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Endogenous Platelet-Activating Factor Is Critically Involved in Effector Functions of Eosinophils Stimulated with IL-5 or IgG

Kathleen R. Barretes, Shannon McKinney, Gerald J. Gleich, and Hirohito Kita

Eosinophil activation and subsequent release of inflammatory mediators are implicated in the pathophysiology of allergic diseases. Eosinophils are activated by various classes of secretagogues, such as cytokines (e.g., IL-5), lipid mediators (e.g., platelet-activating factor (PAF)), and Ig (e.g., immobilized IgG). However, do these agonists act directly on eosinophils or indirectly through the generation of intermediate active metabolites? We now report that endogenous PAF produced by activated eosinophils plays a critical role in eosinophil functions. Human eosinophils produced superoxide when stimulated with immobilized IgG, soluble IL-5, or PAF. Pretreating eosinophils with pertussis toxin abolished their responses to these stimuli, suggesting involvement of a metabolite(s) that acts on G proteins. Indeed, PAF was detected in supernatants from eosinophils stimulated with IgG or IL-5. Furthermore, structurally distinct PAF antagonists, including CV6209, hexanolamine PAF, and Y-24180 (israpafant), inhibited IgG- or IL-5-induced superoxide production and degranulation. Previous reports indicated that exogenous PAF stimulates eosinophil eicosanoid production through formation of lipid bodies. We found in this study that IgG or IL-5 also induces lipid body formation and subsequent leukotriene C4 production mediated by endogenous PAF. Finally, inhibition of cytosolic phospholipase A2, one of the key enzymes involved in PAF synthesis, attenuated both PAF production and effector functions of eosinophils. These findings suggest that endogenous PAF plays important roles in eosinophil functional responses to various exogenous stimuli, such as cytokines and IgG. Therefore, inhibition of PAF synthesis or action may be beneficial for the treatment of eosinophil inflammation. The Journal of Immunology, 1999, 162: 2982–2989.

Eosinophils play important roles in the pathophysiology of bronchial asthma and other allergic diseases (reviewed in Ref. 1). In such diseases, mediators released by T cells, epithelial cells, and other inflammatory cells induce migration of eosinophils from blood into the affected tissues (reviewed in Ref. 2). Subsequently, appropriate stimuli activate the eosinophils, resulting in the local release of several inflammatory mediators, such as leukotrienes (2, 3), superoxide anion (4), and toxic cationic granule proteins (5). Eosinophil infiltration and deposition of released granule proteins are hallmarks of tissues from patients with allergic diseases (6, 7). Although the mechanism(s) of eosinophil activation and mediator release in human diseases still need to be elucidated, a wide range of stimuli can induce eosinophil effector functions in vitro. They include Ig (e.g., immobilized IgA and IgG) (5), lipid mediators (e.g., leukotriene B4 (LTB4)3 and platelet-activating factor (PAF)) (8), cytokines (e.g., IL-5 and granulocyte-macrophage CSF) (9), and complement fragments (e.g., C5a) (10). Structures of receptors for these secretagogues and intracellular signaling molecules associated with these receptors vary considerably. Therefore, the question exists whether these agonists pro-

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Materials and Methods

Reagents

PAF and CV6209 were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA), dissolved in absolute ethanol at 40 and 10 mM, respectively, and stored at −20°C. 1-O-hexadecyl-2-acetyl-sn-glycero-3-phospho-(N,N,N-trimethyl)-hexanolamine (hexanolamine PAF) and PMA were purchased from Calbiochem (La Jolla, CA), dissolved in water and DMSO at 20 mM and 5 mg/ml, respectively, and stored at −20°C. Pertussis toxin (PTX) from Calbiochem was dissolved in water at 100 μg/ml and stored at 4°C. Y-24180 (israpafant) was a gift from Yoshitomi Pharmaceutical Industries (Fukuoka, Japan); it was dissolved in DMSO at 20
mM and stored at 20°C. Mepacrine was purchased from Sigma (St. Louis, MO). Human IL-5, a gift from Schering-Plough Research Institute (Kenilworth, NJ), was diluted in PBS containing 0.1% BSA at 100 µg/ml, and stored at −70°C. All agonists and antagonists were diluted in reaction medium immediately before the experiments. Neither ethanol nor DMSO altered eosinophil functions at the solvent concentrations used in this study (<0.1% v/v). Purified human serum IgG was purchased from Orpegen (Teknika-Cappel, Malvern, PA) and stored at 1 mg/ml in PBS at 4°C. For eosinophil activation, IgG was immobilized onto the wells of tissue culture plates, as described below. The other reagents, including catalase, superoxide dismutase, human serum albumin (HSA), taurine, L-serine, glutathione, and cytochrome c, were purchased from Sigma.

**Eosinophil isolation**

Eosinophil isolation was performed by a magnetic cell separation system (MACS; Becton Dickinson, San Jose, CA), as described previously, with minor modifications (20). Briefly, venous blood anticoagulated with 50 U/ml heparin was obtained from normal volunteers and diluted with PBS at a 1:1 ratio. Diluted blood was overlaid on isotonic Percoll solution (density, 1.085 g/ml; Sigma) and centrifuged at 1000 × g for 30 min at 4°C. The supernatant and mononuclear cells at the interface were carefully removed, and erythrocytes in sediment were lysed by two cycles of hypotonic water lysis. Isolated granulocytes were washed twice with PIPES buffer (25 mM PIPES, 5 mM NaCl, 25 mM NaOH, 5.4 mM glucose, pH 7.4) with 1% defined calf serum (HyClone Laboratories, Logan, UT). An approximately equal volume of anti-CD16-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA) was added to the cell pellet. After 60 min of incubation on ice, cells were loaded onto the separation column positioned in the strong magnetic field of the MACS. Cells were eluted with PIPES buffer containing defined calf serum. The purity of eosinophils counted by Randolph’s stain was regularly greater than 98%. Purified eosinophils were washed and suspended in reaction medium, then used immediately.

**Superoxide anion production**

Eosinophil superoxide production was induced by various stimuli in polystyrene 96-well flat-bottom tissue culture plates (Corning CoStar, Cambridge, MA) and measured by reduction of cytochrome c, as previously described (21, 22). To immobilize human IgG onto the wells of tissue culture plates, 50 µl of human IgG diluted in PBS at 50 µg/ml was added to the wells and incubated overnight at 4°C. The solution was aspirated, and the wells were blocked with 50 µl of 2.5% HSA in PBS for 2 h at 37°C. After incubation, wells were washed twice with 0.9% NaCl and used immediately for the experiments. The tissue culture wells used for eosinophil stimulation with soluble agonists were blocked with HSA without IgG coating. Freshly isolated eosinophils were washed, and resuspended in HBSS with 10 mM HEPES and 200 mM cytochrome c at 5 × 10⁶ cells/ml. Fifty microliters of serial dilutions of inhibitors were added to the wells. One hundred microliters of cell suspension were dispensed onto the wells, and the reactions were initiated by adding 50 µl of soluble stimulants, including IL-5, PMA, and PAF at 25 ng/ml, 1 ng/ml, and 0.3 µM final concentrations, respectively. Wells coated with immobilized IgG received 50 µl of medium alone. In some experiments, cells were pretreated with an optimal concentration of PTX (100 ng/ml) for 2 h at 37°C. After incubation for 1 ha t 37°C and 5% CO₂, the supernatants from each well were carefully removed, and the wells were blocked with 50 µl of 1.085 g/ml and centrifuged at 400 × g. Supernatants and mononuclear cells at the interface were carefully moved, and erythrocytes in sediment were lysed by two cycles of hypotonic water lysis. Isolated granulocytes were washed twice with PIPES buffer (25 mM PIPES, 5 mM NaCl, 25 mM NaOH, 5.4 mM glucose, pH 7.4) with 1% defined calf serum (HyClone Laboratories, Logan, UT). An approximately equal volume of anti-CD16-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA) was added to the cell pellet. After 60 min of incubation on ice, cells were loaded onto the separation column positioned in the strong magnetic field of the MACS. Cells were eluted with PIPES buffer containing defined calf serum. The purity of eosinophils counted by Randolph’s stain was regularly greater than 98%. Purified eosinophils were washed and suspended in reaction medium, then used immediately.

**PAF production**

Generation of PAF by eosinophils was performed in 96-well flat-bottom tissue culture plates, as described previously by van der Bruggen (23), with minor modifications. All incubations for PAF production were performed in enriched HBSS (HBSS supplemented with 1 mM CaCl₂, 5 mM glucose, and 0.5% w/v) HSA and containing superoxide radical scavengers (2.5 mM taurine, 5000 U/ml catalase, and 380 U/ml superoxide dismutase) to prevent lipid degradation. Freshly isolated eosinophils were suspended in enriched HBSS at 2 × 10⁶ cells/ml, and 100-µl aliquots were added to the wells of tissue culture plates prepared as described above. Cells were stimulated with 100 µl of IL-5, PMA, or medium alone. After incubation for 15 or 45 min at 37°C, the plates were briefly centrifuged at 400 × g. Supernatants were collected and stored under nitrogen at −70°C. The concentration of PAF in the samples was determined by a commercial RIA kit (DuPont NEN, Boston, MA), following manufacturer’s protocol. The data were expressed as pg of PAF produced/10⁶ cells. The detection limit of the assay was 100 pg PAF produced/10⁶ cells.

**Eosinophil degranulation**

Eosinophil degranulation was performed in 96-well flat-bottom tissue culture plates, as described previously (21, 22). Eosinophils were washed and suspended in RPMI 1640 medium (Celox, Hopkins, MN), supplemented with 10 mM HEPES and 0.02% HSA, at 5 × 10⁵ cells/ml. Serial dilutions of CV6209 (50 µl) were added to the wells, followed by 100 µl of eosinophil suspension. Cells were stimulated with 50 µl of solubile agonists, and incubated for 180 min at 37°C and 5% CO₂. Eosinophils incubated in the wells coated with IgG received 50 µl of medium alone. After incubation, supernatants were collected and stored at −20°C until they were assayed for eosinophil-derived neurotoxin (EDN). To quantitate eosinophil degranulation, the concentration of EDN in the sample supernatants was measured by RIA. The RIA is a double-Ab competition assay, in which radiolabeled EDN, rabbit anti-EDN, and burro anti-rabbit IgG are used, as reported elsewhere (5). Total cellular EDN contents were measured simultaneously using supernatants from cells lysed with 0.5% Nonidet P-40 detergent. All assays were conducted in duplicate.

**LTC₄ production**

Eosinophil production of LTC₄ was performed in 96-well tissue culture plates, as described previously, with minor modifications (24). Eosinophils were washed with HBSS supplemented with 10 mM HEPES, 20 mM L-arginine, and 2.5 mM glutathione, and resuspended in the same medium at 1 × 10⁹ cells/ml. Serial dilutions of CV6209 or medium (50 µl) were added to the wells of the tissue culture plate, followed by 100 µl of cell suspension. Cells were stimulated by adding 50 µl of soluble agonists. Eosinophils incubated in the wells coated with IgG received 50 µl of medium alone. After incubation for 1 h at 37°C and 5% CO₂, the supernatants from each well were collected and frozen at −70°C or assayed immediately. Concentrations of LTC₄ in the sample supernatants were measured by ELISA using an LTC₄ kit (Cayman Chemical, Ann Arbor, MI), following the procedure recommended by the manufacturer. The sensitivity of the assay was 7.8 pg/ml. All experiments were conducted in duplicate.

**Lipid body formation**

Formation of lipid bodies within the activated eosinophils was examined as described previously (25), with minor modifications. Briefly, wells of a Lab-Tek 16-well chamber slide (Nunc, Naperville, IL) were coated with or without IgG and blocked with 2.5% HSA, as described above. Eosinophils were washed in HBSS with 10 mM HEPES, and resuspended in the same medium at 1 × 10⁵ cells/ml. Serial dilutions of CV6209 or medium (50 µl) were added to the wells, followed by 100 µl of cell suspension. Cells were stimulated by adding 50 µl of soluble agonists. Eosinophils incubated in the wells coated with IgG received 50 µl of medium alone. After incubation for 60 min at 37°C and 5% CO₂, the supernatants were removed by aspiration. Eosinophils were fixed in 3.7% formaldehyde in Ca²⁺/Mg²⁺-free HBSS, pH 7.4, and rinsed in 0.1 M cacodylate buffer, pH 7.4. Cells were stained in 1.5% OsO₄ (30 min), rinsed in water, immersed in 1.0% thioarbohydrazide (5 min), rinsed in 0.1 M cacodylate buffer, restained in 1.5% OsO₄ (3 min), rinsed in water, and dried. Subsequently, chamber walls were removed and the slide was coverslipped. The numbers of lipid bodies were counted with phase-contrast microscope by consecutively scanning 50 eosinophils with ×630 magnification.

**Statistical analysis**

Data are presented as means ± SEM from the numbers of experiments indicated. Statistical significance of the differences between various treatment groups (i.e., with or without inhibitor) was assessed using the Mann-Whitney U test or paired Student’s t test.

**Results**

**Effects of PTX on eosinophil superoxide production**

Previously, eosinophil effector functions, such as superoxide production and degranulation, were shown to be induced by a wide range of stimuli, such as cytokines (e.g., IL-5), lipid mediators (e.g., PAF), and immobilized Ig (e.g., IgG) (8–10, 21). Consistent with these previous findings, Fig. 1 shows that PAF, human IgG immobilized onto tissue culture plates and IL-5, as well as a positive control, PMA, induced eosinophil superoxide production in a time-dependent manner. As reviewed elsewhere, PAF is known to...
mediate its biological effects through activation of a G protein-coupled seven-transmembrane receptor (13). In addition, PTX catalyzes the ADP-ribosylation of certain G protein α-subunits, and treatment of intact cells with PTX results in uncoupling of PTX-sensitive G proteins from cell surface receptors (26). As shown in Fig. 1A, when cells were pretreated with PTX, eosinophil superoxide production stimulated with PMA was abolished, suggesting that eosinophils’ PAF receptor is coupled to PTX-sensitive G protein(s). Surprisingly, as shown in Fig. 1, B and C, superoxide production induced by IgG immobilized onto tissue culture plates or soluble IL-5 was markedly inhibited by PTX pretreatment. In contrast, superoxide production induced by PMA (Fig. 2D) was not affected by pretreatment of cells with PTX. The receptors for IgG and IL-5 belong to the families of FcγR (reviewed in Ref. 27) and hemopoietin receptor (reviewed in Ref. 28), respectively; there is no evidence for coupling of these receptors to PTX-sensitive G proteins. Furthermore, they are structurally distinct from typical G protein-coupled seven-transmembrane receptors. Therefore, in eosinophils, the superoxide production stimulated by IgG or IL-5 is most likely provoked by generation of intermediate metabolite(s) active on G proteins, rather than as a direct consequence of FcγR or IL-5R perturbation.

Production of PAF by eosinophils stimulated with IgG or IL-5

Then, what is the intermediate metabolite(s) that stimulates eosinophil functions in a G-protein-dependent manner? Eosinophils are able to generate several lipid mediators, such as PAF and LTC₄, in response to physiologic and pharmacologic stimuli (3, 12). Eosinophils also contain fourfold higher levels of ether phospholipid, the stored precursor of PAF, than do neutrophils, suggesting that the eosinophil is a good PAF producer (29). As shown in Fig. 2, we found that PAF was detectable in eosinophil supernatants 15 min after stimulation with immobilized IgG or soluble PMA; the amounts of released PAF increased dramatically by 45 min. Smaller but significant amounts of PAF (530.5 ± 127.8 pg PAF/10⁶ cells) were also detectable in supernatants from eosinophils stimulated with soluble IL-5 for 45 min (p < 0.05 compared with no stimulus, n = 4). The kinetics and amounts of PAF released by eosinophils incubated with IgG immobilized onto the tissue culture plates (2908.8 ± 428.3 pg PAF/10⁶ cells) were comparable with those observed previously by eosinophils incubated with IgG immobilized to Sepharose beads (12). Eosinophils incubated in medium alone released minimal levels of PAF at both times. Thus, when stimulated with immobilized IgG or soluble IL-5, eosinophils produce and release PAF in less than 45 min.

Effects of PAF antagonists on eosinophil effector functions

If PAF produced by activated eosinophils played major roles in effector functions of eosinophils themselves, then prevention of PAF binding to its receptor would dampen eosinophil effector functions. To examine this hypothesis, we used a potent and highly selective phospholipid analogue antagonist of PAF, CV6209; CV6209 is a competitive antagonist of PAF receptor (30). In our preliminary study, titration of CV6209 in an eosinophil superoxide production assay using a series of concentrations of PAF as stimuli showed that 50% inhibiting concentrations (IC₅₀) of CV6209 for 0.1, 0.3, 1, and 3 μM of PAF were 0.08, 0.23, 0.50, and 0.95 μM, respectively. As shown in Fig. 3A, CV6209 inhibited eosinophil

![Figure 1](http://www.jimmunol.org/) Kinetics of agonist-induced eosinophil superoxide production in the presence and absence of PTX. Eosinophils were preincubated with (closed circles) or without (open circles) PTX for 2 h and stimulated with 0.3 μM PAF, immobilized IgG (wells precoated with 50 μg/ml human IgG), 25 ng/ml IL-5, or 1 ng/ml PMA. Kinetics of superoxide production was measured by reduction of cytochrome c, as described in Materials and Methods. One representative experiment from a total of four experiments showing similar results is presented.

![Figure 2](http://www.jimmunol.org/) PAF release from eosinophils stimulated by IgG, IL-5, or PMA. Eosinophils were incubated with medium alone, immobilized IgG (wells precoated with 50 μg/ml human IgG), soluble IL-5 (25 ng/ml), or PMA (1 ng/ml) for 15 or 45 min. PAF released into the supernatants was measured by RIA. Results are presented as means ± SEM of three experiments. * Significant difference (p < 0.05) from values in the absence of stimuli.

![Figure 3](http://www.jimmunol.org/) Kinetics of agonist-induced eosinophil superoxide production in the presence and absence of CV6209. Eosinophils were stimulated with PAF (0.3 μM; A), immobilized IgG (wells precoated with 50 μg/ml human IgG; B), soluble IL-5 (25 ng/ml; C), or PMA (1 ng/ml; D) in the presence or absence of serial dilutions of a competitive PAF receptor antagonist, CV6209. The concentrations of CV6209 were 0 (squares), 0.1 (diamonds), 0.3 (circles), or 1 μM (triangles). Kinetics of superoxide production was measured by reduction of cytochrome c, as described in Materials and Methods. One representative experiment is shown; similar results were observed in five experiments.
superoxide production stimulated with 0.3 \( \mu M \) exogenous PAF in a concentration-dependent manner. Interestingly, as shown in Fig. 3, B and C, eosinophil superoxide production induced by immobilized IgG or soluble IL-5 was also inhibited by CV6209, while no exogenous PAF was added to the system. In contrast, superoxide production by eosinophils stimulated with PMA (Fig. 3D) was not inhibited by CV6209, with the exception of a slight change in kinetics.

The experiments were repeated using eosinophils from different donors, and the concentration-response curves of CV6209 are summarized in Fig. 4. Stimulation of eosinophils with PAF, immobilized IgG, IL-5, or PMA consistently induced substantial amounts of superoxide production. Unstimulated eosinophils did not produce detectable levels of superoxide (<0.04 nmol/10^5 cells). When a PAF receptor antagonist, CV6209, was added to the incubation, the eosinophil responses to immobilized IgG and IL-5 as well as those to exogenous PAF were inhibited in a concentration-dependent manner; IC_{50} values for immobilized IgG, IL-5, or PAF were 0.8, 0.3, and 0.3 \( \mu M \), respectively. Notably, CV6209 at 1 \( \mu M \) abolished the eosinophil response to IL-5 and significantly inhibited the response to immobilized IgG by 62% \((p < 0.01, n = 5)\). In contrast, superoxide production induced by PMA was not affected by CV6209 at concentrations up to 1 \( \mu M \). CV6209 showed no effect on eosinophil superoxide production in the absence of any stimuli (data not shown). Thus, eosinophil superoxide production induced by immobilized IgG, soluble IL-5, and exogenous PAF is sensitive to the PAF receptor antagonist, suggesting that endogenous PAF is involved in eosinophil responses to immobilized IgG or soluble IL-5.

To confirm the observation described above, we examined the effects of other PAF receptor antagonists, structurally distinct from CV6209, on IL-5-induced superoxide production. Hexanolamine PAF is a structural analogue of PAF; therefore, it has an advantage over other PAF receptor antagonists in that it may gain access to otherwise unavailable action sites (31). Y-24180, a newly discovered etizolam analogue, is a potent and specific PAF receptor antagonist (32). As shown in Fig. 5, both hexanolamine PAF and Y-24180 inhibited IL-5-induced superoxide production in a concentration-dependent manner; 30 \( \mu M \) hexanolamine PAF and 10 \( \mu M \) Y-24180 completely inhibited the eosinophil response to IL-5.

As described earlier, PAF is one of the strongest secretagogues for eosinophils (9). To investigate the role of endogenous PAF in eosinophil degranulation, purified cells were stimulated with immobilized IgG or soluble IL-5 and the effects of CV6209 were examined. As shown in Fig. 6, both immobilized IgG and soluble IL-5 induced eosinophil EDN release. CV6209, at 1 \( \mu M \), significantly inhibited degranulation of eosinophils stimulated with immobilized IgG and IL-5 by 49% \((p < 0.001, n = 6)\) and 35% \((p < 0.05, n = 6)\), respectively. In the absence of stimuli, CV6209 showed no effect on spontaneous release of EDN (Fig. 6), and it did not inhibit PMA-induced degranulation of eosinophils (data not shown). All in all, these findings indicate that endogenous PAF is involved in effector functions of eosinophils stimulated with immobilized IgG or soluble IL-5. However, this role of endogenous PAF may be different among the functions; for example, superoxide production is likely to be more dependent on endogenous PAF than is degranulation.

FIGURE 4. Inhibition of eosinophil superoxide production by CV6209. Eosinophils were incubated with stimuli in the presence or absence of serial dilutions of CV6209, as described in the legend for Fig. 3. Superoxide production was measured by the reduction of cytochrome c at 3 h. Results are presented as means ± SEM of five experiments (IgG, PMA, and PAF) or three experiments (IL-5). In the absence of stimuli, superoxide production was not detected (<0.04 nmol/10^5 cells). * Significant differences \((p < 0.05)\) from values obtained with eosinophils incubated without CV6209.

FIGURE 5. Effects of structurally distinct PAF receptor antagonists on eosinophil superoxide production induced by IL-5. Eosinophils were incubated with 25 ng/ml IL-5 in the presence of serial dilutions of PAF receptor antagonists, hexanolamine PAF, or Y-24180. Superoxide production was measured by the reduction of cytochrome c at 1.5 h. Results are presented as means ± SEM of four experiments. * and **, Significant differences \((*, p < 0.05; **, p < 0.01)\) from values obtained with eosinophils incubated without PAF antagonists.

FIGURE 6. Effects of CV6209 on eosinophil degranulation. Eosinophils were incubated with medium alone, immobilized IgG (wells precoated with 50 \( \mu g/ml \), or soluble IL-5 (25 ng/ml) in the presence of indicated concentrations of CV6209 for 3 h. The amounts of EDN released in the supernatants were measured by RIA. Results are presented as means ± SEM of six experiments. *, Significantly different \((p < 0.05)\) from values obtained with eosinophils incubated without CV6209.
Effects of CV6209 on lipid body formation and LTC$_4$ release by eosinophils stimulated with IL-5, immobilized IgG, or PAF. Eosinophils were incubated with medium alone, immobilized IgG (wells precoated with 50 μg/ml), soluble IL-5 (25 ng/ml), or PAF (1 μM) in the presence of indicated concentrations of CV6209 for 1 h. A, The numbers of lipid bodies were determined as described in Materials and Methods. Data are presented as means ± SEM from three independent experiments. †, Significant differences (p < 0.05) from values obtained in the absence of stimuli; *, significant differences (p < 0.05) from values obtained with eosinophils incubated without CV6209 in the same stimulus group. B, The amounts of LTC$_4$ released into the supernatants were measured by ELISA. Data are normalized to the values in the absence of CV6209 as 100% and presented as means ± SEM from five independent experiments. * and **, Significant differences (*, p < 0.05; **, p < 0.01) from values obtained with eosinophils incubated without CV6209.

Roles of endogenous PAF in eicosanoid metabolism of eosinophils

Recent studies suggest that intracellular lipid bodies are distinct, inducible, nonnuclear sites for eicosanoid synthesis in granulocytes (33, 34). Lipid body formation in eosinophils is rapidly induced by PAF stimulation, and PAF-induced lipid body production correlates strongly with increased production of eicosanoids (33). Because endogenous PAF was involved in eosinophil functions stimulated with IL-5 or immobilized IgG, we examined whether these agonists may also stimulate lipid body formation and eicosanoid generation in eosinophils. As shown in Fig. 7A, immobilized IgG and soluble IL-5 increased the numbers of intracellular lipid bodies with 1 h, similarly to the effect of exogenous PAF. Two and one-half times more lipid bodies were found in eosinophils stimulated with immobilized IgG, IL-5, or PAF, than in unstimulated eosinophils. Increased numbers of lipid bodies were also accompanied by increased production of eicosanoids, as shown by LTC$_4$ release into the supernatants of activated eosinophils (Table I). As shown in Fig. 7A and B, the PAF antagonist, CV6209, blocked lipid body formation and LTC$_4$ production induced by exogenous PAF. Similarly, CV6209, in concentrations as low as 0.3 μM, significantly inhibited lipid body formation and LTC$_4$ production by eosinophils stimulated with IL-5 or immobilized IgG (p < 0.05, n = 3 and 5, respectively). CV6209, at 1 μM, inhibited lipid body formation by more than 70% and LTC$_4$ production by more than 75%; CV6209 did not affect the number of lipid bodies in eosinophils incubated with medium alone. Thus, endogenous PAF, similarly to exogenous PAF, plays major roles in eicosanoid metabolism of eosinophils.

Blocking of phospholipase A$_2$ inhibits PAF production and eosinophil function

To confirm the involvement of the endogenous PAF pathway in eosinophil activation, we examined the effects of the blockade of PAF generation on eosinophil function. Phospholipase A$_2$ (PLA$_2$), the enzyme that catalyzes glycerol phospholipid to yield lysophosphatide and arachidonic acid, is one of the key enzymes involved in PAF generation (reviewed in Ref. 35). Mepacrine competitively inhibits PLA$_2$ activity by forming a stable complex of drug and phospholipid substrate (36). As expected from its drug action, 100 μM mepacrine inhibited PAF release from eosinophils stimulated with IL-5 or immobilized IgG (Table II). As shown in Fig. 8, mepacrine also significantly inhibited eosinophil superoxide production induced by IL-5 or immobilized IgG (p < 0.05 and p < 0.01 at 30 and 100 μM, respectively, n = 4). In contrast, PMA-induced superoxide production was not affected by up to 100 μM mepacrine. Thus, PLA$_2$ is most likely involved in PAF generation by eosinophils stimulated with immobilized IgG or soluble IL-5, and blockade of this enzyme inhibits superoxide production response to these stimuli.

| Table I. LTC$_4$ production by eosinophils stimulated with various agonists* |
|-----------------|----------------|----------------|
| Stimuli         | Concentration  | Released LTC$_4$ (ng/10$^6$ cells) |
| None            |                | 14.3 ± 6.9   |
| Immobilized IgG |                | 843.5 ± 301.4b |
| IL-5            | 25 ng/ml       | 154.2 ± 37.8b |
| PAF             | 1.0 μM         | 625.1 ± 303.6b |

* Eosinophils were incubated with the agonists as described above for 1 h at 37°C and 5% CO$_2$. Concentrations of LTC$_4$ in the sample supernatants were measured by ELISA using an LTC$_4$ kit. Data are presented as means ± SEM from five independent experiments.

| Stimuli         | Released LTC$_4$ (ng/10$^6$ cells) |
|-----------------|----------------|----------------|
| Expt. 1         |                |                |
| None            | 10.6           | 11.0           |
| IL-5            | 400.9          | 112.7          |
| IgG             | 922.6          | 56.0           |
| Expt. 2         |                |                |
| None            | 11.0           | ND             |
| IL-5            | 112.7          | 42.8           |
| IgG             | 56.0           | 12.1           |

* Eosinophils were incubated with medium alone, 25 ng/ml IL-5, or human IgG immobilized onto tissue culture plates in the absence or presence of 100 μM mepacrine for 45 min at 37°C and 5% CO$_2$. Concentrations of PAF in the sample supernatants were measured by RIA using a PAF RIA kit. ND, not determined.
FIGURE 8. Effects of a PLA₂ inhibitor, mepacrine, on superoxide production by eosinophils. Eosinophils were incubated with immobilized IgG (squares, wells precoated with 50 μg/ml IgG), soluble IL-5 (circles, 25 ng/ml), or PMA (triangles, 1 ng/ml) in the presence of serial dilutions of mepacrine. Superoxide production was measured by reduction of cytochrome c at 1.5 h. Data were normalized to the values in the absence of mepacrine. The amounts of superoxide produced by eosinophils stimulated with immobilized IgG, IL-5, and PMA without mepacrine were 6.1 ± 0.9, 4.2 ± 0.2, and 22.5 ± 1 nmol/10⁶ cells, respectively (means ± SEM, n = 4). Results are presented as means ± SEM of four experiments. * and **, Significant differences (*, p < 0.05; **, p < 0.01) from values obtained with eosinophils incubated without mepacrine.

Discussion

Activation of eosinophils and their subsequent release of inflammatory mediators are implicated in the pathophysiology of various allergic and eosinophilic disorders (reviewed in Ref. 1). Although little is known regarding the mechanisms of eosinophil activation in human diseases, previous studies suggest that the eosinophil is activated in vitro by a wide range of stimuli, such as cytokines, lipid mediators, and immobilized Ig (8–10, 21). Our results indicate that endogenously generated PAF plays important roles in the functional activation of eosinophils stimulated with IL-5 or immobilized IgG. This conclusion is based on several observations. 1) Although FcγR and IL-5R are not coupled to G proteins, eosinophil functional responses to IgG or IL-5 were inhibited by PTX, suggesting that they are mediated by intermediate metabolite(s) active on PTX-sensitive G proteins, rather than being a direct consequence of FcγR or IL-5R ligation. 2) PAF itself was detected in the supernatants of activated eosinophils. 3) Blockade of PAF interaction with its receptor by three PAF receptor antagonists markedly inhibited superoxide production, degranulation, lipid body formation, and LTC₄ release by eosinophils stimulated with IL-5 or immobilized IgG. 4) The inhibitory effects of PAF antagonists are unlikely due to nonspecific cytotoxicity because the IC₅₀ and the slope of concentration-response curves of CV6209 for IL-5 or immobilized IgG were comparable with that for exogenous PAF (Fig. 4), and because each of three structurally distinct PAF antagonists effectively inhibited IL-5 response (Figs. 4 and 5). In addition, the eosinophil responses to PMA were not inhibited by these antagonists, indicating that they do not inhibit the enzymes for cellular functions directly. 5) Finally, inhibition of PAF generation by a PLA₂ inhibitor attenuated eosinophil functional responses to IL-5 or IgG.

Our findings are compatible with a previous report, which demonstrated an important role of PLA₂ in eosinophil functions stimulated by FMLP plus cytochalasin B (37). While these investigators did not examine how PLA₂ is involved in eosinophil functions, our study suggests that PLA₂ was involved in the previous study by inducing the production of endogenous PAF. A number of agonists are known to induce production of PAF by eosinophils. For example, chemotactic factors, such as FMLP and C5a, stimulate rapid (1–3 min) release of PAF from eosinophils (3). In addition, IgG-coated Sepharose beads stimulate eosinophils to produce PAF (12). We also found that eosinophils incubated with soluble IL-5 produced PAF (Fig. 2). Furthermore, PAF provokes various effector functions of eosinophils, including adhesion, degranulation, and generation of superoxide anion and arachidonic acid metabolites (8, 17–19). Altogether, the results suggest that endogenous PAF may play a role in inflammatory responses of eosinophils by acting as a central switch, which bridges activation signals from various cell surface receptors with various effector cell functions. The disruption of the endogenous PAF pathway, either by inhibiting production or by preventing binding to a specific receptor, may profoundly impact eosinophil responses to a variety of stimuli.

The remaining question is how endogenous PAF stimulates effector functions of eosinophils. As shown in Fig. 2, we were able to detect PAF released extracellularly in the supernatants of eosinophils. Therefore, PAF released into the outside medium could stimulate eosinophils in a paracrine manner. However, a caveat for this speculation is whether the concentration of PAF in the extracellular space is high enough to induce eosinophil effector functions. For example, if released PAF is uniformly distributed in the incubation medium, we estimated 5 nM PAF in the eosinophil supernatants stimulated with immobilized IgG. This concentration may not be sufficient to fully activate eosinophils; it usually takes 100 nM or higher concentrations of exogenous PAF to stimulate eosinophil functions (22). However, much higher concentrations can be expected in the microenvironment close to the cells. Therefore, another potential mechanism is that PAF generated by eosinophils may remain within and activate the cells internally, similar to intracellular second messengers. Indeed, in eosinophils stimulated with IgG-coated Sepharose beads, the kinetic study showed that intracellular concentrations of PAF increased quickly after the stimulation, reached a plateau by 15 min, and stayed at the same levels for at least 60 min (12). In addition, Ojima-Uchiyama et al. reported that more intracellular PAF remains than is released extracellularly (38). Furthermore, the presence of intracellular PAF receptors has been suggested in nerve cells and granulocytes (39–41). Because intracellular PAF can be used immediately without involvement of secretory processes or without potential exposure to extracellular catalytic enzymes, this PAF hypothesis provides an economical cellular strategy. Therefore, the traditional plasma membrane receptors may be primarily responsible for mediating paracrine effects of PAF, while intracellular receptors may mediate the autocrine effects of PAF.

Close examination of the kinetics of superoxide production provides some insights regarding the roles of endogenous PAF in eosinophil functions. As shown in Fig. 3B, at early time points (less than 30 min), IgG-induced superoxide production was minimally affected by pretreatment of cells with a PAF receptor antagonist, CV6209. However, the inhibitory effect of CV6209 was pronounced when eosinophils were producing superoxide at later time points (i.e., 60 min). In contrast, the effects of exogenous PAF were completely inhibited by 1 μM CV6209 from the beginning of the cellular response (Fig. 3A). The effect of PTX on PAF- and IgG-induced superoxide production also showed similar findings (Fig. 1, A and B). These delayed onsets for the effects of a PAF receptor antagonist or PTX suggest that, at early time points, small amounts of superoxide may be generated by eosinophils through alternative mechanisms (e.g., signals from IgG Fc receptors) without the effects of endogenous PAF. Thus, at later time points, the dependency on endogenous PAF may become greater when cells are vigorously producing superoxide. Therefore, the role of endogenous PAF may be to enhance the otherwise small responses of eosinophils triggered.
by cytokines or Ig, and to expand to full bloom the effector function of eosinophils.

Eosinophil activation and subsequent release of inflammatory mediators are implicated in the pathophysiology of allergic diseases, such as bronchial asthma. Our study suggests that endogenous PAF plays an important role in mediator release by eosinophils. Furthermore, in addition to its autocrine effects, exogenous PAF exerts various biological activities relevant to bronchial asthma, such as airway constriction, development of bronchial hyperresponsiveness, and induction of microvascular leakage and edema (reviewed in Refs. 14 and 15). Indeed, severe asthma has been positively correlated with a deficiency of PAF acetylhydrolase in a Japanese population (42). Despite the evidence for potential roles of PAF in bronchial asthma, previous studies with PAF receptor antagonists failed to show beneficial effects in patients with asthma (43, 44). However, studies using newly developed and more potent PAF receptor antagonists have shown the effects of these antagonists on bronchial asthma. For example, Y-24180, which is also used in this study, inhibited allergen-induced eosinophilia and physiologic changes in guinea pig lungs (45), and prevented bronchial hyperresponsiveness in patients with asthma (46). Therefore, further studies are needed to elucidate the roles of PAF in bronchial asthma and other allergic diseases. Especially, as shown in this study, PAF antagonists inhibited eosinophil functions induced by a range of physiologic agonists. This wide spectrum of PAF antagonist effects may be advantageous from a therapeutic point of view because a number of eosinophil-active mediators produced by various immunologic or interstitial cells are likely to be involved in the pathophysiology of allergic diseases. The inhibition of only one mediator may be insufficient to inhibit eosinophil functions in such circumstances. Our study suggests that eosinophil activation and functions can be controlled effectively by managing the endogenous PAF pathways, and this concept may open a new avenue for the treatment of patients with allergic or eosinophilic disorders.

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References


