Eosinophil Lipid Bodies: Specific, Inducible Intracellular Sites for Enhanced Eicosanoid Formation

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Summary

The specific intracellular sites at which enzymes act to generate arachidonate-derived eicosanoid mediators of inflammation are uncertain. We evaluated the formation and function of cytoplasmic lipid bodies. Lipid body formation in eosinophils was a rapidly (<1 h) inducible response which was platelet-activating factor (PAF) receptor–mediated, involved signaling through protein kinase C, and required new protein synthesis. In intact and enucleated eosinophils, the PAF-induced increases in lipid body numbers correlated with enhanced production of both lipoxygenase- and cyclooxygenase-derived eicosanoids. All principal eosinophil eicosanoid-forming enzymes, 5-lipoxygenase, leukotriene C_4 synthase, and cyclooxygenase, were immunolocalized to native as well as newly induced lipid bodies in intact and enucleated eosinophils. Thus, lipid bodies are structurally distinct, inducible, nonnuclear sites for enhanced synthesis of paracrine eicosanoid mediators of inflammation.

Leukotrienes (LT)[1], together with PGs, thromboxanes, and lipoxins, are the major constituents of a group of biologically active oxygenated fatty acids known as eicosanoids. Eicosanoids function as paracrine mediators of inflammation as well as intracellular signals. Eicosanoids play major roles in inflammatory responses and have been implicated in the pathogenesis of many inflammatory diseases, including asthma, psoriasis, rheumatoid arthritis, and inflammatory bowel disease (1, 2). The synthesis of eicosanoids is catalyzed by lipoxygenases (LOs) (for LTs, hydroxyeicosatetraenoic acids, and lipoxins) and PG endoperoxide H synthases, also known as cyclooxygenases (for PGs and thromboxanes). Although the enzymatic pathways for eicosanoid formation are well understood, the intracellular sites of action of these enzymes and the cellular sources of arachidonic acid remain less clear. Recent studies have focused on the intracellular localization of eicosanoid-forming enzymes. Cyclooxygenases (COXs) are associated with cellular membranes, including the endoplasmic reticulum and nuclear membrane (3–5). In contrast, 5-LO has been localized to the cytoplasm, the perinuclear membrane, and the euchromatin within the nucleus, according to the cell and activation state used (6–10). While translocation from cytosol to membranes may facilitate interactions of cytosolic enzymes with membrane-bound arachidonate, there is increasing evidence that specific compartmentalization of eicosanoid formation within cells may relate to the different autocrine and paracrine functions of eicosanoids (5, 11). Novel, potential sites for paracrine eicosanoid production within inflammatory cells are lipid bodies.

Lipid bodies are lipid-rich cytoplasmic inclusions which are candidates to play a major role in the formation of eicosanoid mediators during inflammation. Lipid bodies characteristically develop in vivo in cells associated with inflammation; including leukocytes from joints of patients with inflammatory arthritis (12–14), the airways of patients with acute respiratory distress syndrome (15), and casein- or lipopolysaccharide-elicited guinea pig peritoneal exudates (16). In eosinophils, increased lipid body numbers have been observed in patients with the hypereosinophilic syndrome (HES) (17, 18), in biopsies from Crohn's disease (19), and the blood of airway antigen-challenged asthmatic patients (Weller, P.F., unpublished observations). Lipid bodies are sites of esterified arachidonate localization in cells including neutrophils and eosinophils (17, 20). In human eosinophils, by electron microscopic autoradiography and biochemical analysis of purified lipid bodies, lipid bodies have been shown to incorporate [^{3}H]arachidonic acid into specific phospholipid classes (17). In addition, up-

[1]*Abbreviations used in this paper:* $cPLA_2$, cytosolic phospholipase A_2; COX, cyclooxygenase; HES, hypereosinophilic syndrome; LT, leukotriene; LO, lipoxygenase; PAF, platelet-activating factor; PKC, protein kinase C; TBS-BSA, Tris-buffered saline containing 0.1% BSA.

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stream enzymes involved in arachidonic acid release, MAP kinases, and cytosolic phospholipase A_2 ($cPLA_2$) (Yu, W., P.T. Bozza, D.M. Tzizik, J.P. Gray, J. Cassara, A.M. Dvorak, and P.F. Welter, manuscript submitted for publication) as well as COX (21–23) have been localized to lipid bodies in several types of leukocytes and other cells. Moreover, we have demonstrated recently that stimuli-elicited compartmentalization of lipids to form new lipid bodies is associated with enhanced capacity for eicosanoid generation, suggesting that the cellular responses leading to lipid body formation may be important in the formation of eicosanoid mediators of inflammation (24, 25).

In this study we have evaluated mechanisms involved in lipid body formation and function in human eosinophils. We demonstrate that platelet-activating factor (PAF) rapidly induces lipid body formation in eosinophils in a receptor-dependent fashion, with subsequent activation of protein kinase C (PKC) and protein synthesis. By means of immunocytochemistry, electron microscopic immunogold localization, and/or subcellular fractionation with Western blotting, the major eicosanoid-forming enzymes of eosinophils, 5-LO, LTC_4 synthase, and COX, are present within native and induced eosinophil lipid bodies. Furthermore, PAF-elicited lipid body formation is associated with enhanced generation of eicosanoids by both intact and enucleated eosinophils, suggesting that lipid bodies may be important inducible sites for enhanced paracrine eicosanoid mediator production during inflammation.

Materials and Methods

PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine), lyso-PAF (1-*O*-alkyl-*sn*-glyceryl-3-phosphorylcholine), chelerythrine, pertussis toxin, actinomycin D, cytochalasin B, and A23187 were from Calbiochem Novabiochem Corp. (La Jolla, CA). Cycloheximide was obtained from Sigma Chemical Co. (St. Louis, MO). 1-acyl-2-(7-octyl BODIPY™-1-pentanoyl)-*sn*-glycerol was obtained from Molecular Probes (Eugene, OR). WEB 2086 was a gift from Boehringer-Ingelheim (Ingelheim, Germany) and recombinant 5-LO was a gift of Dr. Jilly Evans, Merck Frosst (Pointe Claire-Dorval, Quebec, Canada). Antibodies used included rabbit anti–5-LO (LO32, courtesy of Dr. Jilly Evans), affinity-purified rabbit IgG anti-LTC_4 synthase (26), and affinity-purified rabbit anti-COX (Cayman Chemicals Co., Inc., Ann Arbor, MI). Specificity of anti–5-LO antiserum was confirmed by Western blotting with affinity-purified recombinant 5-LO.

Human Eosinophil Purification. Human eosinophils were purified as previously described (27). In brief, fresh human blood was obtained by venipuncture from healthy adult volunteers and collected into acidified citrate. After addition of 6% dextran 70 (McGaw, Irvine, CA), RBCs were allowed to sediment for 1 h at room temperature. The leukocyte-rich supernatant was overlaid onto an equal volume of Ficoll-Paque gradient (Pharmacia Biotech, Piscataway, NJ) and centrifuged at 400 *g* for 20 min. Granulocytes were recovered from the pellet and washed in Ca^{2+}/Mg^{2+}-free HBSS. Residual RBCs were lysed with hypotonic saline. Eosinophils (>95% pure) were negatively selected with anti-CD16 immunomagnetic beads (Miltenyi Biotec Inc., Auburn, CA) to remove neutrophils using the MACS system (Miltenyi Biotec).

Cytoplast Preparation. Cytoplasts were prepared by the method of Roos et al. (28). Briefly, eosinophils were mixed with 12.5% (wt/vol) Ficoll 70 containing 20 μM cytochalasin B and incubated for 5 min at 37°C. After incubation, eosinophils were layered over a discontinuous gradient of 16% and 25% Ficoll 70 containing 20 μM cytochalasin B which had been prewarmed to 37°C. The cells were centrifuged at 82,000 *g* for 35 min at 35°C. Cytoplasts (>90% pure) were recovered from the layer formed at the 12.5/16% and 16/25% Ficoll interfaces and washed five times in Ca^{2+}/ Mg^{2+}-free HBSS. Nuclei and granule containing karyoplasts were in the pellet.

Lipid Body Induction and Treatments. Human eosinophils (10^6 cells/ml) were incubated with varying concentrations of PAF, lyso-PAF, or vehicle at 37°C in a 5% CO_2, 95% O_2 atmosphere; after the incubation period eosinophils (10^5/slide) were cytocentrifuged (550 rpm, 5 min) onto glass slides. During inhibitor studies, eosinophils were pretreated for 1 h with varying concentrations of receptor antagonist, enzyme inhibitors, or vehicle as indicated. When PKC and protein synthesis inhibitors were used, the preincubation time was reduced to 30 min to avoid toxic effect to the cells. The cell viability, determined by trypan blue dye exclusion at the end of each experiment, was always >90%. In selected experiments, apoptosis of eosinophils was monitored by fluorescence microscopy of cells exposed to propidium iodide (10 μg/ml) and annexin V-FITC (1 μg/ml) according to the manufacturer's recommendation (Apoalert apoptosis kit; Clontech, Palo Alto, CA). Stock solutions for A23187, WEB 2086, and actinomycin D were prepared in DMSO and stored at −20°C. Aliquots were diluted in Ca^{2+}/Mg^{2+}-free HBSS to the indicated concentration immediately before use. The final DMSO concentration was always <0.1% and had no effect on lipid body numbers. Cycloheximide, chelerythrine, and pertussis toxin were diluted in Ca^{2+}/Mg^{2+}-free HBSS. Stock solutions of PAF and lyso-PAF were prepared in Ca^{2+}/Mg^{2+}-free HBSS containing 0.1% BSA.

Lipid Body Staining and Enumeration. While still moist, eosinophils on cytospun slides were fixed in 3.7% formaldehyde in Ca^{2+}/Mg^{2+}-free HBSS, pH 7.4, rinsed in 0.1 M cacodylate buffer, pH 7.4, stained in 1.5% OsO_4 (30 min), rinsed in dH_2O, immersed in 1.0% thiocarbohydrazide (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 phase-contrast microscopy with an ×100 objective lens in 50 consecutively scanned eosinophils.

LTC_4 and PGE_2 Measurement. Human eosinophils or eosinophil cytoplasts (10^6 cells/ml) were stimulated with PAF (10^{-8}–10^{-6} M) or vehicle at 37°C for 1 h for lipid body formation. After incubations, samples were taken for lipid body enumeration and eosinophils were washed in Ca^{2+}/Mg^{2+}-free HBSS. Eosinophils or cytoplasts were resuspended in 1 ml of HBSS containing Ca^{2+}/Mg^{2+} and then stimulated with A23187 (0.5 μM) for 15 min. Reactions were stopped on ice, and the samples were centrifuged at 500 *g* for 10 min at 4°C. LTC_4 and PGE_2 in the supernatants were assayed by ELISA according to the manufacturer's instructions (Cayman Chemical Co., Inc.).

Immunogold Staining. Electron microscopic preparation of samples and immunogold staining was performed as described previously (21, 29). In brief, eosinophils were fixed in suspension for 1 h at 20°C in a dilute mixture of aldehydes in sodium cacodylate buffer, washed overnight at 4°C, centrifuged through molten agar, postfixed in collidine-buffered osmium tetroxide for 2 h at 20°C, stained en bloc with uranyl acetate, dehydrated in a graded series of alcohols, infiltrated, and embedded and polymer-

ized in a propylene oxide-Epon sequence. Postembedding immunogold staining was performed in the following sequence: (*a*) freshly cut thin sections on either gold or nickel grids either unmasked in 2% sodium metaperiodate for 30 min or permeabilized on 0.1% Triton X-100 in 0.1 M Tris-buffered saline, pH 7.6, for 10 min; (*b*) three washes of 10 min each in Tris-buffered saline containing 0.1% BSA (TBS-BSA); (*c*) block with TBS-BSA containing 5% normal goat serum for 30 or 60 min; (*d*) incubation at room temperature for 60 min in a 1:10 dilution of rabbit polyclonal antiserum to 5-LO (LO32 from Merck Frosst) in TBS-BSA containing normal goat serum and 0.1% Tween 20; (*e*) three washes of 10 min each in TBS-BSA; (*f*) incubation for 120 min at room temperature in a 1:20 dilution of 20-nm gold-labeled goat anti–rabbit IgG (E.Y. Laboratories, Inc., San Mateo CA); (*g*) two washes of 10 min each in TBS-BSA; (*h*) two washes for 5 min in distilled water and dried overnight, and (*i*) staining with dilute lead citrate for 10 min. Nonimmune rabbit serum or 5-LO solid phase absorbed anti–5-LO antiserum, prepared as described (19), at the same dilution as the 5-LO antiserum, were used as controls.

Immunocytochemistry. Human eosinophils or eosinophil cytoplasts (10^6 cells/ml) were stimulated with PAF (10^{-6} M) at 37°C for 1 h for lipid body formation. In some experiments, 1 μM of the fluorescent fatty acid–containing diglyceride, 1-acyl-2-(7-octyl BODIPY™-1-pentanoyl)-*sn*-glycerol (30), was added to the incubation in order to fluorescently label lipid bodies. After the incubation, cells were washed twice in Ca^{2+}/Mg^{2+}-free HBSS, cytospun onto slides, and fixed in 3% formaldehyde at room temperature for 10 min. Fixed cells were permeabilized with 0.05% saponin/HBSS solution and then blocked with 10% normal goat serum. After washing, cytospin preparations were incubated for 1 h at room temperature with the following primary antibodies which were diluted in 0.05% saponin/HBSS solution: rabbit polyclonal serum anti–5-LO (1:150 dilution), affinity-purified rabbit anti-COX IgG (5 μg/ml), or affinity-purified rabbit anti-LTC_4 synthase IgG (5 μg/ml). Nonimmune rabbit serum or purified rabbit IgG, at the same concentration as the primary antibodies, were 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 (Vector Labs. Inc., Burlingame, CA). The immunoreactive COX, 5-LO, and LTC_4 synthase in cells were then identified by an ABC Vectastatin glucose-oxidase kit following the manufacturer's instructions (Vector Labs. Inc.). The glucose-oxidase immunostaining was visualized under light microscopy, and fluorescent lipid bodies were identified under FITC filter.

Subcellular Fractionation and Western Blotting of 5-LO. Eosinophils were disrupted and subjected to centrifugation to isolate buoyant lipid bodies and other subcellular fractions by a modification of a previous method (17). In brief, eosinophils, purified from an HES donor, were preincubated with PAF (1 μM) or vehicle for 1 h at 37°C before being washed and resuspended in 3 ml of disruption buffer (25 mM Tris-HCl, 100 mM KCl, 3.5 mM $MgCl_2$, 1 mM EDTA, 5 mM EGTA, pH 7.4) supplemented with 5 μg/ml cytochalasin B, 10 μg/ml leupeptin, 0.7 μg/ml pepstatin A, 50 μg/ml *N*α-*p*-tosyl-L-lysine chloro-methyl ketone, and 0.1 mM PMSF. After 15 min at 4°C, cells were disrupted by nitrogen cavitation at 800 psi for 10 min. The cavitate was collected dropwise and mixed with an equal volume of disruption buffer containing 1.08 M sucrose. After centrifugation at 1,500 *g* for 10 min to pellet the nuclei, the supernatants were overlaid with 1.5 ml of 0.27 M sucrose buffer, 1.5 ml of 0.135 M sucrose buffer, and 1.5 ml of Top solution (25 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, pH 7.4). After centrifugation at 150,500 *g* for 60 min, four discrete fractions were collected sequentially from top to bottom: the buoyant lipid body fraction (2.5 ml), the mid-zone between lipid bodies and cytosol (2.5 ml), the cytosol (5 ml), and the microsomal pellet. The lipid body and mid-zone fractions contained 2.5% and <6% of cytosolic LDH activity, respectively, and essentially no microsomal sulfatase C activity, indicating that these fractions were essentially free of cytosolic and microsomal contaminants. The microsomal and nuclear pellets were washed and resuspended in Top solution by sonication.

Proteins from subcellular fractions were concentrated by precipitation with 10% TCA overnight at 4°C. The precipitates were then washed twice with cold acetone. Protein concentrations were normalized in each fraction after micro BCA assay (Pierce Chemical Co., Rockford, IL). Samples (15 μg) of each fraction were then prepared in reducing and denaturing conditions and separated by electrophoresis in 10% SDS-PAGE gels. After transfer onto nitrocellulose membranes, nonspecific binding sites were blocked with 5% nonfat dry milk (Bio-Rad, Hercules, CA) in Tris-buffered saline–Tween (TBST; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) at room temperature for 1 h. The membranes were then probed with anti-5-LO serum (1:5,000 dilution) followed by horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution) in TBST with 2% nonfat dry milk. Detection of antigen–antibody complexes was performed by Supersignal chemiluminescence (Pierce Chemical Co.).

Statistical Analysis. Results were expressed as mean ± SEM and were analyzed statistically by means of ANOVA followed by the Newman-Keuls Student test with the level of significance set at $P < 0.05$. Correlation coefficients were determined by linear regression, and correlation analysis was performed by Fisher's r to z transformation with the level of significance set at $P < 0.05$.

Results

Mechanisms of PAF-induced Lipid Body Formation in Eosinophils. Lipid bodies, although small in number, are normal constituents of leukocytes, including eosinophils. Increased lipid body formation occurs in vivo and can be induced in vitro. In eosinophils obtained from normal donors, within 1 h PAF stimulated a dose-dependent induction of lipid body formation in vitro (Fig. 1 *A*). In response to 1 μM PAF, lipid body numbers persisted at similar numbers at 1, 4, and 8 h (14.1 ± 0.5, 15.9 ± 0.7, and 9.5 ± 0.5 lipid bodies ± SEM/eosinophil, respectively) with no evidence of enhanced cell death or apoptosis (<7% apoptotic eosinophils at 8 h with and without PAF stimulation). Several findings indicated that the actions of PAF were receptor mediated. First, lyso-PAF failed to induce lipid body formation (Fig. 1 *A*). Second, the PAF-receptor antagonist WEB 2086 dose-dependently inhibited PAF-induced lipid body formation (Fig. 1 *B*). Moreover, earlier treatment of cells with pertussis toxin (100 ng/ml, 1 h before PAF) to block PAF-receptor G-protein–mediated signals completely prevented PAF-induced lipid body formation in human eosinophils (Fig. 1 *B*).

Additional intracellular signaling pathways involved in PAF-induced lipid body formation in human eosinophils were studied. As shown in Table 1, staurosporine (1–10 μM) and H-7 (25–50 μM) significantly inhibited the in-

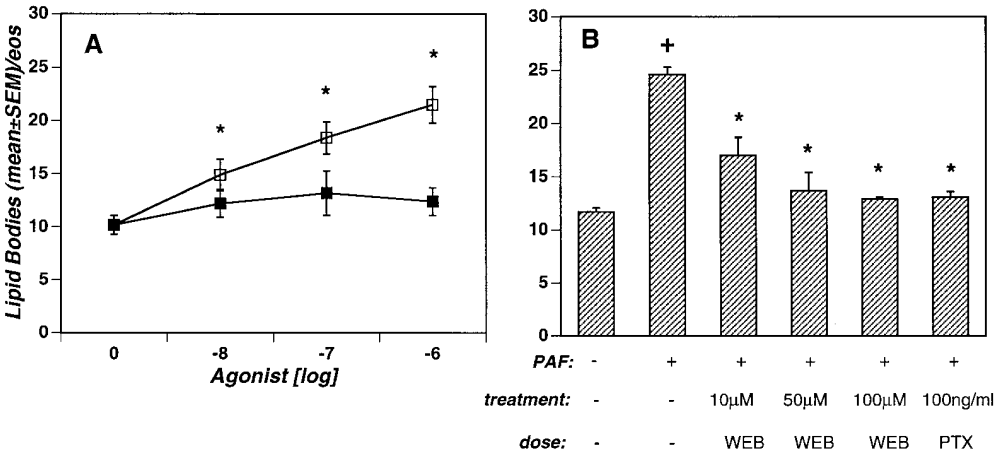

Figure 1. PAF receptor–mediated induction of lipid body formation in eosinophils. (*A*) Human eosinophils, at a concentration of 10^6 cells/ml, were treated with PAF (10^{-8}–10^{-6}M) (*white squares*) or lyso-PAF (10^{-8}–10^{-6} M) (*black squares*) for 1 h at 37°C. (*B*) Human eosinophils at a concentration of 10^6 cells/ml were pretreated with WEB 2086 (10–100 μM), pertussis toxin (100 ng/ml), or vehicle for 1 h at 37°C. The cells were then treated with PAF (1 μM) or vehicle for 1 h at 37°C. Lipid bodies were enumerated using light microscopy after osmium staining. Each point represents the mean ± SEM of lipid bodies on 50 consecutive eosinophils from three to five independent assays using different donors. *Statistically significant difference between PAF and the vehicle. +Statistically significant differences caused by pretreatment with WEB 2086 or pertussis toxin.

creases in lipid body numbers induced by PAF in human eosinophils, thus suggesting a role for PKC in this process. The involvement of PKC in PAF-induced lipid body formation was confirmed by the ability of two selective PKC inhibitors, chelerythrine (1–10 μM) and calphostin C (0.5–1 μM), to almost completely block the induction of lipid bodies (Table 1). Pretreatment with inhibitors of mRNA and protein synthesis, actinomycin D (1 μM) and cycloheximide (1 μM), respectively, significantly inhibited PAF-induced lipid body formation (Table 1), suggesting that eosinophil lipid body induction depends on gene expression and de novo protein synthesis.

Immunolocalization of Eicosanoid-forming Enzymes to Eosinophil Lipid Bodies. Since human eosinophils form LTC_4 as

Table 1. *Effects of PKC and Protein Synthesis Inhibitors on PAF-induced Lipid Body Formation in Eosinophils*

Treatment	Dose	Lipid bodies (mean ± SEM)/eosinophils	Percentage of inhibition
	μM		
Staurosporine	0	21.8 ± 2.1	–
	1	14.9 ± 1.4*	58
	10	12.8 ± 0.9*	83
H-7	0	21.8 ± 2.1	–
	25	14.9 ± 1.0*	58
	50	11.8 ± 1.1	75
Calphostin	0	21.8 ± 2.1	–
	0.5	13.1 ± 1.3*	65
	1	11.6 ± 0.9*	85
Chelerythrine	0	21.8 ± 2.1	–
	1	15.7 ± 1.6*	51
	10	13.0 ± 1.3*	73
Actinomycin	0	11.8 ± 0.8	–
	1	7.9 ± 0.3*	60
Cycloheximide	0	11.8 ± 0.8	–
	1	7.4 ± 0.2*	68

Eosinophils (10^6/ml) were pretreated with PKC or protein synthesis inhibitors for 30 min and then stimulated with PAF (1 μM) or vehicle for 1 h. Results are mean ± SEM from three to five experiments with different donors. Percentage of inhibition was calculated using the following formula: percentage of inhibition = 100 − (net No. of PAF-induced lipid bodies in treatment group × 100) / (net No. PAF-induced lipid bodies with PAF alone). Basal mean numbers of lipid bodies ranged from 5.3 to 9.8/eosinophil with different donors and have been subtracted to determine the net induced lipid body numbers. *Statistically significant differences between agonists alone and treated groups.

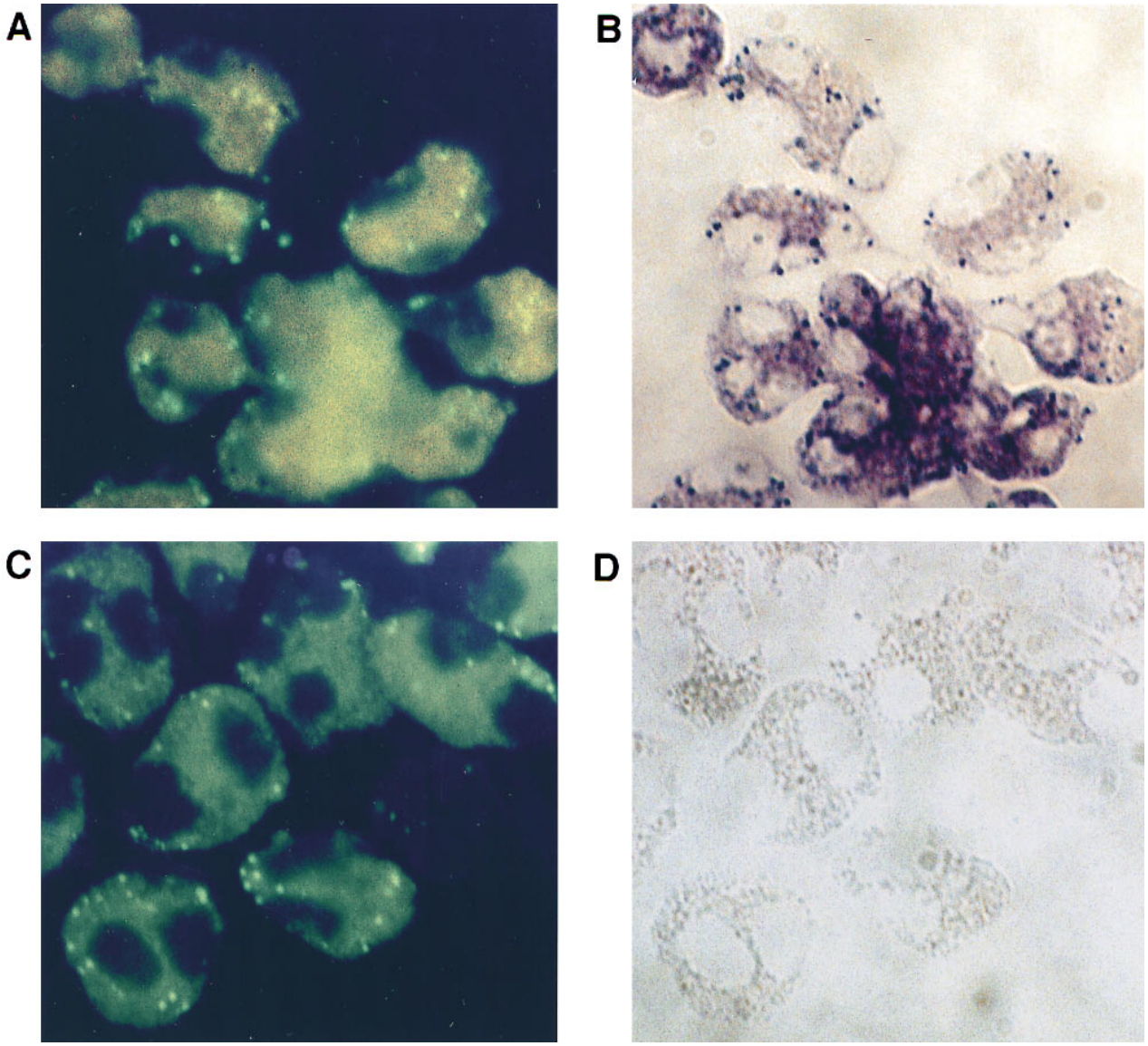

Figure 2. Immunolocalization of 5-LO to lipid bodies of human eosinophils. (*A* and *C*) Human eosinophils from normal volunteers were incubated with PAF (1 μM, for 1 h) to induce lipid body formation, and lipid bodies were visualized by endogenous labeling with the fluorescent fatty acid–containing diglyceride, 1-acyl-2-(7-octyl BODIPY™-1-pentanoyl)-*sn*-glycerol (1 μM, for 1 h). Fluorescent fatty acid–labeled lipid bodies were visualized as green punctate intracytoplasmic inclusions under FITC excitation. (*B*) With specific anti–5-LO rabbit antiserum and glucose-oxidase immunocytochemistry, there was 5-LO staining diffusely in the cytoplasm as well as at punctate lipid bodies (which matched those in *A*). (*D*) With control nonimmune rabbit serum there was no lipid body or cytoplasmic staining.

their principal 5-LO pathway product and form lesser amounts of COX-derived eicosanoids, we evaluated whether the key eicosanoid-forming enzymes were localized at eosinophil lipid bodies, both in naturally formed lipid bodies in eosinophils from HES patients and in lipid bodies induced to form in vitro. The compartmentalization of 5-LO, LTC_4 synthase, and COX to lipid bodies was analyzed by immunocytochemistry using conditions of cell fixation and permeabilization that prevent dissolution of lipid bodies. Human eosinophils were stimulated with PAF (1 μM, for 1 h) to induce lipid body formation, and lipid bodies were visualized by endogenous labeling with the fluorescent fatty acid–containing diglyceride, 1-acyl-2-(7-octyl BODIPY™-1-pentanoyl)-*sn*-glycerol (1 μM) (30). Fluorescent fatty acid-labeled lipid bodies were visualized as green punctate intracytoplasmic inclusions (Fig. 2, *A* and *C*). Eosinophils stained with rabbit anti–5-LO antiserum, in addition to perinuclear membrane and cytosolic staining, showed focal punctate cytoplasmic staining that coincidently matched with fluorescent fatty acid–labeled lipid bodies (Fig. 2 *B*). There was no immunoreactivity when control, normal rabbit serum was used instead of the 5-LO antiserum (Fig. 2 *D*), although fluorescent fatty acid-labeled lipid bodies were strongly visualized (Fig. 2 *C*). Analogously, eosinophils

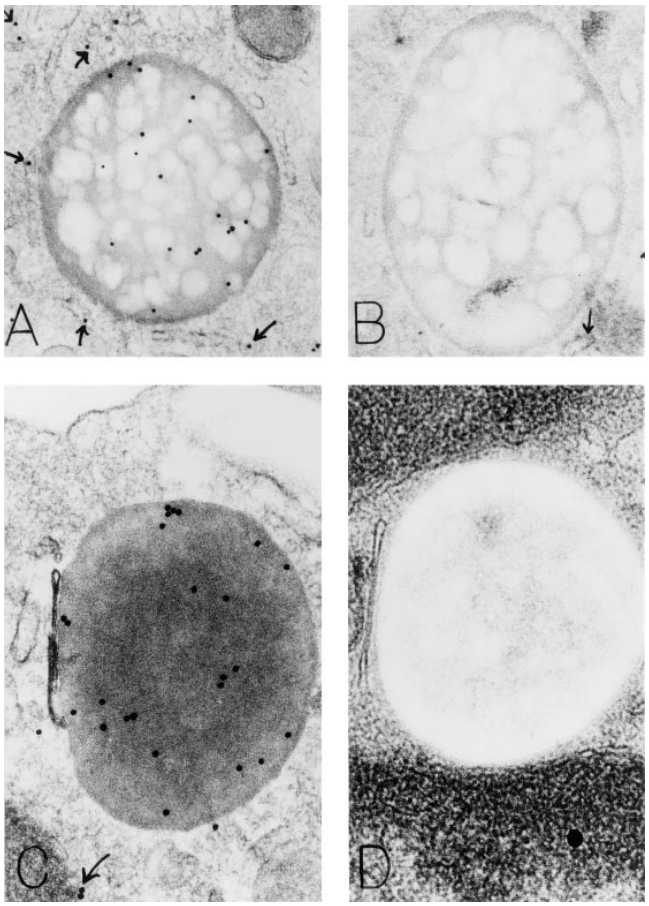

Figure 3. Immunogold localization of 5-LO in freshly isolated, unstimulated eosinophils from an HES donor shows gold label indicating 5-LO in lipid bodies (*A* and *C*), which was absent from lipid bodies stained with control solid phase 5-LO–absorbed anti–5-LO antisera (*B*) or nonimmune control serum (*D*). In *A*, cytoplasmic tubules and vesicles of smooth endoplasmic reticulum adjacent to the lipid body also have gold particles associated with them (*arrows*) which are absent in the 5-LO absorption control (*arrow*, *B*). The lipid bodies in *A* and *B* are not homogeneously dense and reveal numerous small, round areas which are electron lucent and nonmembrane bound. In contrast, lipid bodies in *C* and *D* appear more homogeneous in their content. In *C*, several gold particles reside in the perinuclear cistern (*arrow*); the nonimmune antibody control has one gold particle attached to electron-dense chromatin in the nucleus. *A*, ×29,640; *B*, ×36,480; *C*, ×44,080, *D*, ×45,600.

from eosinophilic donors contained lipid bodies exhibiting specific anti–5-LO staining by glucose oxidase immunocytochemistry (not shown). The immunolocalization of 5-LO within lipid bodies was confirmed by immunogold electron microscopy. Freshly isolated, unstimulated eosinophils from an HES donor, examined by postembedding immunogold with anti–5-LO antiserum, contained lipid bodies which were extensively labeled with 20 nm gold particles (Fig. 3, *A* and *C*). Substitution of the specific anti–5-LO antibody with either control 5-LO absorbed anti–5-LO antisera (Fig. 3 *B*) or nonimmune serum (Fig. 3 *D*) yielded no staining of lipid bodies.

In human eosinophils, LTC_4 is the predominant 5-LO product (31). Recently, human LTC_4 synthase, the enzyme responsible for conjugating glutathione to form LTC_4, has been purified and cloned (26, 32, 33). Using an affinity-purified rabbit anti-LTC_4 synthase IgG (26), this LTC_4-forming enzyme was localized directly to lipid bodies. Eosinophils induced to form lipid bodies and stained with anti-LTC_4 synthase IgG showed focal cytoplasmic staining (Fig. 4 *B*) which matched the fluorescent fatty acid–labeled lipid bodies (Fig. 4 *A*). Likewise, eosinophils from an HES donor showed LTC_4 synthase localization at native lipid bodies (not shown). The enzyme COX was also shown to colocalize to PAF-induced lipid bodies in eosinophils (Fig. 4, *C* and *D*), in agreement with previous immunogold electron microscopic localization of COX to lipid bodies in freshly isolated eosinophils (21). Specificity of anti-COX and anti-LTC_4 staining was demonstrated by the absence of immunoreactivity when nonimmune rabbit IgG was used instead of the specific primary antibody, under conditions where fluorescent fatty acid–labeled lipid bodies were present (Fig. 3, *E* and *F*). Thus, 5-LO, LTC_4 synthase and COX were present at lipid bodies within 1 h of their induction as well as in naturally formed lipid bodies in eosinophils from eosinophilic donors.

Involvement of Lipid Bodies in Enhanced Generation of Eicosanoids by PAF-stimulated Eosinophils. Because eosinophil lipid bodies are sites of intracellular localization of eicosanoid-forming enzymes (Figs. 2–4) and also stores of eicosanoid-precursor arachidonic acid (17), we analyzed whether PAF-induced increases in lipid body numbers in eosinophils would correlate with increased LTC_4 and PGE_2 production by human eosinophils. After eosinophils were incubated with various concentrations of PAF for 1 h, lipid bodies were enumerated and replicate eosinophils were stimulated with A23187 (0.5 μM). As shown in Fig. 5, PAF dose-dependently induced concordant increases in both lipid body numbers and priming for enhanced LTC_4 (Fig. 5 *A*) and PGE_2 (Fig. 5 *B*) generation. Increases in lipid body numbers correlated with enhanced production of each eicosanoid ($r = 0.97$, $P < 0.03$, $r = 0.99$, $P < 0.001$ for LTC_4 and PGE_2, respectively). If the hypothesis that lipid bodies have roles in enhanced eicosanoid formation is correct, then inhibition of lipid body formation should result in suppressed eicosanoid formation. To test this hypothesis, we used the inhibitors of protein synthesis, actinomycin D and cycloheximide, because these compounds were effective in blocking lipid body formation induced by PAF (Table 1). Pretreatment of eosinophils with actinomycin D (1 μM) or cycloheximide (1 μM) inhibited not only PAF-induced lipid body formation, but also priming for LTC_4 (by 70 and 40% for actinomycin and cycloheximide, respectively) and PGE_2 (by 40 and 54% for actinomycin and cycloheximide, respectively) release by eosinophils, under conditions where these inhibitors failed to inhibit calcium ionophore–induced LTC_4 and PGE_2 in cells not prestimulated with PAF (not shown).

Lipid Body Formation, Eicosanoid-forming Enzyme Localization, and Priming for Enhanced Generation of Eicosanoids by PAF-stimulated Enucleated Eosinophils. Recently, several studies have focused on the role of the perinuclear environment

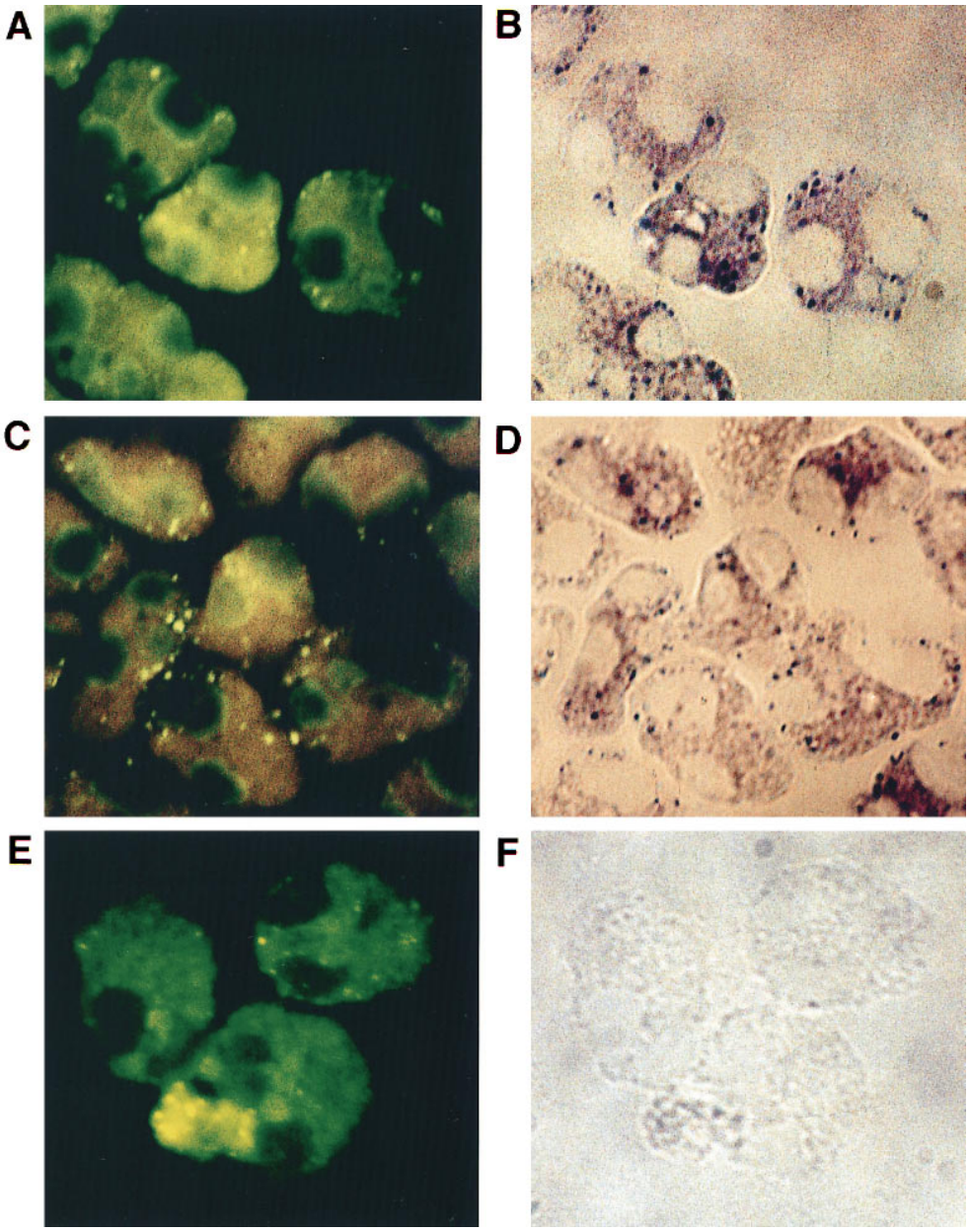

Figure 4. Immunolocalization of LTC_4 synthase and COX to lipid bodies of human eosinophils. Human eosinophils from normal volunteers were incubated with PAF (1 μM, for 1 h) to induce lipid body formation, and lipid bodies were visualized by endogenous labeling with the fluorescent fatty acid–containing diglyceride, 1-acyl-2-(7-octyl BODIPY™-1-pentanoyl)-*sn*-glycerol (1 μM, for 1 h). Fluorescent fatty acid–labeled lipid bodies were visualized as green punctate intracytoplasmic inclusions under FITC excitation (*A*, *C*, and *E*). LTC_4 synthase (*B*) and COX (*D*) were localized to lipid bodies using anti-LTC_4 and anti-COX, respectively, affinity-purified rabbit IgG, and glucose-oxidase immunocytochemistry. Rabbit IgG was used as a control (*F*). Lipid bodies exhibit dark punctate staining in *B* and *D* which matches fluorescent lipids in *A* and *C*, respectively. (Some fluorescent lipid bodies are out of the plane of focus and not visible.)

as a site of eicosanoid-forming enzyme localization and eicosanoid generation (5, 11). In order to evaluate whether lipid bodies may function as nuclear-independent alternative sites of eicosanoid production, we generated enucleated eosinophils, cytoplasts, by modifying the technique of Roos et al. (28) for cytoplast formation in neutrophils. A typical eosinophil cytoplast preparation, devoid of nuclei and specific granules, is shown in Fig. 6. After freshly isolated eosinophils (Fig. 6 *A*) were layered over a discontinuous gradient of Ficoll 70 containing 20 μM cytochalasin B and subjected to centrifugation, cytoplasts (>90% pure, Fig. 6 *B*) were formed by the fusion of the plasma membrane around the cytoplasmic part of the cell and recovered from the layers formed at the 12.5/16% and 16/25% Ficoll interfaces. The nuclei and granules, also surrounded by plasma membrane, migrated to the bottom of the gradient (Fig. 6 *C*) and constituted karyoplasts.

Similar to the reaction observed with intact eosinophils, PAF induced a dose-dependent formation of lipid bodies in eosinophil cytoplasts (Fig. 7). WEB 2086 (10 μM, 1 h before PAF) blocked this PAF-induced lipid body formation by 84%, indicating that PAF-induced lipid body formation was receptor-mediated in cytoplasts as in intact eosinophils. Moreover, PAF-induced lipid body formation in nuclei-free cytoplast strongly correlated with increased LTC_4 (Fig. 7 *A*) and PGE_2 (Fig. 7 *B*) production after submaximal stimulation with A23187 (0.5 μM). The karyoplast fraction of eosinophils was also able to generate increased amounts of LTC_4, but not PGE_2, upon stimulation (data not shown).

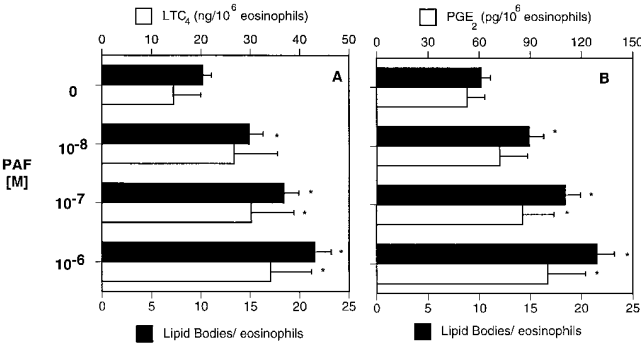

Figure 5. PAF-induced both lipid body formation and priming for LTC_4 (*A*) and PGE_2 (*B*) production by human eosinophils. Eosinophils (10^6/ml) were stimulated for 1 h at 37°C with concentrations of PAF or vehicle alone. Data are means ± SEM of eicosanoids formed by eosinophils and lipid body numbers in eosinophils from six to eight independent experiments. Increasing numbers of lipid bodies correlated with increased production of each eicosanoid ($r \geq 0.97$, $P < 0.03$ for both, Fisher's r to z transformation). *Statistically significant differences ($P < 0.05$, paired *t* test) between PAF and the vehicle. LTC_4 and PGE_2 in supernatants were measured by ELISA after incubation with 0.5 μM A23187 for 15 min.

The presence of 5-LO, LTC_4 synthase, and COX within eosinophil cytoplasts was analyzed by glucose oxidase immunocytochemical staining after stimulation of eosinophil cytoplasts with PAF (1 μM, for 1 h) to induce lipid body formation. Cytoplasts, stained with anti–5-LO polyclonal antiserum (Fig. 6 *D*), affinity-purified rabbit anti-LTC_4 synthase IgG (Fig. 6 *E*) or affinity-purified rabbit anti-COX IgG (Fig. 6 *F*), showed the punctate cytoplasmic immunochemical staining indicative of lipid body localization. The specificities of immunochemical stainings were demonstrated by the absence of immunoreactivity when nonimmune rabbit IgG (Fig. 5 *G*) or normal rabbit serum (Fig. 5 *H*) were used instead of specific antibody.

Subcellular Fractionation and 5-LO Localization at Lipid Bodies. To confirm the localization of 5-LO to lipid bodies, subcellular fractions, including buoyant lipid bodies, cytosol, microsomes, and nuclei, were isolated from disrupted eosinophils. Immunoblotting with anti-5-LO rabbit sera showed that 5-LO was dominantly in the cytosol of eosinophils not stimulated with PAF, whereas after PAF stimulation 5-LO was also strongly present in lipid bodies (Fig. 8). Little 5-LO was present in the microsomal and nuclear fractions. In addition to the dominant 78-kD band of 5-LO, lower molecular mass bands of lesser intensity were noted in lipid body fractions. Since such bands were also present in the recombinant 5-LO standard, they may represent breakdown products of 5-LO. These results corroborate the immunolocalization observed in intact eosinophils, providing evidence of the specificity of the anti–5-LO antisera and demonstrating the lipid body localization of 5-LO in PAF-stimulated eosinophils.

Discussion

In this study we report a series of complementary observations in human eosinophils which provide evidence for novel roles of lipid bodies as specific inducible sites of paracrine eicosanoid mediator formation. First, lipid bodies, prominent in vivo in eosinophils and other leukocytes associated with inflammatory reactions, can be induced to rap-

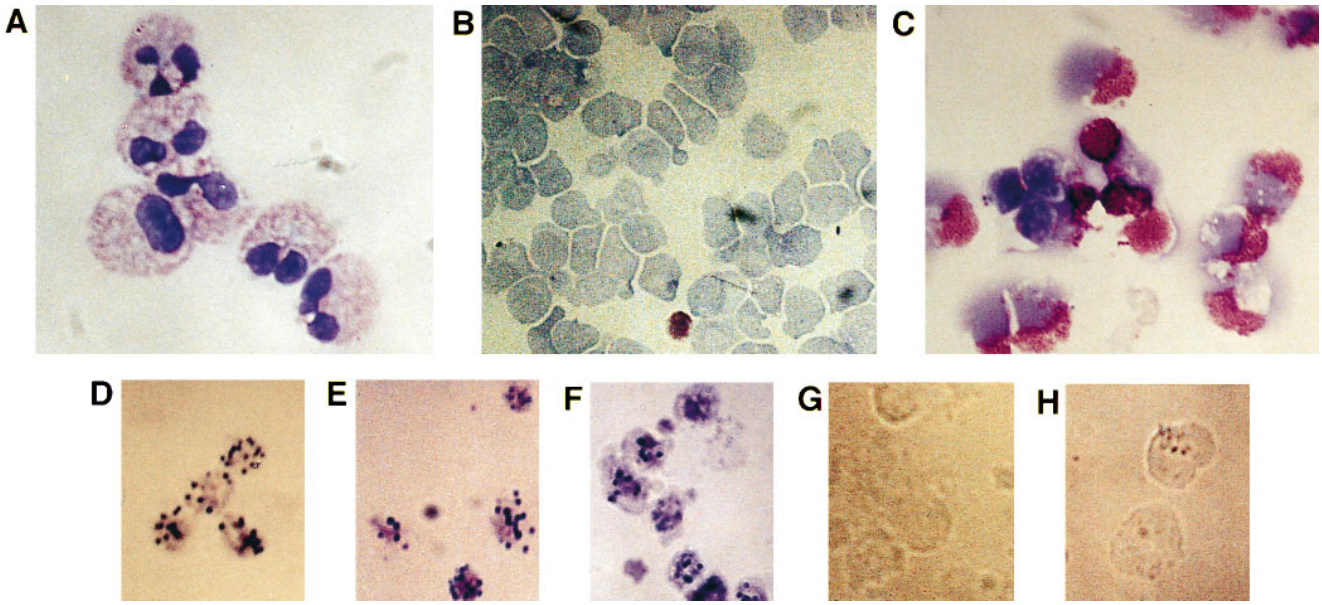

Figure 6. Eosinophil cytoplasts and immunolocalization of eicosanoid-forming enzymes. Freshly isolated eosinophils (*A*), eosinophil cytoplasts (*B*), and karyoplasts (*C*) were fixed in methanol and stained with Diff-Quik (Baxter Healthcare Corp., Miami, FL). Eosinophil cytoplasts were incubated with PAF (1 μM, for 1 h) to induce lipid body formation. 5-LO (*D*), LTC_4 synthase (*E*), and COX (*F*) were localized at cytoplast lipid bodies using specific anti–5-LO rabbit antiserum, affinity-purified rabbit IgG anti-LTC_4 synthase, or anti-COX, respectively, and glucose-oxidase immunocytochemistry. Substitution of the primary antibody by nonimmune rabbit serum (*G*) or rabbit IgG (*H*) were controls.

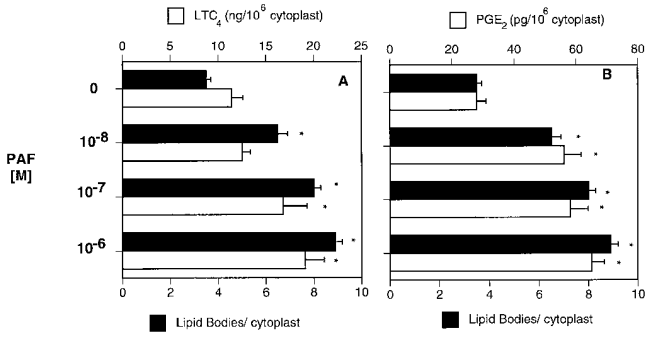

Figure 7. Dose-dependent effect of PAF (10^{-8}–10^{-6}M) on both lipid body formation and priming for LTC_4 (*A*) and PGE_2 (*B*) production by eosinophil cytoplasts. Cytoplasts (10^6/ml) were stimulated for 1 h at 37°C with PAF or vehicle alone. Data are means ± SEM of eicosanoids formed by cytoplasts and lipid body numbers in cytoplasts from three to four independent assays. *Significantly > value without PAF stimulation ($P < 0.05$). LTC_4 and PGE_2 in supernatants were measured by ELISA after incubation with 0.5 μM A23187 for 15 min.

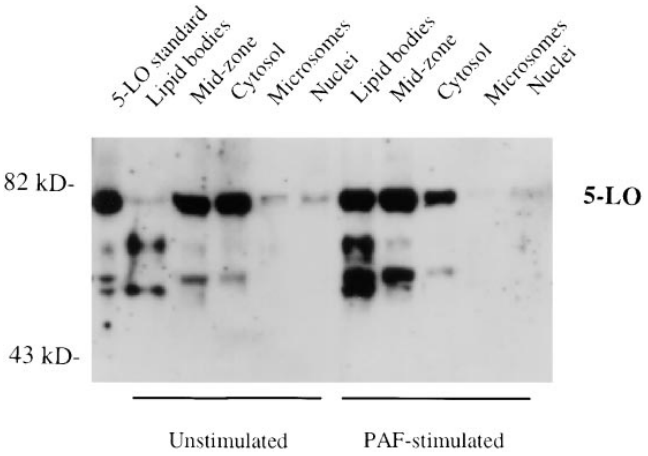

Figure 8. Immunoblotting of 5-LO in lipid bodies and other subcellular fractions of unstimulated and PAF-stimulated eosinophils. Lipid bodies and other subcellular fractions were isolated from unstimulated and PAF-stimulated (1 μM, 1 h) eosinophils as described in Materials and Methods. Proteins (15 μg) from subcellular fractions were electrophoresed on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and immunoblotted with anti–5-LO serum. Purified recombinant 5-LO protein was used as a standard for the Western blot. The anti–5-LO serum recognized a 78-kD protein, which comigrated with the purified recombinant 5-LO standard, in subcellular fractions, prominently including the cytosol and buoyant lipid bodies from PAF-stimulated eosinophils.

idly form in eosinophils. Second, key eicosanoid-forming enzymes are localized directly at eosinophil lipid bodies, both those induced experimentally to form within 1 h and those naturally formed in cells from HES donors. Third, induction of lipid body formation correlates with enhanced generation of LTC_4 and PGE_2. Fourth, even in anucleate eosinophil cytoplasts, lipid bodies are inducible, correlate with enhanced eicosanoid formation, and are discrete sites of eicosanoid-forming enzyme localization. Together, these results point to roles for lipid bodies as distinct, inducible extranuclear domains involved in the generation of eicosanoid mediators of inflammation.

If lipid bodies are to participate in the stimulated generation of eicosanoid paracrine mediators, normal leukocytes with few lipid bodies should be able to rapidly form increased numbers of these cytoplasmic inclusions. In human eosinophils, lipid body formation was a tightly controlled cellular response, which developed rapidly (within 1 h) after in vitro stimulation with PAF. PAF-induced, newly formed eosinophil lipid bodies were morphologically identical to native lipid bodies, and although PAF is a lipid, mechanisms other than the simple incorporation of exogenous lipids are involved in lipid body formation. First, the PAF precursor and metabolite, lyso-PAF, which shares the lipid structure of PAF but has no receptor agonist activity (for review see reference 34), lacked the capacity to induce lipid body formation. In addition, PAF-stimulated eosinophil lipid body formation was dose dependent (10^{-8}–10^{-6} M), with significant lipid body induction at submicromolar PAF concentrations, consistent with a receptor-dependent process. In agreement, PAF-induced lipid body formation was dose-dependently inhibited by the PAF receptor antagonist WEB 2086. Moreover, G protein-coupled PAF receptor signaling is pertussis toxin sensitive (35), and pertussis toxin significantly inhibited PAF-induced lipid body formation. In addition to PAF receptor–initiated signaling, additional downstream intracellular signaling pathways were involved in eosinophil lipid body formation. A role for PKC mediation of lipid body formation has been indicated in studies using neutrophils (25, 36). In eosinophils, the abilities of staurosporine, H-7, and two highly selective PKC inhibitors (chelerythrine, which interacts with the catalytic domain, and calphostin C, which acts on the regulatory domain of PKC) to inhibit lipid body induction are consistent with involvement of PKC in PAF-induced lipid body formation. Further, inhibitors of transcription (actinomycin D) and translation (cycloheximide) significantly inhibited lipid body formation induced by PAF, thus indicating that the stimulated induction of lipid bodies depends on new protein synthesis and suggesting that early response genes are activated during the process of lipid body formation.

The definition that eosinophil lipid bodies can be rapidly induced by PAF-initiated signaling is in accord with studies in neutrophils which also have demonstrated that specific signaling pathways lead to rapid lipid body induction (24, 25). However, these findings do not define how lipid bodies may participate in enhanced eicosanoid synthesis. If lipid bodies are to have roles in eicosanoid mediator formation, then the arachidonic acid present in those lipid-rich structures must be liberated by phospholipases. Consistent with regulated release of arachidonate occurring directly at lipid bodies, we have recently demonstrated the cocompartmentalization of MAP kinases and $cPLA_2$, the upstream enzymes involved in arachidonic acid liberation, within lipid bodies in U937 cells (Yu, W., P.T. Bozza, D.M. Tzizik, J.P. Gray, J. Cassara, A.M. Dvorak, and P.T. Weller, manuscript submitted for publication). Similarly, $cPLA_2$

can be demonstrated by immunocytochemistry at eosinophil lipid bodies (not shown).

If arachidonate is liberated intracellularly at lipid bodies, then either arachidonate must be translocated to sites of eicosanoid synthesis or eicosanoid formation must occur directly at lipid bodies. Previous studies on the intracellular localization of 5-LO have shown that 5-LO localization is cell type specific and may vary according to the activation state of the cell (6–10). 5-LO has been localized within the nuclear environment (perinuclear membrane and euchromatin) of some cell types, including alveolar macrophages and basophilic leukemia cells (9, 10). Using cell fractionation with immunoblotting, 5-LO was found to be predominantly cytosolic in human neutrophils (9) and resting peritoneal macrophages (37), although after activation 5-LO could also be found in the nuclear membranes of these cells. Stimulated leukocytes metabolize arachidonic acid via the 5-LO pathway to form LTA_4, which can degrade nonenzymatically to form 6-*trans* isomers of LTB_4 or can be enzymatically converted to LTB_4 or LTC_4 (for review see reference 1). Upon stimulation, human eosinophils preferentially produce LTC_4 as their 5-LO pathway product (31). LTC_4 synthase is the terminal LTC_4-forming enzyme and is present selectively in eosinophils, basophils, and mast cells (31, 38, 39). In human eosinophils, the coupling of LTA_4 synthesis with LTC_4 synthesis is highly efficient since the 6-*trans* isomers of LTB_4 do not form in ionophore-stimulated eosinophils, as they do in neutrophils (31). Human eosinophils also generate COX-derived eicosanoids (40), albeit in lesser quantities than LTC_4. Although COX localization is undefined in eosinophils, in other cells COX isoforms have been localized to the endoplasmic reticulum and the perinuclear membrane (3–5). Thus, in human eosinophils the key eicosanoid-forming enzymes are 5-LO, LTC_4 synthase (potentially coupled in some fashion with 5-LO to form LTC_4), and COX.

By means of immunocytochemistry and ultrastructural postembedding immunogold, we have now shown that all three key eicosanoid-forming enzymes in human eosinophils, 5-LO, LTC_4 synthase, and COX, are localized directly at lipid bodies in eosinophils. The three enzymes are present both in naturally formed eosinophil lipid bodies and, of pertinence to the capacity of lipid bodies to participate in enhanced eicosanoid mediator formation, in PAF-induced lipid bodies which are formed rapidly (within 1 h) in intact and anucleate human eosinophils. In intact eosinophils, anti–5-LO staining was present in the perinuclear and cytosolic regions, consistent with previous observations in other cells (9, 10), and was specifically found at lipid bodies, identified by their incorporation of fluorescent fatty acids (Fig. 2, *A* and *B*). Ultrastructural immunogold localization of 5-LO in naturally formed lipid bodies in eosinophils from an HES donor corroborated this localization and further suggested that 5-LO was distributed within the lipid bodies and not principally at their periphery (Fig. 3). Similar to that observed for 5-LO, LTC_4 synthase was shown to colocalize at lipid bodies (Fig. 4, *C* and *D*). Immunocytochemistry of PAF-stimulated eosinophils using an anti-COX polyclonal antibody demonstrated diffuse perinuclear and cytoplasmic staining, consistent with previous studies which reported perinuclear and endoplasmic reticulum localization of COX (3–5), as well as distinct punctate anti-COX staining which precisely colocalized with fluorescent fatty acid–labeled lipid bodies (Fig. 4, *C* and *D*). In agreement, COX has been previously localized by immunogold electron microscopy within naturally formed lipid bodies in eosinophils and other cell types (21–23, 41). Leukocytes can express two isoforms of COX: COX-1 (the constitutive enzyme), and COX-2 (an inducible form in most cells). Since the anti-COX antibody used in this study does not discriminate between the two COX isoforms, further studies are necessary to establish the isoform present in PAF-induced lipid bodies. The compartmentalization of three key eicosanoid-forming enzymes at lipid body domains in eosinophils (Figs. 2–4, 6), even within an hour of their induced formation, together with esterified arachidonate substrate (17, 20) and $cPLA_2$, provides in one inducible locale an efficient topographic means to regulate arachidonate release and directly couple it with the requisite eicosanoid-forming enzymes.

Stimuli known to prime leukocytes for enhanced eicosanoid generation, including PKC activators, arachidonate, and PAF (24, 25, 42–44), are also active in inducing lipid body formation (24, 25, 36). Consistent with roles for lipid bodies in enhanced formation and release of eicosanoids, we demonstrated significant correlations between levels of PAF-induced lipid body formation and amounts of enhanced LO- and COX-derived eicosanoids generated by human eosinophils (Fig. 5). Analogous enhancement of eicosanoid production by eosinophils has been observed after lipid body induction by *cis*-fatty acids (24). Conversely, agents which inhibited lipid body formation also resulted in inhibited priming for eicosanoid production. Pretreatment of eosinophils with the protein synthesis inhibitors, actinomycin D or cycloheximide, inhibited not only PAF-induced lipid body formation, but also priming for increased LTC_4 and PGE_2 release by eosinophils. To ascertain that PAF induction of lipid body formation and priming for enhanced eicosanoid formation was truly independent of any nuclear pools of eicosanoid-forming enzymes or lipids, eosinophil enucleated cytoplasts were prepared. As observed with intact eosinophils, PAF induced dose-dependent increases in the number of lipid bodies in eosinophil cytoplasts. Likewise, PAF-induced lipid body formation in nuclei-free cytoplasts strongly correlated with increased LTC_4 and PGE_2 production after submaximal stimulation with A23187 (Fig. 7), and PAF-induced eosinophil cytoplast lipid bodies were sites of 5-LO, LTC_4 synthase, and COX localization (Fig. 6). Further evidence for the localization of 5-LO at lipid bodies was obtained by immunoblotting of isolated eosinophil subcellular fractions. After PAF stimulation of lipid body formation, 5-LO was strongly present in isolated lipid bodies (Fig. 8). In both unstimulated and 5-LO–stimulated eosinophils, 5-LO was abundant in the cytosol but not in the microsomal and

nuclear fractions. Thus, lipid bodies are nuclear-independent sites at which enhanced formation of eicosanoids may occur.

Our findings indicate that lipid bodies are inducible, cytoplasmic sites for eicosanoid-forming enzyme localization and eicosanoid production. Together with recent findings indicating that the nucleus and perinuclear membrane may also function as important sites for eicosanoid metabolism (5, 11), our findings of a distinct role for lipid bodies as extranuclear sites for eicosanoid formation may be indicative of differential intracellular compartmentalization of eicosanoid synthesis related to the autocrine or paracrine activities of the eicosanoids. Eicosanoids formed within or around the nucleus may function as autocrine regulators of transcription or other processes. In contrast, lipid bodies, as rapidly inducible structures which provide sources of arachidonate at sites for regulated arachidonate release coupled intimately with eicosanoid-forming enzymes, are likely to have specific roles in the generation of eicosanoids with paracrine mediator activities. The prominence of lipid bodies in leukocytes in vivo in association with a variety of inflammatory pathological conditions (12–15, 17–19), many of which are known to have enhanced generation of arachidonic acid products, such as LTs and PGs, would be compatible with this role for lipid bodies. In conclusion, our results indicate that lipid bodies are rapidly inducible, specialized cytoplasmic domains for eicosanoid-forming enzyme localization which may have specific roles in enhanced paracrine eicosanoid mediator formation during inflammatory processes.

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