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Mouse and Human Eosinophils Degranulate in Response to Platelet-Activating Factor (PAF) and LysoPAF via a PAF-Receptor–Independent Mechanism: Evidence for a Novel Receptor

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Platelet-activating factor (PAF [1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine]) is a phospholipid mediator released from activated macrophages, mast cells, and basophils that promotes pathophysiologic inflammation. Eosinophil responses to PAF are complex and incompletely elucidated. We show in this article that PAF and its 2-deacetylated metabolite (lysoPAF) promote degranulation (release of eosinophil peroxidase) via a mechanism that is independent of the characterized PAFR. Specifically, we demonstrate that receptor antagonists CV-3988 and WEB-2086 and pertussis toxin have no impact on PAF- or lysoPAF-mediated degranulation. Furthermore, cultured mouse eosinophils from PAFR^{-/-} bone marrow progenitors degranulate in response to PAF and lysoPAF in a manner indistinguishable from their wild-type counterparts. In addition to PAF and lysoPAF, human eosinophils degranulate in response to lysophosphatidylcholine, but not phosphatidylcholine, lysophosphatidylethanolamine, or phosphatidylethanolamine, demonstrating selective responses to phospholipids with a choline head-group and minimal substitution at the *sn*-2 hydroxyl. Human eosinophils release preformed cytokines in response to PAF, but not lysoPAF, also via a PAFR-independent mechanism. Mouse eosinophils do not release cytokines in response to PAF or lysoPAF, but they are capable of doing so in response to IL-6. Overall, our work provides the first direct evidence for a role for PAF in activating and inducing degranulation of mouse eosinophils, a crucial feature for the interpretation of mouse models of PAF-mediated asthma and anaphylaxis. Likewise, we document and define PAF and lysoPAF-mediated activities that are not dependent on signaling via PAFR, suggesting the existence of other unexplored molecular signaling pathways mediating responses from PAF, lysoPAF, and closely related phospholipid mediators. *The Journal of Immunology*, 2010, 184: 000–000.

Platelet-activating factor (PAF [1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine]) is a unique phospholipid secretory mediator, originally identified as an agent released from IgE-sensitized basophils that promotes platelet aggregation (1, 2). Since its discovery, numerous proinflammatory roles have been attributed to PAF, and it has been explored extensively as a crucial pathophysiologic mediator in asthma, anaphylaxis, and other inflammatory states (reviewed in Refs. 3–5). Recently,

heightened interest in the biology of PAF has emerged from the work of Tsujimura and colleagues (6, 7), who implicated PAF as the primary modulator of systemic anaphylaxis resulting from IgG1-activated mouse basophils. Additionally, Vadas et al. (8) presented a positive correlation between serum PAF and the clinical severity of anaphylaxis in human subjects.

In addition to basophils, PAF is synthesized by mast cells and macrophages and is the end-product and presumed sole active metabolite of a biosynthetic-degradation pathway involving the enzymes PAF-acetyltransferase and PAF-acetylhydrolase, respectively (9, 10). The precursor and product of these enzymatic reactions, the 2-hydroxy-deacetylated derivative of PAF (lysoPAF) is generally perceived as inactive or, in some cases, antagonistic to the biological activities of PAF, although the precise biochemical nature of this functional antagonism remains unclear.

Platelets are only one of many cell populations that respond to PAF (reviewed in Ref. 11). Human eosinophils are prominent among this group and have numerous characterized PAF-mediated responses, including chemotaxis, superoxide production, adhesion, and release of cationic granule proteins (12–19). In contrast, mouse eosinophil responses to PAF have not been characterized directly, which is of significant concern, because asthma and anaphylaxis studies focusing on PAF are routinely carried out in mouse model systems (20–22). Human and mouse eosinophils are known for their profound dissimilarities (23), and mouse eosinophils are relatively resistant to stimuli that induce activation and degranulation

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Abbreviations used in this paper: bmEos, mouse eosinophils derived from bone marrow progenitors; EPO, eosinophil peroxidase; FGF, fibroblast growth factor; IL-5Tg, IL-5 transgenic; lysoPAF, 2-deacetylated platelet-activating factor; lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine; PAF, platelet-activating factor; PC, phosphatidylcholine; PDGF, platelet-derived growth factor; PE, phosphatidylethanolamine; PTX, pertussis toxin.

in human eosinophils *in vitro* and in models of inflammation and disease (24–26).

PAFR is a single seven-*trans*-membrane G protein-coupled receptor that has been identified and characterized (27–29), along with numerous biochemical receptor antagonists (30–32). PAFR has been detected in human eosinophils (33, 34) and in the eosinophil-related cell line EoL-1 (35). Only one PAFR has been identified, yet a number of complexities in receptor-mediated signaling have emerged. Most notable among these is the differential pertussis toxin (PTX) sensitivity for eosinophil chemotactic versus degranulation responses, suggesting the possibility of multiple receptors, multiple receptor conformations, and/or differential association with specific G proteins (reviewed in Ref. 36).

In this study, we examined PAF-mediated responses from another perspective; we explored release of the granule protein eosinophil peroxidase (EPO) and, for the first time, the release of proinflammatory cytokines in response to PAF and lysoPAF. In doing so, we identified a distinct and somewhat unanticipated PAF-response pathway that functions independently of the characterized G protein-coupled PAFR.

Materials and Methods

Mice

Six- to eight-week-old wild-type BALB/c and C57BL/6 mice were purchased from Taconic Farms (Rockville, MD). IL-5 transgenic (IL-5Tg) mice on the BALB/c background (37) are maintained on site. PAFR gene-deleted (PAFR^{-/-}) mice [C57BL/6 background (38)] were graciously provided by Dr. Peter Murray and Dr. Elaine Tuomanen (St. Jude's Children's Research Hospital, Memphis, TN); the genotype was confirmed prior to use in this study. This study was reviewed and approved via IL-5Tg Animal Study Proposal LAD-7E.

Reagents

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of PAF (C16-PAF; P4904, 1-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; hereafter referred to as PAF), C16-lysoPAF (L5016, 1-hexadecyl-*sn*-glycero-3-phosphocholine; hereafter referred to as lysoPAF), and C18-PAF (P6537, 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) were prepared at 1 or 10 mM in DMSO and used as indicated; cytochalasin B at 10 mg/ml in DMSO; f-formyl-Met-Leu-Phe at 50 μ g/ml in dimethyl-formamide. Phosphatidylcholine (PC; 850355P, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), lyso-phosphatidylcholine (lysoPC; 855675P, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine), phosphatidylethanolamine (PE; 856705P, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine), lyso-phosphatidylethanolamine (lysoPE; 850705P, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine), and C18-lysoPAF (878120P, 1-*O*-octadecyl-2-hydroxy-*sn*-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids (Alabaster, AL) and were prepared at 10 mM in DMSO. All subsequent dilutions were prepared in RPMI 1640 (Invitrogen, Carlsbad, CA), without phenol red. Cytokines used to challenge eosinophils were purchased from R&D Systems (Minneapolis, MN); stock solutions were prepared in PBS/0.1% BSA and further diluted in RPMI 1640, without phenol red. Stock solutions of PAFR antagonists WEB-2086 and CV-3988 (Enzo Life Sciences, Plymouth Meeting, PA) were prepared at 1 mM in ethanol and diluted in RPMI 1640, without phenol red, for use in degranulation and cytokine release assays. PTX was purchased from Calbiochem (San Diego, CA) and diluted to 100 μ g/ml in H₂O. Cycloheximide was purchased as a 100-mg/ml solution and diluted in RPMI 1640, without phenol red.

Eosinophils from mouse bone marrow progenitor cultures

The method for eosinophil production from unselected bone marrow progenitors was as previously described (39). Briefly, bone marrow cells were suspended in RPMI 1640 (Invitrogen) and seeded at 10^6 /ml in media containing RPMI 1640 with 20% FBS (Cambrex, East Rutherford, NJ), 100 IU/ml penicillin and 10 μ g/ml streptomycin (Mediatech, Manassas, VA), 2 mM glutamine (Invitrogen), 25 mM HEPES, 1 $\times$ nonessential amino acids, 1 mM sodium pyruvate (Invitrogen), 50 μ M 2-ME (Sigma-Aldrich) supplemented with 100 ng/ml stem cell factor (PeproTech, Rocky Hill, NJ), and 100 ng/ml FLT3 ligand (PeproTech) and maintained in this medium from days 0–4. On day 4, the medium containing stem cell factor and FLT3 ligand

was replaced with fresh medium containing 10 ng/ml recombinant mouse IL-5 (R&D Systems). Cultures were used for experimental studies on days 10–16, at >90–100% eosinophils, as determined by visual inspection of Diff-Quik–stained cytopsin preparations.

Isolation of eosinophils from IL-5Tg mice

Single-cell suspensions were obtained by cutting the spleen into small pieces in Hanks' buffered saline solution with 1% FBS and 10 mM HEPES. Fragments were passed through a 100- μ m strainer, followed by a 21-gauge needle. After RBC lysis, T and B cells were removed by binding to Miltenyi Biotec (Auburn, CA) CD90.2- and CD45R/B220-conjugated magnetic beads on a CS column, as per the manufacturer's directions. Purity was assessed at ~80% by visual inspection of Diff-Quik–stained cytopsin preparations; viability, assessed by trypan blue exclusion, was >99%.

Isolation of human peripheral neutrophils and eosinophils

Human eosinophils were isolated from heparinized whole blood collected from normal volunteers (protocol #NIAID 09-I-0049) using a Miltenyi Biotec human eosinophil isolation kit, as previously described (40). Purity was assessed by visual inspection of Diff-Quik–stained cytopsin preparations and was routinely >95%, with viability >99%, as determined by trypan blue exclusion. Initially, eosinophils were maintained in 25 ng/ml recombinant human IL-5 for 18–24 h at 37°C prior to performing the degranulation assay to detect the release of EPO. Cytokine release was assayed immediately after eosinophil isolation (i.e., cells were not exposed to IL-5), and additional EPO-release experiments were performed on freshly isolated eosinophils in the absence of IL-5. Neutrophils were obtained by lysis of RBCs following separation from whole blood using lymphocyte separation medium (MP Biomedicals, Solon, OH). Neutrophil purity was assessed by visual inspection of Diff-Quik–stained cytopsin preparations and was routinely >90%, with viability at >99%, as determined by trypan blue exclusion.

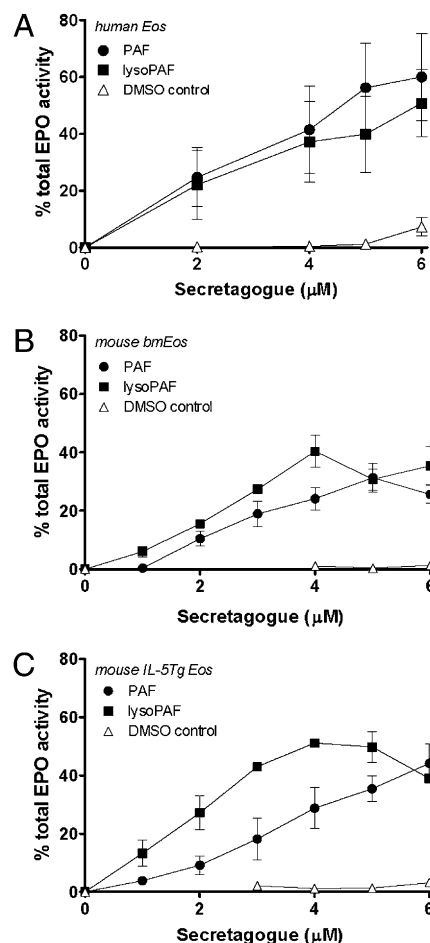


FIGURE 1. Eosinophils degranulate in response to challenge with PAF and lysoPAF. EPO release from human peripheral blood eosinophils (A), bmEos (39) (B), and splenic eosinophils (C) from IL-5Tg mice.

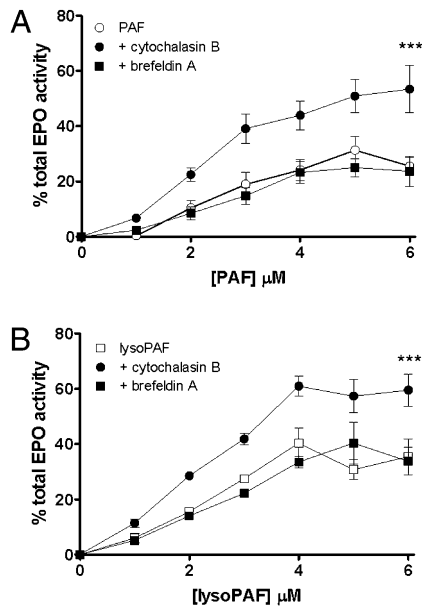


FIGURE 2. Eosinophil degranulation in response to PAF and lysoPAF is augmented by cytochalasin B but is unaffected by brefeldin A. bmEos challenged with increasing concentrations of PAF (A) or lysoPAF (B) in the presence of 5 μ g/ml cytochalasin B or 1 μ g/ml brefeldin A. *** p < 0.001, EPO release in the presence versus the absence of cytochalasin B.

Degranulation of EPO

Detection of EPO released in response to challenge with PAF was essentially as described (41). Cells were collected by centrifugation and resuspended in RPMI 1640, without phenol red, at 250,000 cells/ml; 100 μ l was used per well, unless otherwise indicated. One microliter of secretagogue or vehicle

control was added to achieve the indicated concentrations, and cells were incubated at 37°C, 5% CO₂ for 30 min. The assay was developed using 100 μ l *o*-phenylenediamine reagent (800 μ l 5 mM *o*-phenylenediamine in 4 ml 1 M Tris [pH 8], 5.2 ml H₂O, and 1.25 μ l 30% H₂O₂). The reaction was terminated by the addition of 100 μ l 4 M H₂SO₄ to each well and read at 492 nm. In addition to the wells containing the secretagogue to be evaluated, each plate contained a set of cells that remained untreated and a set of wells in which the cells were lysed in 0.2% NaDodSO₄ (SDS; KD Medical, Columbia, MD) to determine the total EPO content. Data are reported as the percentage of total EPO [(absorbance of stimulated sample – no treatment) $\times$ 100/total EPO from SDS-lysed cells]. All data are presented as mean $\pm$ SEM. Each experiment represents data from at least three different subjects. PAFR antagonists WEB-2086 and CV-3988 were introduced at 10 μ M final concentration, and cells were preincubated for 30 min at 37°C prior to the addition of PAF or lysoPAF. In experiments using PTX, the cells were incubated for 1 h with 100 ng/ml PTX prior to the addition of PAF.

Cytokine release

Cells were collected by centrifugation and resuspended in RPMI 1640, without phenol red, at 10⁶ cells/ml; 100 μ l were used per well. One microliter of PAF, lysoPAF, or vehicle control was added to achieve the 5 μ M concentrations. Cells were incubated at 37°C, 5% CO₂ for 60 min, and the cell-free supernatant was stored at –80°C until assayed for cytokine content. Cytokines were assayed by using the Milliplex (Millipore, Bedford, MA) or the Bioplex (Bio-Rad, Hercules, CA) multibead cytokine assays or by ELISA (R&D Systems), according to the manufacturer's directions. Human peripheral blood eosinophils were isolated from normal donors; each isolate was assayed in triplicate, and the data obtained from each donor are reported separately. The data collected from mouse eosinophils were averaged from three different experiments, with each trial performed at least three times. Human cytokines assayed include IL-1 β ; IL-1R antagonist; IL-2, -4, -5, -6, -7, -8, -9, -10, -12(p70), -13, -15, and -17; eotaxin/CCL11; basic FGF; G-CSF; GM-CSF; IFN- γ ; inflammatory protein-10; MCP-1; MIP-1 α and -1 β ; platelet-derived growth factor (PDGF)-bb; RANTES/CCL5; TNF- α ; and vascular endothelial growth factor. Mouse cytokines assayed included IL-1 α , -1 β , -2, -3, -4, -5, -6, -7, -8, -9, -10, -12(p40), -12(p70), -13, -15, and -17; eotaxin/CCL11; G-CSF; GM-CSF; IFN- γ ; inflammatory protein-10; KC/CXCL1; MCP-1; MIP-1 α

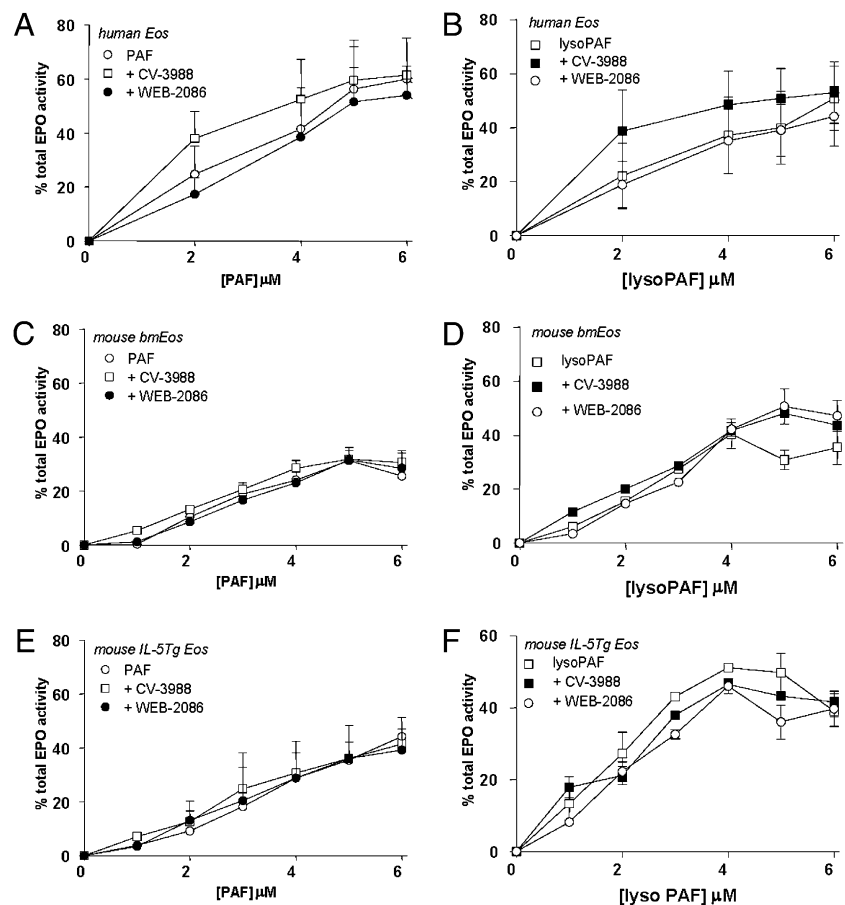


FIGURE 3. PAFR antagonists have no impact on eosinophil degranulation in response to PAF or lysoPAF. Human eosinophils (A, B), bmEos (C, D), and eosinophils (E, F) from IL-5tg mice challenged with increasing concentrations of PAF (A, C, E) or lysoPAF (B, D, F) in the absence or presence of 10 μ M PAFR antagonists CV-3988 and WEB-2086.

and β ; RANTES/CCL5; and TNF- α . The PAFR antagonists CV-3988 and WEB-2086 were introduced at 10 μ M for 30 min at 37°C prior to the addition of PAF to stimulate cytokine release. Cycloheximide was introduced at 5 μ g/ml immediately prior to the addition of PAF to inhibit de novo protein synthesis. Recombinant murine IL-6 and eotaxin (R&D Systems) were used at 20 ng/ml to stimulate cytokine release from mouse eosinophils cultured from bone marrow progenitors (bmEos).

Measurement of intracellular Ca^{2+} mobilization

Purified human neutrophils and eosinophils were loaded into Biocoat polylysine 96-well plates (BD Biosciences, San Jose, CA) at 1.3×10^5 cell/well and centrifuged at $100 \times g$ for 5 min to form a monolayer. Intracellular Ca^{2+} mobilization was measured using the FLIPR calcium 3 assay kit (Molecular Devices, Sunnyvale, CA), according to the manufacturer's guide. Briefly, the cells were loaded with fluorescence dye for 50 min at 37°C in 5% CO_2 . The PAFR inhibitors WEB-2086 and CV-3988 were gently added, to a final concentration of 10 μ M, to the appropriate wells and incubated for an additional 10 min. Serial dilutions of PAF and lysoPAF were prepared separately at 10-fold concentrations and added automatically during the calcium detection by the FLEX Station II (Molecular Devices) instrument. Relative fluorescence units were recorded for 180 s, and the maximum fluorescence signal minus the basal minimum signal after agonist addition was used as the response to that agonist. Results are reported as the percentage of control due to the variability in response among the three donors.

Statistical analysis

All analyses were performed using GraphPad Prism 5 (GraphPad, La Jolla, CA). Each data set was examined using ANOVA, followed by the Bonferroni or Tukey posttest.

Results

Human and mouse eosinophils degranulate in response to PAF and lysoPAF

PAF has been characterized as a chemotactic agent and secretagogue for human eosinophils (reviewed in Ref. 36). In this study, we demonstrate that human eosinophils degranulate in response to PAF and to its deacetylated metabolite lysoPAF; each promoted the dose-dependent release of up to 60% of the total cellular content of the granule protein EPO (Fig. 1A). Mouse eosinophils are typically resistant to agents that are known to activate human eosinophils (24, 25) (Supplemental Fig. 1); however, bmEos and splenic eosinophils isolated from IL-5Tg mice degranulate in response to the same concentrations of PAF and lysoPAF that activate human eo-

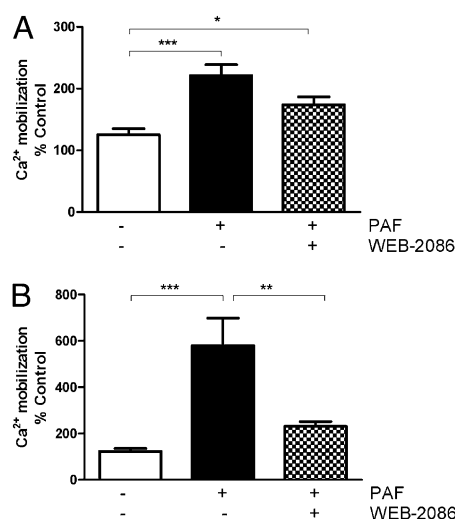


FIGURE 4. WEB-2086 antagonizes PAF-mediated activation of human neutrophils but not eosinophils. Ca^{2+} mobilization in eosinophils (A) and neutrophils (B) stimulated with 5 μ M PAF in the presence of the PAFR antagonist WEB-2086 (10 μ M). Data are pooled from three separate experiments using different donors and presented as percentage of Ca^{2+} mobilization in untreated cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

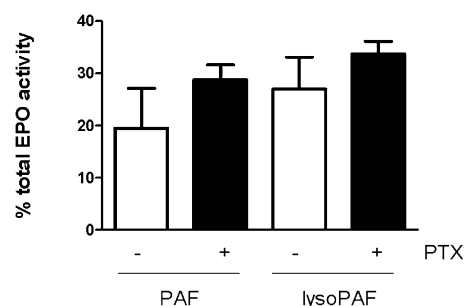


FIGURE 5. Eosinophil degranulation in response to PAF is not sensitive to PTX. Release of EPO from human eosinophils in response to 5 μ M PAF or lysoPAF following pretreatment for 1 h with 100 ng/ml PTX.

sinophils (Fig. 1B, 1C, respectively). Similar to what was reported for human eosinophils (42), mouse eosinophil degranulation in response to PAF is augmented by the fungal metabolite, cytochalasin B, which disrupts microfilament formation and facilitates the release of granule proteins (Fig. 2A); cytochalasin B also results in augmented degranulation in response to lysoPAF (Fig. 2B). In contrast, treatment with brefeldin A, which disrupts the Golgi apparatus and results in diminished release of IL-4 from human eosinophil granules (43), has no impact on PAF or lysoPAF-mediated EPO release from mouse eosinophils.

PAF and lysoPAF promote degranulation via PAFR-independent mechanism(s)

The seven-trans-membrane G protein-coupled PAFR has been detected on human eosinophils (33, 34), but its existence on mouse eosinophils has only been inferred from in vivo studies. Transcript-encoding PAFR was detected in cultured bmEos by quantitative PCR (data not shown). The synthetic PAFR antagonists WEB-2086 and CV-3988, both characterized as blocking human eosinophil effector functions in vivo as well as in vitro (44–46), had no impact on PAF- or lysoPAF-mediated release of EPO from human eosinophils (Fig. 3A, 3B). Likewise, neither antagonist had any effect on degranulation of mouse bmEos or on eosinophils from IL-5Tg mice in response to PAF or lysoPAF (Fig. 3C–F). Consistent with earlier findings (36), we observed PAF-induced calcium mobilization in human eosinophils, as well

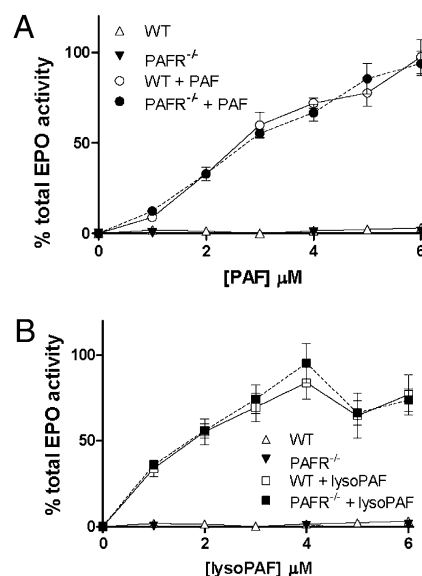


FIGURE 6. Eosinophils from PAFR gene deleted (PAFR^{-/-}) mice degranulate in response to PAF and lysoPAF. Wild-type and PAFR^{-/-} bmEos challenged with increasing concentrations of PAF (A) or lysoPAF (B).

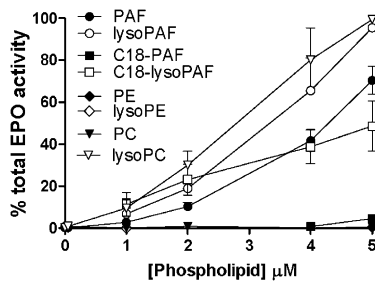


FIGURE 7. Human eosinophil degranulation in response to a select subset of phospholipid mediators. Release of EPO in response to increasing concentrations of PAF, lysoPAF, C18-PAF, C18-lysoPAF, PE, lysoPE, PC, and lysoPC. Pooled data from experiments performed in triplicate with eosinophils from three or four normal donors.

as in neutrophils (Fig. 4A, 4B, respectively). However, calcium mobilization was inhibited by 10 μ M WEB-2086 in neutrophils only. Pretreatment with PTX for 1 h had no impact on EPO release from human eosinophils in response to PAF or lysoPAF (Fig. 5). Finally, bmEos generated from PAFR gene-deleted mice (PAFR^{-/-}) responded to PAF and lysoPAF (Fig. 6A, 6B, respectively), releasing EPO in a manner that was indistinguishable from that of bmEos derived from PAFR-sufficient wild-type mice.

Human eosinophils degranulate in response to a specific subset of phospholipid mediators

In an effort to characterize a potentially novel receptor or pattern-recognition molecule mediating responses to PAF and lysoPAF, we challenged human eosinophils with a series of structurally related phospholipid mediators (Fig. 7). In addition to PAF and lysoPAF, eosinophils released EPO in response to C18-lysoPAF and lysoPC over the same range of concentrations (1–5 μ M); no degranulation was observed in response to PE, lysoPE, PC, or C18-PAF. These results suggest that eosinophils respond selectively to phospholipids with a quaternary saturated amine (i.e., choline) head-group and no (lysoPC, lysoPAF, and C18-lysoPAF) or minimal substitution (PAF) at the *sn*-2 hydroxyl (see www.avantilipids.com for detailed structures). Additional characterization is necessary to explain why C18-PAF is inactive in this assay.

Human eosinophils experienced no loss of viability in response to challenge with any of the oxidized phospholipids evaluated (PAF and lysoPAF, Supplemental Fig. 2A; others, data not shown). In contrast, the viability of mouse bmEos (wild-type and PAFR^{-/-}) was reduced to ~10% in response to PAF challenge in experiments carried out using 25,000 cells/100 μ l. Loss of viability in response to PAF or lysoPAF was less profound when cells were maintained at concentrations of 50,000 or 100,000/100 μ l, and the

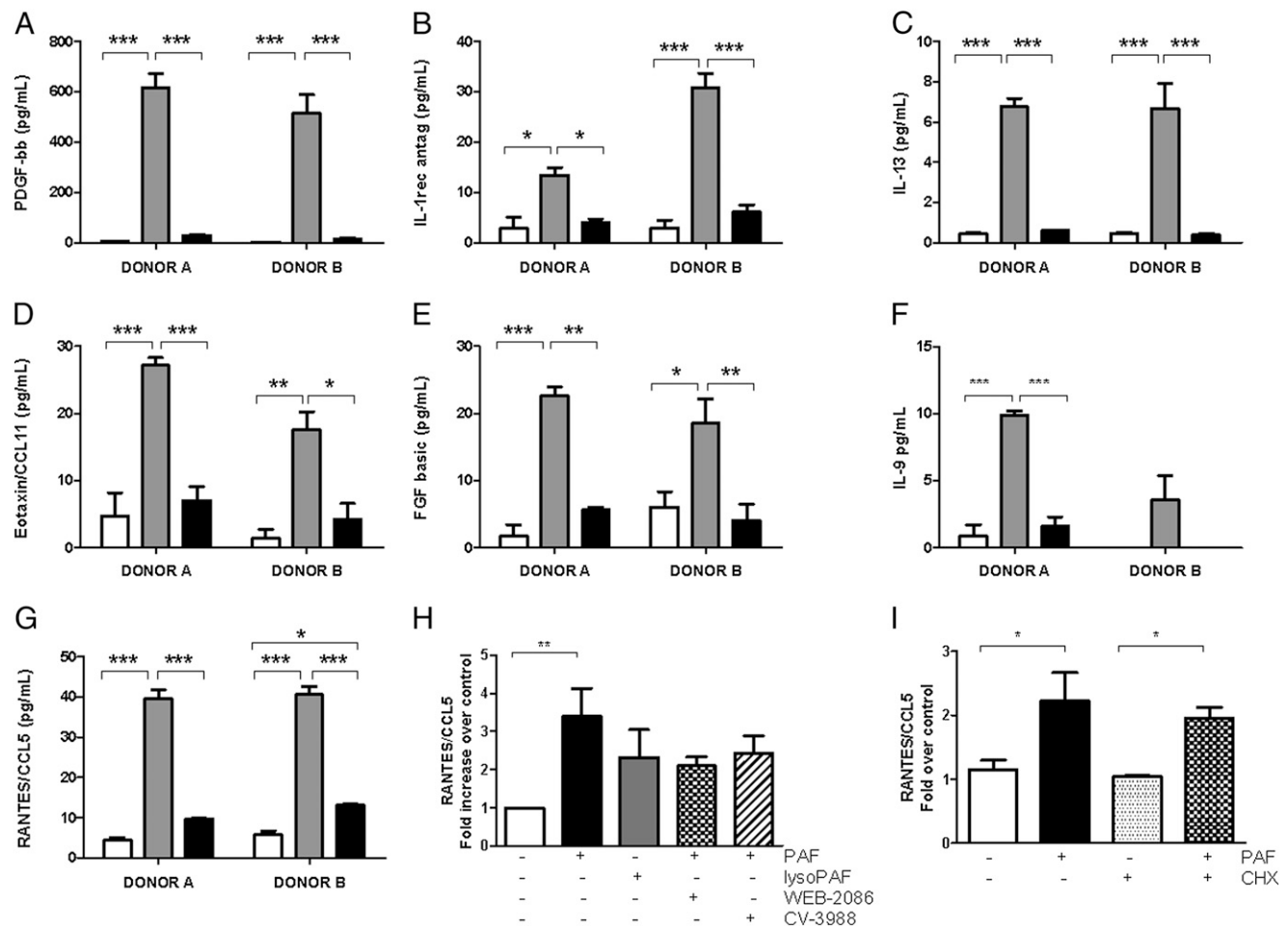


FIGURE 8. Cytokines are released from human eosinophils in response to PAF but not lysoPAF. Cytokines released in response to 5 μ M PAF (gray bars) or lysoPAF (black bars) or DMSO control (white bars) include PDGF-bb (A), IL-1R antagonist (B), IL-13 (C), eotaxin-1 (D), basic FGF (E), IL-9 (F), and RANTES/CCL5 (G). Data collected from two independent donors, each assayed in triplicate. H, RANTES/CCL5 release in response to 5 μ M PAF or lysoPAF in the absence or presence of 10 μ M PAFR antagonists (pooled data from 9–12 separate donors). I, RANTES/CCL5 release in response to 5 μ M PAF in the presence or absence of 5 μ g/ml cycloheximide. Significance was determined by one-way ANOVA, followed by the Tukey multiple comparison; * p < 0.05; ** p < 0.01; *** p < 0.001.

extent of PAF-mediated degranulation of cultured mouse eosinophils increased with enhanced viability (Supplemental Fig. 2B). However, degranulation was not merely a function of loss of viability, because cells at 25,000/100 μ l challenged with lysoPAF did not undergo substantial loss of viability, and the extent of degranulation in response to PAF and lysoPAF at this cell density was indistinguishable (Fig. 1).

Human eosinophils release cytokines in response to PAF but not lysoPAF

Numerous cytokines have been localized to the specific granules of human eosinophils (reviewed in Refs. 47, 48); recent work indicated that they are mobilized and released via distinct intracellular signaling mechanisms (49–52). Among the cytokines released in response to PAF are IL-13 and -9, IL-1R antagonist, eotaxin/CCL11, basic FGF, RANTES/CCL5, and PDGF (Fig. 8A–G). No significant increase in cytokine release over vehicle control was observed from human eosinophils in response to lysoPAF, with the exception of one donor who released a small amount of RANTES/CCL5 in response to this mediator (Fig. 8G). In a further exploration using eosinophils from 9–12 independent donors, PAF consistently induced the release of RANTES/CCL5, whereas lysoPAF did not. Receptor antagonists CV-3988 and WEB-2086 had no impact on PAF-mediated RANTES/CCL5 release (Fig. 8H). Additionally, treatment with cycloheximide had no effect on PAF-stimulated RANTES release, consistent with the interpretation that PAF induces cytokine release from preformed granule stores rather than inducing synthesis *de novo* (Fig. 8I). In contrast, bmEos did not release any cytokines in response to PAF, and they released minimal amounts of IL-9 only in response to lysoPAF (data not shown). However, these cells were capable of cytokine release in response to other mediators (Fig. 9). bmEos challenged with IL-6 released significant quantities of IL-1 β , -9, and -12(p70); IFN- γ ; TNF- α ; and CCL2/MCP-1. In contrast, little to no cytokine release was observed in response to challenge with eotaxin/CCL11.

Discussion

Given the recent findings implicating PAF in the pathophysiology of acute anaphylaxis (8, 20), the biological impact of this mediator has come under renewed scrutiny (4, 53). PAF is a phospholipid secretory mediator synthesized by basophils, mast cells, and macrophages and is the presumed sole active metabolite of a biosynthetic and degradation pathway involving the enzymes PAF-acetyltransferase and PAF-acetylhydrolase, respectively (9, 10). The precursor and product of these enzymatic reactions, the 2-hydroxy-deacetylated derivative of PAF known as lysoPAF, is generally considered inactive or, in some cases, antagonistic to the activities of PAF, although the precise biochemical nature of this antagonism remains unclear. Recently, Welch et al. (54) demonstrated that the opposing actions of PAF and lysoPAF on neutrophils are clearly not classic single receptor-based competitive antagonism, because neither classic biochemical antagonists nor PAFR gene deletion had any impact on the antagonistic or opposing actions carried out by lysoPAF. In eosinophils, we found that PAF and lysoPAF promote dose-dependent degranulation responses, and both responses occurred independently of biochemical antagonists or PAFR gene deletion. The fact that eosinophils degranulate equally effectively after challenge with PAF and lysoPAF may explain some of the clinically unsatisfying results achieved with cloned PAF-acetylhydrolase, an agent intended to hydrolyze PAF to lysoPAF systemically and explored for its effectiveness as an antiasthma therapeutic (55).

In addition to PAF and lysoPAF, human eosinophils degranulate in response to C18-lysoPAF and lysoPC, phospholipids with a choline

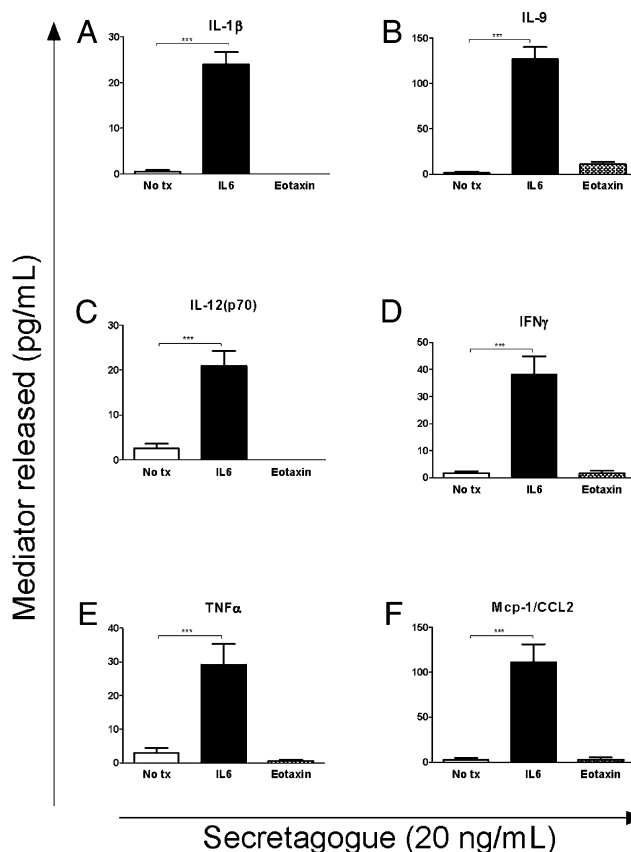


FIGURE 9. Cytokines are released from mouse eosinophils in response to proinflammatory mediators other than PAF and lysoPAF. Cytokine release in response to 20 ng/ml IL-6, eotaxin, or no treatment was assessed in three independent experiments, each including bmEos generated from three different mice. Significance determined by one-way ANOVA followed by the Tukey multiple comparison; *** $p < 0.001$.

head-group and minimal or no fatty acid substitution at the *sn*-2 hydroxyl. Phospholipids without the saturated quaternary amine (i.e., ethanolamine in place of choline) or with extensive *sn*-2 substitution do not promote degranulation. The identity of this receptor or pattern-response molecule is not immediately apparent. PAFR is the only known receptor that responds to PAF, although the family of lipid receptors is large, and there are quite a few orphan receptors that remain unexplored and incompletely characterized (reviewed in Ref. 53). G2AR was initially characterized as responding to lysoPC, but this finding has recently been reconsidered (56). Likewise, several groups reported that the actions of lysoPC are dependent on the presence of the receptor GPR4 (57, 58); however, the receptor itself has been characterized alternatively as ligand independent (59) or proton sensing (60). However, it is interesting to note that the PAF biosynthetic enzyme PAF acetyltransferase can acetylate lysoPAF and lysoPC (61); this suggests that there may be one or more phospholipid receptors with a similarly flexible ligand/substrate specificity.

We demonstrated for the first time that PAF promotes the release of cytokines from human eosinophils; the subset released includes IL-9 and -13, IL-1 receptor antagonist, eotaxin-1, basic FGF, RANTES, and PDGF. Most of these cytokines have been identified previously as components of human eosinophils (47, 48). Of those that have not, IL-1R antagonist has been associated with eosinophilia (62), but it has not previously been identified directly as an eosinophil component. This is the first report of basic FGF as an endogenous eosinophil secretory mediator. Interestingly, we detected no release of IL-4 or IFN- γ in response to PAF, which are among the most prominent cytokines present in human eosinophil

granules (63). Although many cytokines have been localized to the eosinophil granule, they are not all released in response to a single given stimulus. The current model for cytokine release involves receptor sorting and differential mobilization of specific cytokine components in response to individual eosinophil-active agents; this was described in detail for the release of IL-4 in response to eotaxin (49–52, 63, 64) and is the molecular basis for the electron microscopic description of piecemeal degranulation (51). Given the role implicated for PAF in the pathogenesis of asthma (4, 65), one might envision shared PAF-induced signals that mobilize basic FGF, IL-13, and PDGF, which are all implicated in fibrosis and tissue remodeling (66–68). Similarly, eotaxin and RANTES are eosinophil chemoattractants (69, 70) that could be mobilized in response to PAF and then serve to promote further eosinophil recruitment toward sites in need of remodeling and repair.

It is intriguing and important to note that although EPO release is more or less equivalent and indistinguishable when comparing human and mouse eosinophils, cytokine release in response to PAF is not observed in mouse eosinophils—a substantial caveat for researchers studying mouse models of eosinophil pathobiology and of particular importance with respect to mouse models invoking PAF in the pathogenesis of asthma (4, 65). There are many differences between human and mouse eosinophils (reviewed in Ref. 23), including the evolutionary divergence of the human and mouse eosinophil secretory ribonucleases and cell surface Siglec proteins, the absence of the prominent galectin-10 protein and high-affinity IgER in the mouse eosinophils, and distinct responses to chemotactic cytokines and agents stimulating degranulation. Differential cytokine release in response to PAF can now be recognized as among the prominent differences between human and mouse eosinophils.

In summary, human and mouse eosinophils degranulate, releasing EPO in a dose-dependent fashion in response to PAF and also to its deacetylated metabolite lysoPAF. Studies with receptor antagonists, signaling pathway inhibitors, and gene-deleted mice clearly document that eosinophil degranulation is distinct from other PAF-mediated responses and is not dependent on signaling via the characterized G protein-coupled PAFR. In addition to PAF and PAF derivatives, human eosinophils degranulate in response to lysoPC but not in response to other closely related phospholipid mediators, including PC or PE and their derivatives. Taken together, our results suggest that eosinophil degranulation in response to PAF and related phospholipid ligands is specific and selective, involving an uncharacterized receptor or pattern-recognition molecule.

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Disclosures

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