the production of LTB_4 in macrophages stimulated with calcium ionophore, an effect that correlated with the increase in lipid body numbers (Fig. 8).

PPARγ agonists synergize with oxLDL and C4-PAF to induce lipid body formation

PPARγ ligands induce lipid droplet accumulation in monocytic cells (29). Recently, a potent agonist for PPARγ, azPC, was found in phospholipid fractions of oxLDL (27). In this context, we considered that PPARγ agonists could induce lipid body formation in our model. We incubated peritoneal macrophages with the PPARγ agonists, BRL 49653 or azPC, for 1 h and observed that those substances did not induce lipid body formation at this time. However, incubation of macrophages for 6 h with azPC induced an increase in lipid body numbers as compared with control macrophages (from 4.1 $\pm$ 0.3 lipid bodies/cell in vehicle-stimulated cells to 7.3 $\pm$ 0.4 lipid bodies/cell in azPC-stimulated cells). oxLDL (200 μg/ml) alone or in combination with BRL 49653 induced lipid body formation in cultured peritoneal macrophages, but no synergism between oxLDL and BRL 49653 was noted (9.0 $\pm$ 0.8 lipid bodies/cell in oxLDL-stimulated cells and 9.5 $\pm$ 0.5 lipid bodies/cell in BRL and oxLDL-stimulated cells). Nevertheless, when a suboptimal concentration of oxLDL (50 μg/ml) was used, lipid body formation was seen only when oxLDL was present with BRL 49653 or azPC (Fig. 9*A*). These data clearly demonstrate that signaling through PPARγ has a synergistic role with oxLDL in the induction of lipid body formation. In addition, we tested the possibility that the synergistic effect of azPC with oxLDL reflected the action of the bioactive phospholipid, C4-PAF. We incubated peritoneal macrophages with suboptimal concentrations of C4-PAF (0.1 nM) and showed that, at this concentration, C4-PAF did not increase lipid body numbers in macrophages. In contrast, if azPC or BRL 49653 was incubated together with suboptimal concentrations of C4-PAF, a significant increase in lipid body numbers was noted (Fig. 9*B*). This shows that PPARγ agonists and PAF mimetics cooperate to induce lipid body formation. Interestingly, the synergistic effect of azPC and BRL 49653 was specific for oxLDL and C4-PAF because those compounds failed to increase lipid body numbers when associated with suboptimal concentrations of arachidonic acid (Fig. 9*C*).

Discussion

Oxidatively modified LDL has a well-recognized proinflammatory activity and plays an essential role in atherosclerotic plaque formation (5, 6, 35, 36). In this study, we characterized the lipid body

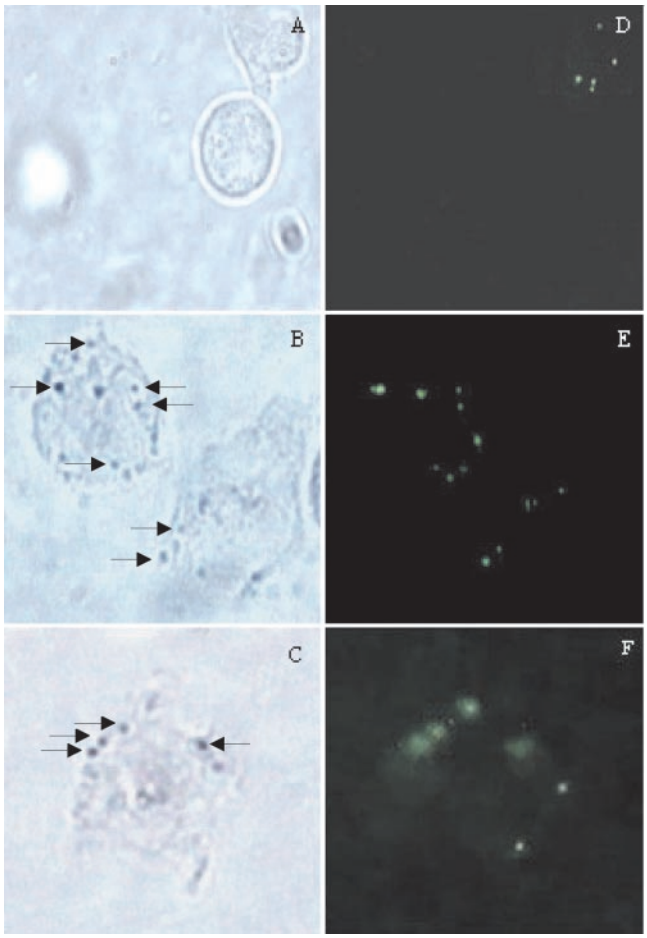

FIGURE 6. COX-2 (*B*) and 5-LO (*C*) colocalize in lipid bodies stained with fluorescent fatty acids (*E* and *F*). Leukocytes were recovered from the pleural cavity of mice 6 h after the i.t. injection of C4-PAF (1 μg/cavity). Negative control with nonimmune serum is depicted on *A* and *D*.

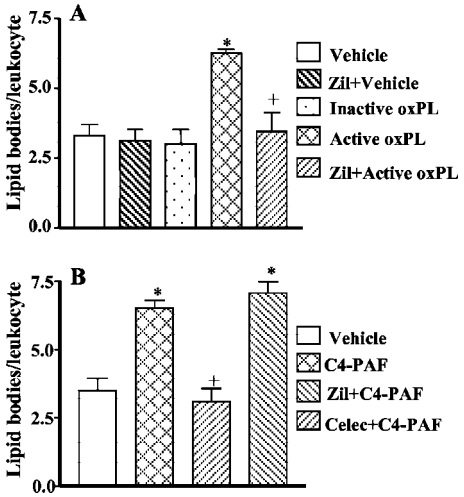

FIGURE 7. Zileuton (Zil), a specific 5-LO inhibitor, but not celecoxib (Celec), a specific COX-2 inhibitor, inhibits leukocyte lipid body formation induced by i.t. injection of bioactive HPLC fractions obtained from oxLDL phospholipids (oxPL) (*A*) or C4-PAF (*B*) in the mice pleural cavity. The animals received i.t. injection of HBSS/BSA (vehicle), inactive (2, 3, 10), active (6, 7, 8) HPLC fractions of oxidized phospholipids or C4-PAF (1 μg/cavity). The cells were recovered from the pleural cavity 6 h after stimulation. Zileuton was administered i.t. at 50 μg/cavity immediately before the stimulus, and celecoxib (5 mg/kg) was administered i.p. 1 h before stimulation. Each bar is the mean from at least six animals, and vertical lines represent SEM. $*$, Indicates statistically significant differences when compared with vehicle, and $+$, when compared with oxLDL phospholipids (6, 7, 8) or C4-PAF.

formation in leukocytes stimulated by oxLDL and its phospholipids.

We observed that oxLDL induced lipid body formation in macrophages in vitro. This cytoplasmic inclusion is constitutive in several myeloid cells, including eosinophils, neutrophils, and monocytes, and increased numbers of lipid bodies are found in cells associated with natural or experimentally induced inflammatory process (22, 37–42). Lipid body formation can be induced by different stimuli, including CC chemokines through activation of CCR3 (43), arachidonic acid, oleic acid, and PAF (20, 21, 44). This latter agonist acts through its receptor on the leukocyte membrane to increase lipid body numbers in neutrophils (20) and eosinophils (21, 22).

PAF is a phospholipid mediator with several proinflammatory effects (45). Recently, PAF mimetics were shown to be formed during oxidative modification of LDL and to reproduce several of the inflammatory effects attributed to the oxLDL particle (12, 13). In this context, we have shown that the enzyme rhPAF-AH and PAF antagonists inhibited the increase in lipid body numbers in macrophages exposed to oxidized LDL. This shows that the generation of PAF mimetics during oxidation of LDL is directly involved in stimulating lipid body formation by oxLDL. Indeed, bioactive phospholipid fractions extracted from oxLDL or the C4-PAF, found to be present in those phospholipid fractions (13), induced lipid body formation through the activation of the PAF receptor and were sensitive to degradation by rhPAF-AH. These results confirm that oxLDL phospholipids have PAF-like activity and implicate C4-PAF as the oxLDL component responsible for lipid body formation in macrophages in vitro. We also demonstrated that oxLDL phospholipids or synthetic C4-PAF potently induced lipid body formation in pleural leukocytes in vivo, acting through the PAF receptor.

Lipid bodies are sites for localization of arachidonate (34) and eicosanoid-forming enzymes, such as COX (21, 25) and lipoxygenase (21, 22). In fact, we detected increased COX-2 expression in leukocytes from the pleural cavity of mice exposed to oxidized phospholipid or C4-PAF. In addition to perinuclear COX-2 localization, we observed cytoplasmic distribution of this enzyme in lipid bodies that costained with fluorescent lipids at early time points (6 h). This is in agreement with previous results showing localization of COX-2 in lipid bodies in macrophages after LPS stimulation and also showing that the effect of LPS depends on the activation of the PAF receptor (38). At later time points (24 h), the immunostaining for COX-2 showed a more diffuse cytoplasmic pattern, which might be related to the decrease in lipid body numbers at this time. Increased expression of COX-2 in response to oxLDL has been previously reported in human monocyte-derived macrophages (46). This effect was mediated by hexadecyl azPC, a phospholipid in the oxLDL particle with potent PPARγ agonist activity, and dependent on the upstream PPAR-responsive element of COX-2. On contrast, we also find increased COX-2 expression in cells recovered from the pleural cavity of animals injected with synthetic C4-PAF. This might reflect the increased complexity of leukocyte response in an inflammatory reaction in vivo in which a diversity of secondary mediators is formed to influence the final phenotype of the response. COX-2 is expressed in atherosclerotic lesions, particularly in infiltrating macrophages (47, 48), and its

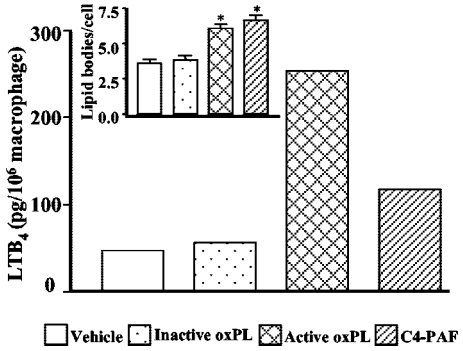

FIGURE 8. LTB_4 production in the supernatant of the pleural leukocytes stimulated with calcium ionophore (0.5 μM) and its correlation with lipid body numbers. Leukocytes were recovered after 6 h from the pleural cavity of mice injected with inactive (2, 3, 10), bioactive HPLC fractions from oxLDL phospholipids (oxPL) (6, 7, 8), C4-PAF, or vehicle. Lipid body numbers present at the same population of leukocytes are shown in the *inset*. The amount of LTB_4 was determined by ELISA in duplicate wells from at least four animals.

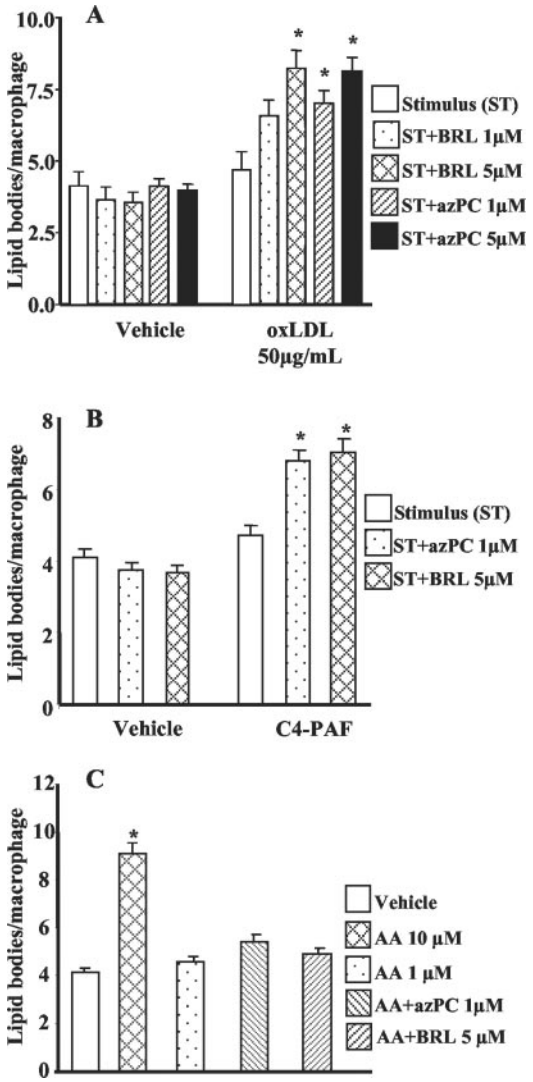

FIGURE 9. PPARγ agonists, BRL 49653 (BRL) and hexadecyl azPC, synergize with oxLDL (*A*) and C4-PAF (*B*) to induce lipid body formation in mice peritoneal macrophages. This synergic effect was not seen with arachidonic acid (AA) (*C*). Lipid body enumeration was performed 1 h after stimulation with the agonists in 50 cells. Each column is the mean, and vertical lines are SEM from at least three animals. *, Indicates statistically significant differences when compared with oxLDL- or C4-PAF-untreated group.

expression is also increased after vascular injury (49). It is conceivable that both oxLDL PAF-like compounds and azPC contribute to increased COX-2 expression in atherosclerotic lesions.

We observed 5-LO localization in lipid bodies in macrophages stimulated with oxidized phospholipids or C4-PAF. We also demonstrated that treatment with the 5-LO inhibitor, zileuton, decreased lipid body formation in pleural leukocytes in our pleurisy model, suggesting requirement of 5-LO activity in the lipid body formation induced by oxidized phospholipids or C4-PAF. Bozza et al. (20) reported that PAF induced lipid body formation in leukocytes obtained from wild-type, but not 5-LO knockout mice, reinforcing the role of 5-LO products in lipid body formation elicited by PAF. Moreover, we have demonstrated that monocyte chemoattractant protein 1 (MCP-1) is also required for lipid body formation induced by C4-PAF because leukocytes from MCP-1-deficient mice do not show increased lipid body numbers when stimulated with oxLDL bioactive phospholipids (33). Interestingly, MCP-1 is apparently upstream of 5-LO, as leukocytes from MCP-1-deficient mice also fail to increase LTB_4 production (33). These results, together with the observations by Aiello et al. (50), showing that MCP-1 and LTB_4 interact to induce the progression of atherosclerotic lesions, highlight the relevance of our findings of 5-LO expression in lipid bodies induced by oxLDL to the pathophysiology of atherosclerosis. We suggest that C4-PAF activated 5-LO and stimulated lipid body formation in leukocytes, and these lipid bodies, in turn, are sites for localization of newly generated 5-LO. Because macrophages produce the chemotactic factor LTB_4 (51), it is reasonable to hypothesize that macrophages stimulated with oxidized phospholipids or C4-PAF may synthesize LTB_4 via 5-LO, resulting in lipid body formation. This would create a positive loop, enhancing the capacity for LT generation by these cells. Indeed, increased levels of LTB_4 were detected in pleural fluid of mice stimulated with oxidized phospholipids (33). However, previous studies indicate that lipid body formation may involve other 5-LO products, such as 5-hydroxyeicosatetraenoic acid, because LTB_4 does not directly stimulate lipid body formation in human neutrophils and a specific LTB_4 receptor antagonist showed no effect on PAF-induced lipid body formation (20).

In addition to sites for 5-LO localization, lipid bodies increase the capacity for LTB_4 synthesis in macrophage primed by PAF-like oxLDL phospholipids and then stimulated with suboptimal concentration of calcium ionophore. Accordingly, Bozza et al. (20) have shown that this increase in lipid body numbers induced by PAF correlates with increased LTB_4, and PGE_2 release by neutrophils when activated by submaximal concentrations of A23187. Conversely, agents that inhibit lipid body formation in vitro inhibit the priming response for enhanced eicosanoid release (20, 43, 44, 52). Moreover, a direct demonstration of the involvement of lipid bodies as sites of LT production is provided by intracellular immunofluorescent localization of newly formed LTC_4 within lipid bodies in chemokine-stimulated human eosinophils (43, 53).

The recent identification of azPC by Davies et al. (27) in the phospholipid fractions obtained from oxLDL together with the observations by Tontonoz et al. (29) that PPARγ agonists induce lipid droplet accumulation in leukocytes prompted us to ask whether azPC would induce lipid body formation. We found that azPC alone induced an increase in the number of lipid bodies in peritoneal macrophages 6 h after stimulation. Although PPARγ is not expressed in monocytes in the basal state, this receptor can be

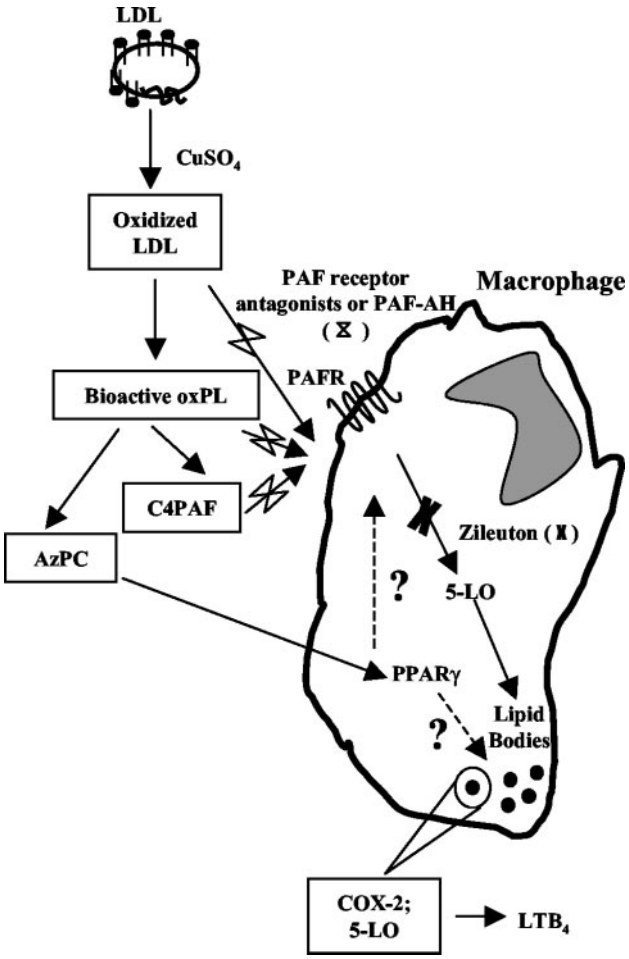

FIGURE 10. Schematic diagram of the mechanism of lipid formation induced by oxLDL phospholipids (oxPL). oxLDL induces lipid body formation in leukocytes. This oxidative modification of LDL results in the formation of PAF-like phospholipids with biological activity, including C4-PAF, with ability to induce lipid body formation in leukocytes in a 5-LO-dependent mechanism. These cytoplasmic inclusions are sites for COX-2 and 5-LO localization and LTB_4 synthesis. The oxLDL phospholipid and PPARγ agonist, azPC, also induced lipid body formation at later time points and synergized with suboptimal concentrations of oxLDL or C4-PAF.

rapidly induced (27) and accumulates during monocyte differentiation to macrophages (54), which is consistent with the effect seen with azPC in our experiments using peritoneal macrophages. Furthermore, azPC and another PPARγ agonist, BRL 49653, induced acute (i.e., 1 h poststimulation) lipid body formation when coupled with suboptimal concentrations of oxLDL or C4-PAF. This indicates a synergistic effect of azPC and BRL 49653 with oxLDL and its PAF mimetics. This result is somewhat puzzling if we consider that PPARγ is a nuclear receptor that controls expression of select genes, and so the phenotypic manifestation of PPARγ activation should only be noted at later time points. The activator-dependent differences in the time frame of lipid body formation (i.e., 1 h for oxLDL or oxLDL plus azPC and 6 h for azPC alone) may reflect differences in the intracellular pathway implicated in the formation of lipid bodies after each stimulus. For instance, lipid bodies formed 6 h after stimulation with PPARγ agonists may be the result of the activation of transcription of genes related to lipid metabolism and under control of PPAR-responsive element, although PPARγ-independent effects have already been described for some PPARγ agonists such as thiazolidinediones (55). In contrast, lipid bodies seen 1 h after stimulation with PAF mimetics might be the consequence of activation of G protein pathways coupled to the PAF receptor. These assumptions implicate the possibility that lipid bodies formed under those two different situations might have similar morphological characteristics, but different lipid and even protein composition. Importantly, the synergistic effect of PPARγ agonists with oxLDL or its PAF-like components is a specific effect, because the combination of PPARγ agonists with suboptimal concentrations of arachidonic acid, another stimulus for lipid body formation (44, 56), did not increase the number of lipid bodies in peritoneal macrophages. One interesting possibility is that PPARγ agonists are increasing PAF receptor expression levels and therefore increasing sensibility to PAF mimetics and oxLDL. In contrast, down-regulation of the PAF receptor has been shown to occur in monocytes and macrophages exposed to PPARα agonists (57). Whether activation of different PPAR subtypes has opposite effects on the expression of the PAF receptor and the biological significance of this possibility is now under investigation by different research groups.

According to our results, oxidation of LDL generated biologically active PAF-like phospholipids, including C4-PAF, with ability to induce lipid body formation. These organelles are sites for COX-2 and 5-LO localization and LTB_4 synthesis (see schematic model in Fig 10). Moreover, lipid body formation depended on 5-LO activation. The oxLDL phospholipid azPC also induced lipid body formation in association with suboptimal concentrations of oxLDL or C4-PAF. We suggest that biologically relevant lipid body formation induced by oxLDL can be attributed to synergistic cooperation between phospholipids generated during LDL particle oxidation, including C4-PAF and azPC.

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