

CUTTING EDGE

Cutting Edge: Prostaglandin D₂ Enhances Leukotriene C₄ Synthesis by Eosinophils during Allergic Inflammation: Synergistic In Vivo Role of Endogenous Eotaxin¹

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In addition to the well-recognized ability of prostaglandin D₂ (PGD₂) to regulate eosinophil trafficking, we asked whether PGD₂ was also able to activate eosinophils and control their leukotriene C₄ (LTC₄)-synthesizing machinery. PGD₂ administration to presensitized mice enhanced in vivo LTC₄ production and formation of eosinophil lipid bodies—potential LTC₄-synthesizing organelles. Immunolocalization of newly formed LTC₄ demonstrated that eosinophil lipid bodies were the sites of LTC₄ synthesis during PGD₂-induced eosinophilic inflammation. Pretreatment with HQL-79, an inhibitor of PGD synthase, abolished LTC₄ synthesis and eosinophil lipid body formation triggered by allergic challenge. Although PGD₂ was able to directly activate eosinophils in vitro, in vivo PGD₂-induced lipid body-driven LTC₄ synthesis within eosinophils was dependent on the synergistic activity of endogenous eotaxin acting via CCR3. Our findings, that PGD₂ activated eosinophils and enhanced LTC₄ synthesis in vivo in addition to the established PGD₂ roles in eosinophil recruitment, heighten the interest in PGD₂ as a target for antiallergic therapies. The Journal of Immunology, 2006, 176: 1326–1330.

Leukotriene C₄ (LTC₄)³ and prostaglandin D₂ (PGD₂) are key lipid mediators of allergic airway inflammation that are released following allergen exposure in patients with asthma (1–4). LTC₄ and PGD₂ exert a variety of asthma-relevant actions, including bronchoconstriction and infiltration of eosinophils (1, 4), one of the principal cell types recruited to and activated at sites of allergic inflammation.

Eosinophils represent a major source of cysteinyl leukotrienes (cysLTs; LTC₄/D₄/E₄) (5, 6). The regulated synthesis of cysLTs is initiated by phospholipase A₂-mediated release of arachidonic acid (AA) from phospholipids. Released AA binds to 5-li-

poygenase (5-LO)-activating protein (FLAP) enabling AA to be oxygenated by 5-LO to form LTA₄. In eosinophils, a GST, LTC₄ synthase, catalyzes the adduction of glutathione to LTA₄ to form LTC₄. Although the enzymatic pathways by which eosinophils synthesize LTC₄ have been characterized, the pathophysiologic mechanisms that regulate these pathways during allergic conditions remain to be fully elucidated. Endogenous allergy-relevant stimuli, specialized intracellular compartments, and regulatory mechanisms are key features of allergen-induced LTC₄ synthesis that remain to be characterized. Recently, we have demonstrated that allergic inflammation triggers in vivo formation of new lipid bodies, which compartmentalize LTC₄ synthesis within infiltrating eosinophils. The enhanced formation of LTC₄ at newly formed lipid bodies can be mediated by eotaxin/RANTES acting via CCR3 receptors (7), but the role of other eosinophilotactic mediators in the induction of eosinophil LTC₄ synthesis during allergic inflammation has not been ascertained.

PGD₂ is a major mast cell derivative that appears to be critical for the pathogenesis of eosinophilic airway inflammation (3, 4) because of the following: 1) it is a potent chemoattractant for eosinophils both in vitro (8, 9) and in vivo (10); 2) overexpression of PGD₂ synthase or inhalation of PGD₂ enhanced eosinophilic airway inflammation in mice following allergen exposure (11, 12); and 3) disruption of the *DP* gene abolished eosinophil influx in a mouse model of asthma (13). Such PGD₂ role in eosinophil trafficking is associated with PGD₂'s ability to induce a variety of migration-related responses, including Ca²⁺ influx and actin polymerization (9, 14). Of note, in vitro data support an additional role for PGD₂ as an eosinophil activator (14, 15). However, there is no evidence as yet indicating that PGD₂-triggered signaling is important in vivo for activation/function of eosinophils, beyond its role in cell recruitment.

In this study, the interplay between two crucial lipid mediators of allergen-triggered eosinophilic inflammation, LTC₄ and

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³ Abbreviations used in this paper: LTC₄, leukotriene C₄; PGD₂, prostaglandin D₂; cysLTs, cysteinyl leukotrienes; AA, arachidonic acid; 5-LO, 5-lipoxygenase; i.p., intraperitoneal; ADPR, adipocyte-related protein.

PGD₂ was investigated. Specifically, we studied PGD₂'s ability to activate LTC₄-synthesizing machinery in eosinophils, by analyzing both LTC₄ generation and the biogenesis of the cytoplasmic LTC₄-synthesizing compartments (lipid bodies) of eosinophils in *in vivo* models of eosinophilic inflammation.

Materials and Methods

Animals

Swiss mice of 16–20 g from both sexes were used as approved by the institutional Animal Welfare Committee.

Allergic pleurisy in sensitized mice

Mice were sensitized with a s.c. injection (0.2 ml) of OVA (50 µg) and Al(OH)₃ (5 mg) in 0.9% NaCl solution (saline) at days 1 and 7. Allergic challenge was performed at day 14 by means of an intrapleural (i.pl.) injection of OVA (12 µg/cavity; 0.1 ml) (7). Alternatively, sensitized or naive mice were challenged with saline or PGD₂ (15–55 pmol/cavity; Cayman Chemicals). Mice were euthanized by CO₂ inhalation 24 h after challenge. Pleural cavities were rinsed with 1 ml of PBS.

Lung allergic inflammation in sensitized mice

Mice were sensitized with i.p. injection of OVA (10 µg/mouse) and Al(OH)₃ (10 mg/ml) in 0.2 ml of saline on days 1 and 10. From day 19 to day 24, mice were challenged for 20 min with OVA (5% in PBS) or PBS by aerosol (7). Mice were euthanized by CO₂ 24 h after the last aerosol. Bronchoalveolar lavage was collected by washing mice lungs with 1 ml of PBS.

Leukocyte and lipid body counts

Total leukocytes (diluted with Turk's 2% acetic acid fluid) were counted using a Neubauer chamber. Differential counts were performed using cytopins stained by the May-Grunwald-Giemsa method. Lipid bodies were enumerated by microscopy in 50 consecutively scanned cells, in cytospin slides fixed in 3.7% formaldehyde and stained and contrasted with osmium (16).

Treatments

Using the pleurisy model, animals were pretreated with i.p. injections of PGD₂ synthase inhibitor HQL-79 (1 mg/kg; Cayman Chemicals) (4) or anti-mCCR3 mAb (10 µg/cavity; clone 83103.111; R&D Systems) 30 min before allergic challenge. Animals pretreated with an irrelevant Ab showed no alteration of allergic response (data not shown). Using the asthma model, animals were pretreated (i.p.) four times with HQL-79 (1 mg/kg) 30 min before the four last aerosols.

In vitro stimulation of human blood eosinophils

Peripheral blood was obtained with informed consent from six normal donors, and eosinophils were isolated by negative selection using StemSep system (StemCell Technologies) (purity >99%; viability >95%) (17). Eosinophils (2×10^6 cells/ml in HBSS^{-/-}) were incubated for 1 h (37°C) with PGD₂ (5, 25, or 625 nM), recombinant human eotaxin (12 nM; R&D Systems), or a combination of eotaxin (0.1 nM) plus PGD₂ (5 nM). For mechanistic studies, eosinophils were pretreated for 30 min with anti-mCCR3 mAb (10 µg/ml; clone 61828.111; R&D Systems). After samples were taken for lipid body analysis, eosinophils were resuspended in HBSS^{+/+} and stimulated with 0.1 µM A23187 (Sigma-Aldrich) for 15 min (37°C). Each experiment was repeated at least three times with eosinophils purified from distinct donors.

Quantification of eicosanoids and eotaxin

cysLTs, PGE₂, and PGD₂ levels were measured by EIA kits according to Cayman Chemicals' protocols. Eotaxin levels were measured by the mEotaxin Duo Set (R&D Systems).

Intracellular immunodetection of newly formed LTC₄

To localize LTC₄ at its sites of formation, leukocytes were recovered from the pleural cavity with 500 µl of HBSS, and mixed with 500 µl of 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (1% in HBSS), before its immunofluorescent detection with rabbit anti-LTC₄ Ab (1/5 dilution; Cayman Chemicals) or nonimmune rabbit IgG plus Cy2-labeled anti-rabbit IgG Ab (1/500) (7) (18). To specifically localize leukocyte lipid bodies, a guinea pig antiadipocyte-related protein (ADRP) Ab (1/300 final dilution) plus Cy3-labeled anti-guinea pig IgG Ab (1/1000 final dilution) were added. Fluorescent microscopy was imaged by Cool Snap digital camera and Image Pro Express program (Media Cybernetics).

Statistical analysis

Results were expressed as means ± SEM and analyzed by ANOVA followed by the Newman-Keuls-Student's test ($p \leq 0.05$).

Results and Discussion

In this study, we studied whether PGD₂, an eosinophilotactic mediator, is also able to trigger the activation of recruited eosinophils and control eosinophil LTC₄-synthesizing machinery *in vivo*. To this end, we first developed a mouse model of PGD₂-induced eosinophilia. Similar to other models (10, 12), administration of PGD₂ in naive mice did not elicit eosinophil migration (Fig. 1A). In contrast, i.pl. injection of PGD₂ (15, 35, or 55 pmol/cavity) into sensitized mice induced eosinophil accumulation with a bell-shaped dose-response curve (Fig. 1A). PGD₂-induced pleural eosinophilia in sensitized mice was apparent within 6 h, reached maximum levels within 24 h, and was not associated with an influx of neutrophils or mononuclear cells (data not shown). It is noteworthy that PGD₂'s ability to induce local eosinophilia had already been shown in other models of inflammation, which used different strategies to create a proper PGD₂-sensitive environment (10, 12, 19). The mechanism involved in the PGD₂ responsiveness acquired after

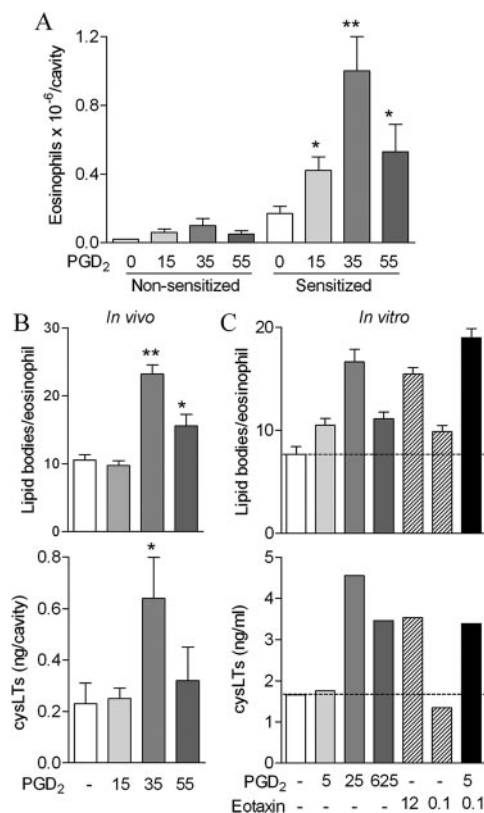


FIGURE 1. PGD₂ induces pleural eosinophil accumulation (A) plus eosinophil activation *in vivo* (B) and *in vitro* (C). In A and B, naive or sensitized mice received an i.pl. injection of PGD₂ (15, 35, or 55 pmol/cavity). Analysis of eosinophil influx, lipid body formation and cysLTs production were performed 24 h after PGD₂ administration. Results were expressed as the means ± SEM from at least eight animals. *, $p \leq 0.05$ and **, $p \leq 0.01$, compared with saline-injected sensitized animals. In C, human eosinophils were stimulated with PGD₂ (5, 25, or 625 nM), eotaxin (0.1 or 12 nM), or costimulated with eotaxin (0.1 nM) and PGD₂ (5 nM) *in vitro*. Analysis of lipid body formation was performed 1 h after PGD₂ incubation. For *in vitro* cysLTs assays, PGD₂-stimulated eosinophils were activated with A23187 (0.5 µM) for 15 min. Results are representative of three independent experiments.

sensitization has not been characterized but may involve differential expression of PGD₂ receptors. In fact, in vitro functional studies revealed that whereas eosinophils from both naive and sensitized mice respond equally to PGD₂ chemotactic stimulation, PGD₂ was able to trigger in vitro chemotaxis of resident pleural mononuclear cells recovered from sensitized, but not from naive mice (data not shown).

Human eosinophils upon stimulation preferentially synthesize LTC₄ as their 5-LO product. LTC₄ and its derivatives, LTD₄ and LTE₄, play a critical role in the pathogenesis of allergic conditions (1, 2). In vitro studies have identified a variety of allergy-relevant stimuli capable of inducing LTC₄ synthesis by human eosinophils (20). In vivo, eosinophils appear to play a central role in LTC₄ secretion during allergic inflammatory reactions (6, 7); however, the endogenous stimuli and regulatory mechanisms that trigger LTC₄ production in vivo by recruited/activated eosinophils have not been fully characterized. PGD₂ administration induced in vivo secretion of LTC₄ (Fig. 1*B*), but not PGE₂ (data not shown), in the pleural fluid of sensitized mice, indicating that PGD₂ activates preferentially, the 5-LO- and not COX-dependent pathway of eicosanoid synthesis in vivo. Confirming a previous observation (14), PGD₂ was also able to prime human eosinophils in vitro for an enhanced, A23187-activated LTC₄ synthesis in a concentration-dependent manner (Fig. 1*C*). Similar to the in vivo responses, in vitro stimulation with PGD₂ failed to enhance PGE₂ synthesis (data not shown), indicating that PGD₂-elicited intracellular signaling in eosinophils is preferentially coupled to activation of leukotriene- but not prostanoid-synthesizing mechanisms. Of note, human eosinophils are capable of enhanced PGE₂ synthesis upon proper stimulation, such as platelet-activating factor (16).

Eosinophils attracted to the site of PGD₂-induced inflammatory reactions exhibited signs of cell activation. In vivo eosinophil activation-mediated by PGD₂ was confirmed by analyzing the biogenesis of new cytoplasmic lipid bodies within infiltrating eosinophils (Fig. 1*B*). Lipid bodies are not conventional membrane-bound organelles, which represent specialized intracellular domains whose induced biogenesis is centrally related to activating mechanisms within the cells. Lipid body numbers are characteristically increased in eosinophils following in vitro activation, as well as in in vivo inflammatory disorders, and can be used as a marker of eosinophil activation (20). Administration of PGD₂ in sensitized mice promoted formation of new lipid bodies within the eosinophils recruited to the inflammatory site in a dose-dependent (bell-shaped curve; Fig. 1*B*) and cell type-selective manner, because PGD₂ failed to induce lipid body formation within resident mononuclear cells (data not shown). Moreover, as observed for LTC₄ production, in vitro stimulation of human eosinophils with PGD₂ also induced a rapid, dose-dependent biogenesis of cytoplasmic lipid bodies (Fig. 1*C*). In vivo dose-response curves shown for exogenous PGD₂-induced eosinophil influx, lipid body formation, and LTC₄ synthesis in sensitized mice are quite narrow (Fig. 1, *A* and *B*), but tends to be extended by synergistic interactions with local produced mediators during allergic inflammatory reactions (*vide infra*). Moreover, the mechanisms of PGD₂ bell-shaped curve may depend on counterregulatory effects of the PGD₂ dual receptor system (currently under investigation).

Based on prior findings with other stimuli (20), such increases in lipid body numbers may represent part of the mech-

anism of PGD₂'s ability to directly prime eosinophils for an enhanced LTC₄ production. Concurrent up-regulation of LTC₄S expression does not seem to represent a complementary mechanism contributing to the PGD₂-driven LTC₄ synthesis, because this can be a very rapid event (detected within 1 h). In an elegant study, Mandal et al. (21) have established that successful LTC₄ production is not merely determined by AA availability and kinetic properties of the relevant enzymes, but requires spatial interactions between specific biosynthetic proteins, FLAP and LTC₄-synthase. In addition to proper assembly of enzymatic complexes, it is now well recognized that LTC₄ biosynthesis also depend on the intracellular localization of these complexes. Within activated eosinophils, two distinct intracellular domains may compartmentalize such molecular organization for LTC₄ synthesis: the nuclear membrane (22, 23) and/or lipid bodies (7, 16, 18). Such putative role for eosinophil lipid bodies as specific sites for LTC₄ formation was validated by the direct intracellular immunofluorescent localization of newly formed LTC₄ within eosinophil lipid bodies by a new methodology that cross-linked LTC₄ at its sites of synthesis (17, 18). By using the same technique, we verified that the cell population responsible for LTC₄ production during PGD₂-induced inflammatory reaction corresponds to the infiltrating eosinophils (Fig. 2, *top panels*). Because PGD₂ is able to induce in vitro chemotaxis of mononuclear cells from sensitized mice (data not shown), the failure by resident mononuclear cells in synthesizing LTC₄ (Fig. 2, *top panels*) indicates that the signaling pathways engaged by PGD₂ in mononuclear cells are distinct from those involved

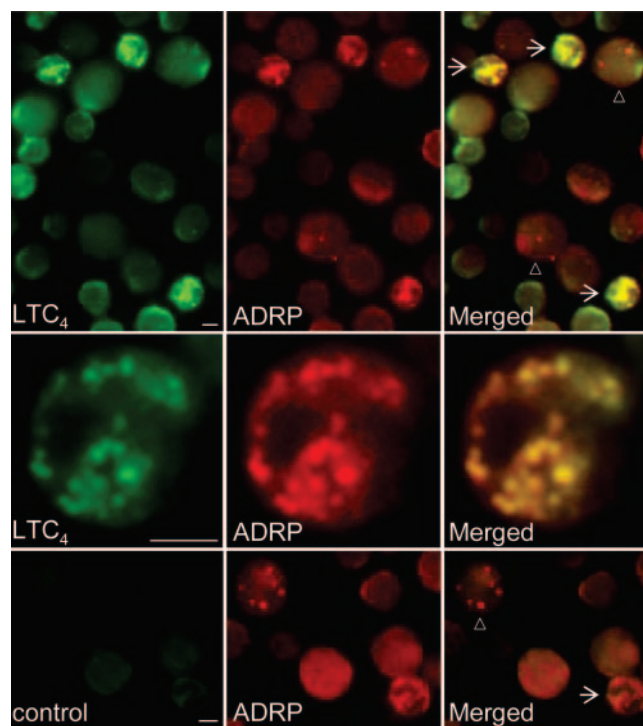


FIGURE 2. Cytoplasmic lipid bodies of recruited eosinophils compartmentalize LTC₄ synthesis during PGD₂-induced inflammatory reaction. In *top panels*, anti-LTC₄ was merged with identical field of anti-ADRP fluorescent images of pleural leukocytes recovered from sensitized mice 24 h after PGD₂ administration. *Middle panels* show LTC₄-immunoreactive lipid bodies (as identified by anti-ADRP) of a PGD₂-recruited eosinophil. In *bottom panels*, a nonimmune rabbit serum replaced anti-LTC₄ Ab. Arrow-heads, macrophages with ADRP-positive lipid bodies; arrows, eosinophils; bars, 5 μ m.

The mechanisms involved in PGD₂-elicited eosinophil lipid body-driven LTC₄ synthesis *in vivo* may have direct and indi-

In evidence of the PGD₂/eotaxin functional interactions, treatment with HQL-79 (1 mg/kg) abolished allergen-induced eotaxin production detected 24 h after challenge (from 17.6 ± 2.8 to 3.7 ± 0.5 ng/cavity of eotaxin in nontreated vs HQL-79-treated group, respectively; $p \leq 0.05$; $n = 8$). Increased levels of eotaxin were found in the pleural fluid of sensitized mice given injections with PGD₂ (35 pmol/cavity) (data not shown), identifying endogenous PGD₂ as a stimulus for the production of eotaxin during allergic reaction. Therefore, one can speculate that part of PGD₂'s effects on lipid body-driven LTC₄ production *in vivo* may be due to eotaxin activity. In fact, treatment with a neutralizing Ab against eotaxin receptor CCR3 inhibited PGD₂-induced lipid body formation and LTC₄ generation *in vivo* (Fig. 3C). Which cell secretes eotaxin in response to PGD₂ stimulation is now under investigation, but does not seem to be the eosinophils themselves. Interestingly, *in vitro* CCR3 neutralization failed to affect PGD₂-induced lipid body formation and priming for enhanced LTC₄ production by human eosinophils (data not shown), indicating that eotaxin autocrine activity played no role in PGD₂ *in vitro* stimulatory effect. Of note, autocrine activation of eosinophils through CCR3 by eotaxin is a phenomenon that can mediate lipid body-driven LTC₄ production *in vitro* (17). Anti-CCR3 also failed to inhibit *in vitro* PGD₂-induced lipid body formation by mouse eosinophils (data not shown), indicating that species differences are not the reason for such phenomenon and strengthens the hypothesis that human or mouse eosinophils do not secrete eotaxin under *in vitro* PGD₂ stimulation and therefore this chemokine cannot autocrinally regulate an *in vitro* effect. Altogether, lipid body-driven LTC₄ production triggered by PGD₂ *in vivo* seems to be mediated by eotaxin, acting in a paracrine fashion on eosinophils, because PGD₂ seems not to be able to trigger eotaxin secretion by eosinophils. Finally, suboptimal concentrations of eotaxin (0.1 nM) and PGD₂ (5 nM) cooperated to induce lipid body formation and prime human eosinophils for enhanced LTC₄ synthesis *in vitro* (Fig. 1C). *In vivo*, similar cooperation between eotaxin and PGD₂ (15 or 150 pmol/cavity) is also able to trigger lipid body formation within eosinophils (data not shown). Thus, physiological local levels of eotaxin and PGD₂ *in vivo* may synergize to effectively activate recruited eosinophils. Altogether, the mechanism of PGD₂-induced eosinophil activation that leads to lipid body-driven LTC₄ synthesis *in vivo* may involve complementary activities,

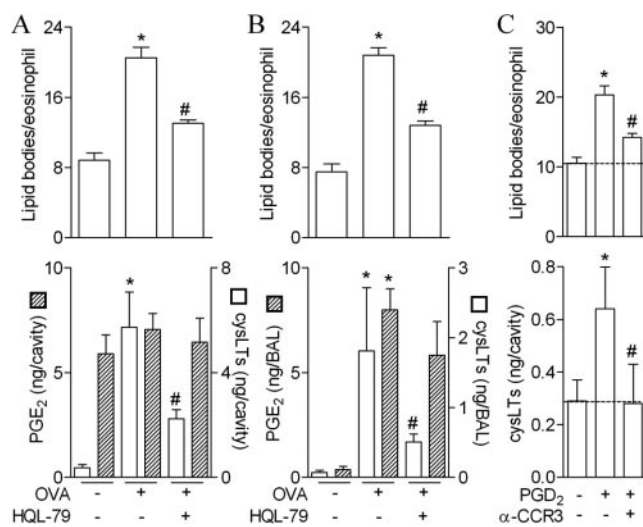


FIGURE 3. PGD₂ mediates lipid body formation and LTC₄ synthesis induced by allergic challenge in sensitized mice. Pretreatment with HQL-79 (1 mg/kg) (*A* and *B*) or anti-CCR3 (10 μg/cavity) was performed 30 min before OVA or PGD₂ (35 pmol/cavity) challenges (as indicated) in pleurisy (*A* and *C*) or asthma (*B*) models. Analyses of lipid body formation, cysLTs, and PGE₂ production were performed 24 h after allergic challenge. Results were expressed as the means ± SEM from at least eight animals. *, *p* ≤ 0.05, compared with control group. #, *p* ≤ 0.05, compared with allergen-challenged mice.

including direct stimulation of recruited eosinophils and indirect effects dependent on a paracrine/synergic activity of endogenous eotaxin acting on CCR3.

PGD₂ is a major candidate to mediator of allergic inflammatory disorders, such as asthma. Because PGD₂ modulates key aspects of this prevalent pathology, including the recruitment of eosinophils to inflamed tissue, and as described in this study eosinophil activation, production of chemokines, and synthesis of cysLTs, PGD₂ emerges as a key target for antiallergic treatments. In future years, PGD₂ receptor antagonists or inhibitors of synthesis may provide further therapeutic options in the management of allergic airway diseases.

Disclosures

The authors have no financial conflict of interest.

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