

Endothelium-derived prostaglandin I₂ controls the migration of eosinophils

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Background: Enhanced eosinophil migration from the blood into the tissue is a hallmark of allergic diseases. Prostaglandin (PG) I₂ is the major prostanoid released by endothelial cells. Mice deficient in PGI₂ receptors (IPs) show exaggerated eosinophilic inflammation in response to allergen.

Objective: We set out to determine the role of PGI₂ in eosinophil trafficking.

Methods: Human lung microvascular endothelial cells and purified human eosinophils were used to study adhesion and transendothelial migration. Morphologic studies were performed with fluorescence microscopy.

Results: PGI₂ markedly attenuated the migration of eosinophils through cell-free filters but had no effect on neutrophil migration. The inhibitory effect of PGI₂ on eosinophils was prevented by the IP antagonist Cay10441 and the adenylyl cyclase inhibitor SQ22536. Similarly, PGI₂ prevented the adhesion of eosinophils to fibronectin and the rapid upregulation and activation of the adhesion molecule CD11b. IP expression on eosinophils was confirmed by means of flow cytometry and Western blotting. Furthermore, when endothelial cells were treated with the COX inhibitor diclofenac to abolish PGI₂ production, adhesion of eosinophils to endothelial monolayers and subsequent transendothelial migration were markedly enhanced. Similarly, the IP antagonist enhanced eosinophil adhesion to endothelial cells. Inhibition of PGI₂ biosynthesis decreased the electrical resistance of endothelial monolayers and compromised the texture of adherent junctions, as visualized by means of VE-cadherin and F-actin staining.

Conclusion: We propose that endothelium-derived PGI₂ might be fundamental for the maintenance of the endothelial barrier function against infiltrating cells. These results suggest that

selective IP agonists might have beneficial effects in allergic inflammation. (*J Allergy Clin Immunol* 2010;■■■:■■■-■■■.)

Key words: Endothelial cells, eosinophils, COX, prostaglandins, migration, adhesion, allergy

Eosinophils are important effector cells in allergic inflammation. They play important roles in late-phase reactions by releasing bronchoconstrictor mediators, such as leukotriene C₄, and other chemoattractants that cause further influx of inflammatory cells into the tissue. Eosinophils produce and release proinflammatory cytokines and growth factors, including the T_H2 cytokines IL-4, IL-5, IL-10 and IL-13.¹ Mucosal damage in patients with chronic asthma is associated with cytotoxic mediators that are released by activated eosinophils, including reactive oxygen species, major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin. Consequently, eosinophils also play a role in airway remodeling and angiogenesis in chronically inflamed tissue.²

Several agonists can mediate eosinophil locomotion and recruitment, in particular those of the CC chemokine family, such as eotaxin (CCL11), which act through the CC-chemokine receptor 3.³ In addition, activated complement factor 5 (C5a) and lipid mediators, such as platelet-activating factor, leukotriene B₄, leukotriene C₄, 5-oxo-6,8,11,14-eicosatetraenoic acid, and prostaglandin (PG) D₂, are also potent chemoattractants for eosinophils.⁴⁻⁷

PGI₂ is the major prostanoid released by endothelial cells, but its role in allergic inflammation has not been elucidated in depth. The effects of PGI₂ are mediated by a single G protein-coupled receptor, IP, which is coupled to G_s proteins and stimulates adenylyl cyclase. Mice deficient for the IP receptors show augmented allergic responses in the lung and skin, enhanced airway remodeling,^{8,9} and prolonged inflammation after inoculation with respiratory syncytial virus.¹⁰ Using IP-deficient mice, it has been shown that activation of IP receptors by endogenous PGI₂ attenuates the antigen-induced cytokine production by splenocytes⁸ and abrogates dendritic cell function through cyclic AMP (cAMP) induction and downregulation of nuclear factor κB.¹¹ Recent studies also demonstrated that PGI₂ attenuates the migration of murine T_H2 cells¹² and inhibits cytokine production and T-cell stimulatory function of murine bone marrow-derived dendritic cells.¹¹ Treatment of endothelial cells with PGI₂ reduces subsequent adhesion of neutrophils,¹³ and neutrophil superoxide generation is attenuated by a PGI₂ analogue.¹⁴ In monocytes and macrophages, PGI₂ analogues prevent the LPS-induced expression of IL-18 and TNF-α,^{15,16} but vascular endothelial growth factor production in lung fibroblasts is enhanced by PGI₂.¹⁷ In alveolar and peritoneal macrophages PGI₂ analogues inhibit phagocytosis, bacterial killing, and TNF-α generation.¹⁸

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Abbreviations used

cAMP: Cyclic AMP

C5a: Activated complement factor 5

IP: Prostaglandin I₂ receptor

PG: Prostaglandin

The aim of the present study was to test the hypothesis that PGI₂ is involved in eosinophil-endothelial interaction. We observed that endothelium-derived PGI₂ inhibits, whereas inhibition of COX enhances, eosinophil adhesion and migration. Therefore endothelial dysfunction might be a mechanistic factor in eosinophilic inflammation, such as bronchial asthma, and PGI₂ analogues might be of therapeutic potential.

METHODS**Reagents**

All laboratory reagents were from Sigma (Vienna, Austria), unless specified. Assay buffer was made from Dulbecco modified PBS (with 0.9 mmol/L Ca²⁺ and 0.5 mmol/L Mg²⁺; Invitrogen, Vienna, Austria), 0.1% BSA, 10 mmol/L HEPES, and 10 mmol/L glucose, pH 7.4. Human eotaxin was from Peprotech (London, United Kingdom). PGD₂, polyclonal anti-IP antibody, the corresponding blocking peptide, and the IP antagonist CAY10441 were purchased from Cayman (Ann Arbor, Mich). Anti-rabbit IgG secondary antibody conjugated with Alexa fluor-488 was from Invitrogen. Monoclonal antibody 24 was from Hycult (Uden, The Netherlands). The adenylyl cyclase inhibitor SQ22536 was supplied by Biomol (Hamburg, Germany). CellFix and FACS-Flow were from Becton Dickinson (Vienna, Austria). Fixative solution was prepared by adding 9 mL of distilled water and 30 mL of FACS-Flow to 1 mL of CellFix. Phalloidin-Texas Red was from Molecular Probes (Eugene, Ore). Vectashield/DAPI was from Vector Laboratories (Burlingame, Calif). Drugs were dissolved in ethanol or dimethyl sulfoxide and further diluted in assay buffer to produce a final concentration of the solvents of less than 0.1%.

Preparation of human peripheral blood eosinophils

Blood was sampled from healthy nonatopic volunteers who were not taking any medication after informed consent was provided, according to a protocol approved by the Ethics Committee of the Medical University of Graz. Polymorphonuclear leukocytes (containing eosinophils and neutrophils) were prepared by means of dextran sedimentation of erythrocytes, followed by centrifugation on Histopaque gradients, as previously described.⁷ Purified eosinophils were prepared by using negative magnetic selection with antibody cocktails (CD2, CD14, CD16, CD19, CD56, and glycophorin A) and colloidal magnetic particles from StemCell Technologies (Vancouver, Canada). The resulting purity and viability of eosinophil and neutrophil preparations was typically greater than 95%.

Culture of endothelial cells

Human lung microvascular endothelial cells cryopreserved as tertiary cultures were purchased from Lonza (Verviers, Belgium) and were maintained in EGM-2 MV Bullet medium (Lonza) with 5% FCS. All culture surfaces were precoated with 1% gelatin for 1 hour at 37°C to promote endothelial cell attachment and growth. Endothelial cells were passaged at 80% to 90% confluence and were used within 4 passages.^{19,20} PGI₂ was measured as its stable metabolite, 6-keto-PGF_{1α}, by using an enzyme immunoassay (Cayman). PGE₂ was quantified by using an in-house RIA.²¹

Migration assays

Chemotaxis of eosinophils or neutrophils (10⁵ per well) was determined by using a 48-well micro-Boyden chamber with a 5-μm pore-size PVP-free

polycarbonate filter (NeuroProbe, Inc, Gaithersburg, Md) at 37°C for 1 hour. Cells that had migrated into the bottom wells of the chamber were counted with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, Calif), and eosinophils or neutrophils were distinguished from contaminating cells by means of forward-scatter/side-scatter gating and autofluorescence, as previously described.^{7,22}

Endothelial cells were grown to confluence in 3-μm filters in 6.5-mm Transwell inserts (Corning, NY) to study eosinophil transmigration through endothelial monolayers. Confluence of the monolayers was confirmed by measuring transendothelial electrical resistance with an Endohm device (WPI, Sarasota, Fla).¹⁹ The inserts were placed into 24-well culture plates containing medium or eotaxin in the bottom well and 3 × 10⁵ eosinophils in the top well. The plates were incubated at 37°C in a humidified incubator for 4 hours, and eosinophils that had migrated to the bottom wells were enumerated by means of flow cytometry.^{19,20}

Adhesion assays

Adhesion of purified eosinophils to fibronectin was determined by incubating the eosinophils (10⁵ per well) for 30 minutes at 37°C in flat-bottom 96-well plates that had been coated with 5 μg/mL fibronectin for 2 hours.²³ After transferring the plates to ice, nonadherent cells were removed, and adherent eosinophils were harvested with an EDTA (3 mmol/L) buffer. Both nonadherent and adherent cells were enumerated by means of flow cytometry. Eosinophils were distinguished from endothelial cells on the basis of forward-scatter/side-scatter characteristics.

Adhesion of eosinophils to endothelial monolayers, which had been grown to confluence in 48-well plates, was investigated by allowing purified eosinophils (3 × 10⁵/well) to become adherent for 30 minutes at 37°C.²⁴ After transferring the plates to ice, nonadherent cells were removed, and adherent eosinophils were harvested with an EDTA (3 mmol/L) buffer. The cells were enumerated by means of flow cytometry, which allows the distinction of eosinophils from endothelial cells on the basis of forward-scatter/side-scatter characteristics.

For the staining of F-actin in eosinophils, chamber slides were coated with 5 μg/mL fibronectin for 2 hours, and purified eosinophils were then allowed to adhere for 30 minutes at 37°C. After removal of nonadherent cells, adherent eosinophils were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% BSA, stained with phalloidin-Texas Red (5 U/mL) for 20 minutes at room temperature in the dark, and mounted with Vectashield/DAPI.

Upregulation and activation of eosinophil adhesion molecules

Polymorphonuclear leukocyte preparations were incubated with agonists for 30 minutes at 37°C and then stained with anti-CD11b (fluorescein isothiocyanate, 1:30) and anti-CD16 (phycoerythrin, 1:50) antibodies. CD11b expression on CD16⁺ eosinophils was quantified by means of flow cytometry and expressed as the fold increase over baseline expression.²⁵

Eotaxin-induced activation of β₂-integrins was determined by means of indirect immunofluorescence flow cytometry with mAb 24, which recognizes activated CD11/CD18.²⁶ Purified eosinophils were incubated with vehicle or eotaxin (3 nmol/L) for 30 minutes at 37°C and then stained with mAb 24 (1:50) or isotype control antibody followed by Alexa fluor 488-labeled secondary antibody (1:500).

Flow cytometric staining of IP receptors and Western blotting

IP expression of purified human eosinophils was determined by using indirect immunofluorescence flow cytometry with polyclonal IP antibody. The nonspecific binding sites were blocked with 1% BSA for 30 minutes at 4°C. Samples were then incubated with the following antibodies, each for 30 minutes at 4°C: 20 μg/mL polyclonal rabbit IP antibody or IP antibody, the binding sites of which had been blocked with 5-fold amount of the corresponding immunogenic sequence of the IP receptor as control. Finally, 4 μg/mL goat anti-rabbit IgG secondary antibody conjugated with Alexa fluor-488 was added and cells were fixed and analyzed with flow cytometry.⁴

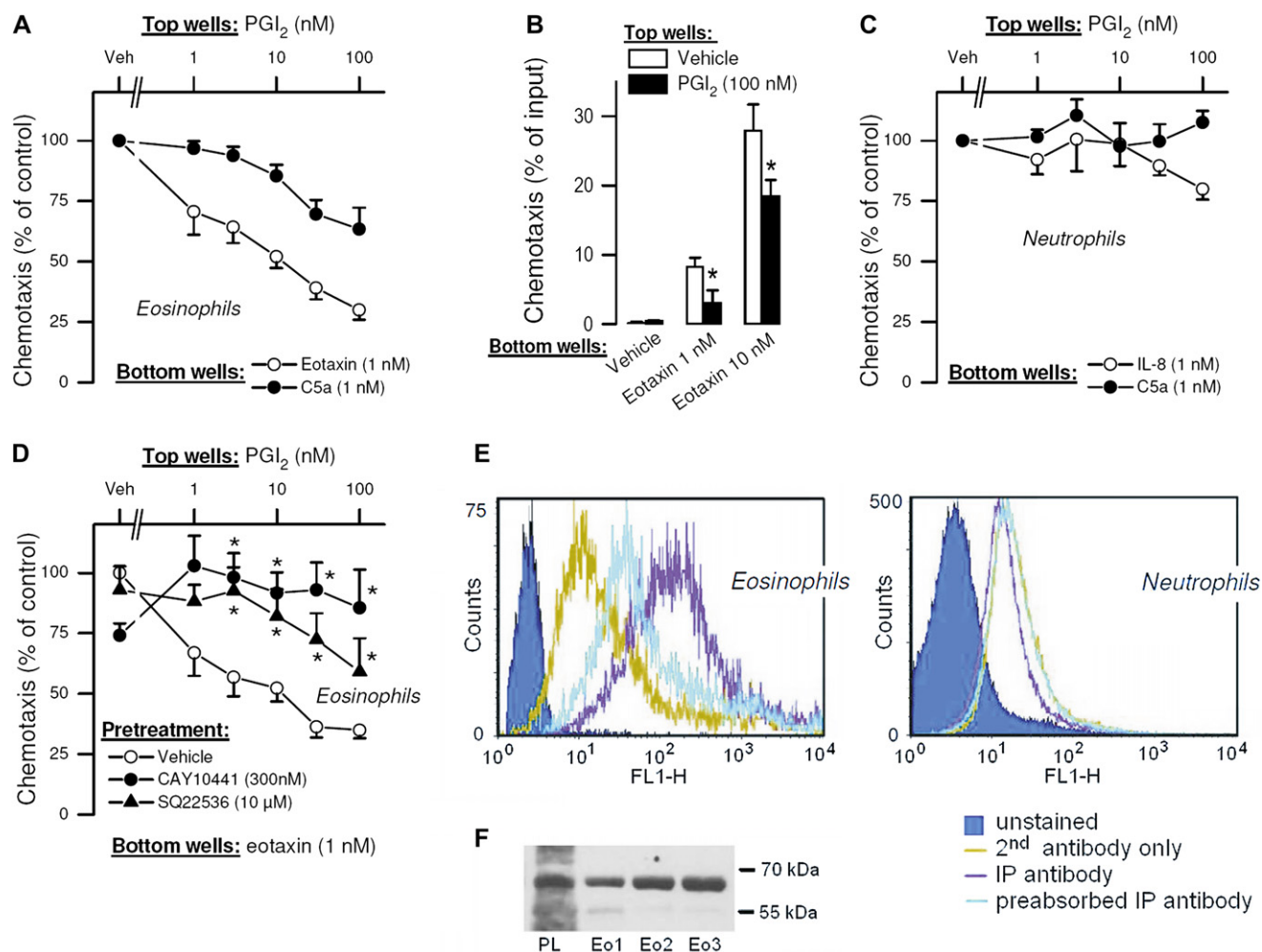


FIG 1. PGI₂ inhibits the migration of eosinophils through activation of IP receptors and adenylyl cyclase. The migration of human purified eosinophils (**A** and **B**) or neutrophils (**C**) toward eotaxin, C5a, or IL-8 was investigated in the presence of PGI₂. **D**, Eosinophils were pretreated with vehicle, the IP antagonist CAY10441, or the adenylyl cyclase inhibitor SQ22536, and migration toward eotaxin was determined in the presence of PGI₂. **E** and **F**, Expression of IP receptors on purified eosinophils was investigated by means of flow cytometry or Western blotting. Eo1-3, Eosinophils from 3 donors; PL, platelets. n = 4-8. *P < .05 versus vehicle.

For Western blot visualization of IP receptors purified eosinophils were lysed in a buffer containing 50 mmol/L Tris-HCl, 25 mmol/L KCl, 5 mmol/L MgCl₂, and 0.2% Nonidet P-40 supplemented with protease inhibitors, as previously described.¹² Protein samples (40 μg) were resolved on 10% Tris-HCl polyacrylamide gels and subsequently transferred to a nitrocellulose membrane. Membranes were probed with rabbit polyclonal IP antibodies (20 μg/mL), followed by horseradish peroxidase-conjugated anti-rabbit secondary antibodies (4 μg/mL) and Amersham ECL Plus (GE Healthcare, Vienna, Austria) detection reagents.

Fluorescence staining of endothelial cells

Endothelial monolayers were fixed (1.5% methanol and 3.7% formaldehyde in PBS) and then incubated with 100 mmol/L glycerol each for 30 minutes, followed by permeabilization with 0.1% Triton X-100 in PBS for 15 minutes at room temperature. The nonspecific binding sites were blocked with 5% goat serum in PBS for 60 minutes. Incubation with the mouse monoclonal VE-cadherin specific primary antibody (1 μg/mL) or isotype-matched control antibody was performed in PBS for 3 hours at room temperature, followed by staining with Alexa fluor 488-conjugated secondary antibody (4 μg/mL). For F-actin staining, samples were incubated with Texas Red-labeled phalloidin (5 U/mL) for 30 minutes.²⁷ After being mounted with Vectashield/DAPI

medium, the samples were analyzed on an Olympus IX70 fluorescence microscope and an Olympus UPlanApo-60x/1.20 lens using an Olympus DP50-CU digital camera and Olympus Cell^P software (Olympus, Lake Success, NY).

Statistical analyses

Data are shown as means ± SEMs for *n* observations. Comparisons of groups were performed by using 1-way ANOVA or 2-way ANOVA for repeated measurements, and *P* values of less than .05 were considered statistically significant.

RESULTS

PGI₂ inhibits eosinophil chemotaxis and adhesion

Purified human eosinophils were mixed with vehicle or serial dilutions of PGI₂ for 5 minutes at room temperature and allowed to migrate for 60 minutes in a micro-Boyden chemotaxis chamber toward eotaxin or C5a (1 nmol/L each) placed in the bottom wells. Fig 1, A, shows that PGI₂ markedly inhibited the migration of human eosinophils toward eotaxin and C5a achieving 50% inhibition at concentrations of 3 to 10 nmol/L. On average, migration

stimulated by 1 nmol/L eotaxin amounted to 10% of input cells. When 10 nmol/L eotaxin was used, which induced the migration of 20% to 30% of eosinophils added to the top wells of the chemotaxis chamber, the inhibitory effect of PGI₂ was still evident (Fig 1, B). In contrast, the same concentrations of PGI₂ did not affect the migration of neutrophils toward IL-8 (1 nmol/L) or C5a (1 nmol/L; Fig 1, C). Pretreatment of eosinophils for 15 minutes with an IP antagonist, CAY10441 (300 nmol/L), or an inhibitor of adenylyl cyclase, SQ22536 (10 μ mol/L), prevented the PGI₂ inhibition of eosinophil chemotaxis (Fig 1, D). Accordingly, eosinophils expressed the cognate PGI₂ receptor IP, as determined by means of indirect flow cytometric immunostaining. Specificity of the IP antibody was demonstrated by preabsorption of the specific binding sites of the antibody with the immunogenic peptide sequence of the IP receptor, which markedly reduced the binding of the antibody to the cells (Fig 1, E). In contrast, neutrophils did not express IP receptors. The presence of IP receptors on eosinophils was also confirmed by means of Western blotting with the same anti-IP antibody, yielding a band with the predicted molecular size of 67 kD (Fig 1, F). These data showed that PGI₂ attenuates eosinophil chemotaxis by activating an IP/cAMP signaling pathway.

The effect of PGI₂ on eosinophil adhesion to fibronectin was investigated by mixing purified eosinophils with vehicle or PGI₂ (100 nmol/L), which resulted in a short preincubation of eosinophils with PGI₂ at room temperature. Eosinophils were then applied to fibronectin-coated 96-well plates containing increasing concentrations of eotaxin (0.3-3 nmol/L) and allowed to become adherent for 30 minutes. Fig 2, A, demonstrates that PGI₂ reduced adhesion of eosinophils to fibronectin by 40% to 60%. F-actin staining of adherent eosinophils revealed distinct morphologic alterations after PGI₂ treatment. Fig 2, B, shows that unstimulated eosinophils were round but acquired a polarized elongated shape and formed discrete protrusions containing dense F-actin filaments that appeared consistent with podosomes on activation with 3 nmol/L of eotaxin. PGI₂ pretreatment of eosinophils did not prevent the eotaxin-induced polarization of the cells but abrogated their ability to form podosome-like structures (Fig 2, B). Quantitatively, eotaxin-stimulated eosinophils expressed an average of 19.9 ± 1.9 of these structures (eosinophil preparations from 3 different donors), whereas PGI₂ reduced their number to 6.6 ± 1.7 ($P < .01$). Because increased podosome formation and adhesiveness of eosinophils after allergen challenge has been linked to the adhesion molecule CD11b/CD18,²⁸ we investigated whether PGI₂ was also able to block the rapid upregulation of CD11b on the cell membrane of eosinophils, a process that might be important for mediating cell adhesion and activation. Fig 2, C, shows that stimulation of eosinophils with eotaxin (0.01-10 nmol/L) concentration-dependently upregulated the cell-surface expression of CD11b and that PGI₂ pretreatment (100 nmol/L) of eosinophils attenuated this response. The effect of PGI₂ was mimicked by the adenylyl cyclase activator forskolin (20 μ mol/L), which likewise blunted the eotaxin-stimulated CD11b upregulation. Moreover, PGI₂ prevented the eotaxin-induced activation of CD11b/CD18 complexes, as revealed by flow cytometric staining of eosinophils with mAb 24 antibodies that specifically bind to β_2 -integrins in the active conformation (Fig 2, D). These data suggest that PGI₂ blocks the mobilization of intracellular pools of integrins and their activation and prevents the morphologic changes of eosinophils that are required for effective adhesion.

Endogenous PGI₂ attenuates eosinophil-endothelial interaction

The role of endogenous PGI₂ production in eosinophil adhesion and transmigration was studied. Human lung microvascular endothelial cells produced significant amounts of PGI₂, as estimated by its stable metabolite 6-keto-PGF_{1 α} in the supernatants (133 ± 38 pg/mL in 5 hours, $n = 4$) but not PGE₂ (data not shown). Treatment of endothelial cells with the COX inhibitor diclofenac (10 μ mol/L) at a concentration that corresponds to plasma levels in patients²⁹ attenuated the PGI₂ biosynthesis by 90% to 95% (12 ± 5 pg/mL, $n = 4$). Purified eosinophils were then added to confluent endothelial monolayers in the absence or presence of various concentrations of eotaxin (0.3-3 nmol/L) or PGD₂ (10-100 nmol/L) to study eosinophil adhesion. The portion of adherent eosinophils relative to the total numbers of eosinophils added was determined after 30 minutes' incubation at 37°C. Fig 3 demonstrates that the diclofenac-induced abrogation of PGI₂ biosynthesis or blocking IPs markedly enhanced the adhesiveness of eosinophils to the endothelial monolayers, both under basal conditions and after stimulation of eosinophils with eotaxin or PGD₂. The effect of diclofenac on eosinophil adhesion to endothelial cells appeared to be a dual one: first, baseline adhesion increased (Fig 3, A and C), and second, the responsiveness of eosinophils to stimulation was enhanced, which became apparent when data were expressed as a percentage increase over baseline value (Fig 3, D). Moreover, pretreatment of eosinophils with the IP antagonist CAY10441 (300 nmol/L) mimicked the effect of diclofenac to enhance eosinophil adhesion to endothelial cells (Fig 3, B).

In further experiments we assessed the effect of endogenous PGI₂ on eosinophils undergoing transendothelial migration. To this end, the endothelial cells were grown to confluent monolayers in Transwell inserts. Confluence of the monolayers was confirmed by measuring transendothelial electrical resistance. Mean electrical resistance of endothelial monolayers increased to values of 75 to 106 Ω and remained constant thereafter. Diclofenac (10 μ mol/L) or its solvent were supplemented to the cell medium 2 or 5 hours before purified eosinophils were added to the top wells of the Transwell inserts. Eosinophils were allowed to migrate toward vehicle or eotaxin (1 nmol/L) in the bottom wells for 4 hours. Unexpectedly, transendothelial electrical resistance of the endothelial monolayers was reduced after diclofenac treatment (Fig 4, A). Eotaxin enhanced the portion of eosinophils that had migrated through the endothelial monolayer into the bottom wells by 3- to 4-fold compared with vehicle, and pretreatment of the monolayers with diclofenac further enhanced the chemotactic response to eotaxin (Fig 4, B). Interestingly, the duration of COX inhibition in endothelial cells was correlated with the enhancement of eosinophil transendothelial migration, with 5 hours of diclofenac treatment being more effective than 2 hours of treatment (Fig 4, B). Under these conditions, approximately 6% of input eosinophils could be recovered from the bottom wells. Moreover, transendothelial electrical resistance of the endothelial monolayers was even further decreased after eosinophil transmigration (Fig 4, A). These observations suggested that the loss of endogenous PGI₂ not only augmented eosinophil transmigration but also affected the barrier function of the endothelial monolayer.

Cells were grown on glass cover slips and treated with diclofenac for 2 and 5 hours to investigate in more detail whether inhibition of COX altered the barrier function of the endothelial

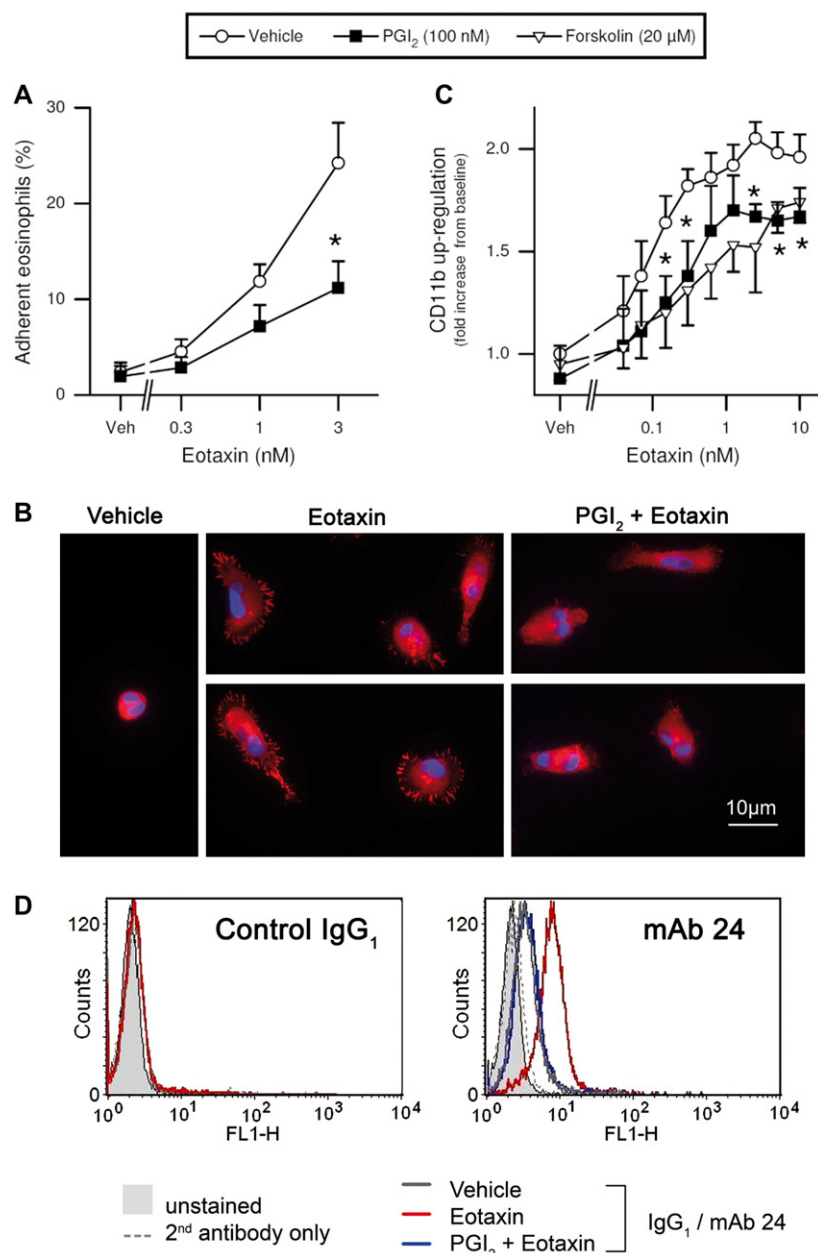


FIG 2. PGI₂ inhibits eosinophil binding to fibronectin and attenuates the upregulation and activation of the adhesion molecule CD11b in eosinophils. **A**, Adhesion to fibronectin-coated 96-well plates was stimulated by eotaxin, and results were expressed as the portion of adherent cells relative to eosinophils added. **B**, Representative micrographs of eosinophils in the absence or presence of eotaxin (3 nmol/L) and PGI₂ (100 nmol/L) adhering to fibronectin. Cells were stained with phalloidin–Texas Red and the nuclear dye DAPI (blue). **C**, Eotaxin-induced upregulation of CD11b is shown as a fold increase of baseline expression. **D**, Flow cytometric staining with isotype control antibody or mAb 24, an antibody directed against activated CD11b/CD18, of eosinophils incubated in the absence or presence of eotaxin (3 nmol/L), PGI₂ (100 nmol/L), or both. *n* = 4–8. **P* < .05, PGI₂ versus vehicle (*Veh*).

monolayers. Fluorescence staining of VE-cadherin, a major determinant of endothelial permeability by forming adherens junctions, and F-actin was carried out. The vehicle-treated endothelial monolayers presented with tight, closed cell junctions and subtle F-actin staining mainly distributed to the subcortical actin rim (Fig 5). Diclofenac treatment disrupted the endothelial cell junctions, and after 5 hours of diclofenac treatment, even pore formation between endothelial cells was detected. As a result

of blocked PGI₂ biosynthesis, more intense actin polymerization was observed, with formation of F-actin stress fibers after 2 hours and redistribution of dense F-actin filaments to the subcortical region after 5 hours. Coincubation of endothelial cells with diclofenac plus the stable PGI₂ analogue iloprost (300 nmol/L) prevented the loss of tight adherent junctions and gap formation and also delayed the reorganization of the actin cytoskeleton (Fig 5).

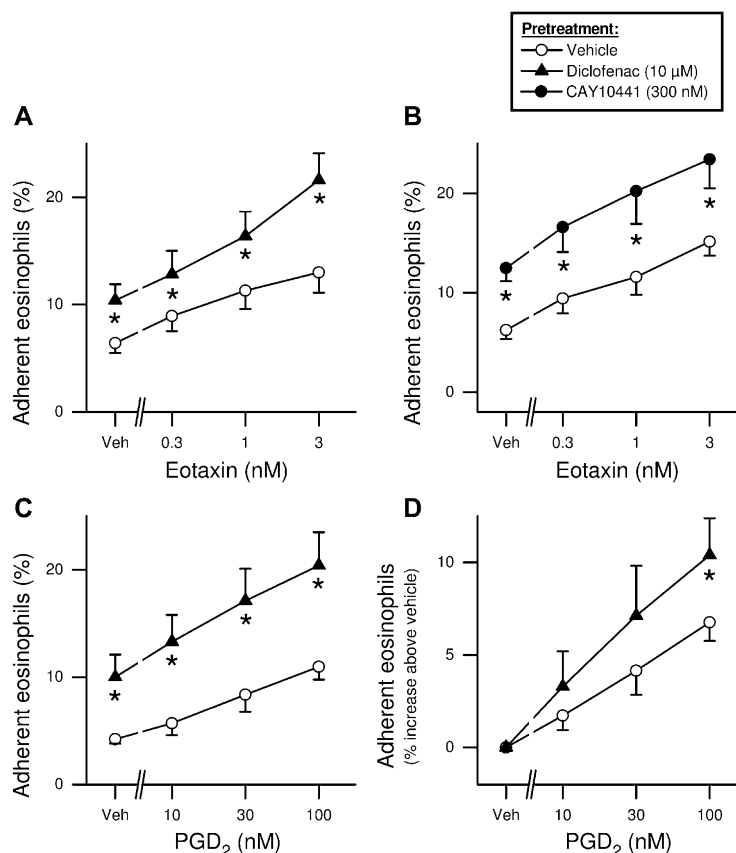


FIG 3. Endogenous prostaglandins attenuate the adhesion of eosinophils to endothelial cells. Endothelial cells were pretreated with vehicle (*Veh*) or the COX inhibitor diclofenac, or eosinophils were pretreated with the IP antagonist CAY10441 or its vehicle. Eosinophils were added to the cultures in the absence or presence of various concentrations of eotaxin (**A** and **B**) or PGD₂ (**C** and **D**). In **Fig 3, D**, Data from **Fig 3, C**, were replotted as the percentage increase above baseline (ie, in the presence of diclofenac or its vehicle but in the absence of eotaxin or PGD₂). $n = 6-8$. * $P < .05$ versus vehicle.

DISCUSSION

In this study we have shown that endothelium-derived PGI₂ is an effective modulator of eosinophil locomotion by attenuating eosinophil chemotaxis, adhesion, and transmigration through endothelial monolayers. From our data, it appears that the effect of PGI₂ is brought about by a dual action (1) by directly targeting eosinophils through IP receptors and activating adenylyl cyclase and (2) by strengthening endothelial barrier function. The latter effect became evident after inhibition of PGI₂ biosynthesis with a COX inhibitor, which disrupted the texture of endothelial monolayers and markedly augmented eosinophil transmigration through the endothelium. These observations suggested that the endothelium plays a key role through PGI₂ as a gatekeeper by limiting the extravasation of eosinophils from the circulation into the tissue and that endothelial dysfunction, where PGI₂ biosynthesis is compromised, might contribute to eosinophilic inflammation in patients with allergic disease.

In detail, we observed that eosinophils express the PGI₂ receptor IP and that exogenously added PGI₂ markedly inhibited the migration of eosinophils through polycarbonate filters toward the chemoattractants eotaxin and C5a. The involvement of IP receptors was confirmed by a selective IP antagonist, CAY10441, which completely prevented the inhibitory effect of PGI₂ on eosinophil migration but had no effect on the inhibitory effect of

PGE₂ on eosinophil migration toward eotaxin (data not shown). The inhibitory effect of PGI₂ appeared to be selective for eosinophils because neutrophil migration toward IL-8 was not attenuated by PGI₂. We have recently observed that monocyte-derived PGE₂ attenuates the locomotion of eosinophils through stimulation of the PGE₂ receptors EP2 and EP4.⁴ Although activation of both these receptors has been shown to stimulate cAMP production, the inhibitory effect of PGE₂ was not reversed by the adenylyl cyclase inhibitor SQ22536 or the protein kinase A inhibitor H-89 but was brought about by phosphatidylinositol 3-kinase and protein kinase C.⁴ In contrast, the current data have shown that the inhibitory effect of PGI₂ is largely mediated by cAMP increase because the adenylyl cyclase inhibitor SQ22536 reversed the PGI₂-induced attenuation of eosinophil chemotaxis.

In further experiments we observed that PGI₂ inhibited the adhesion of eosinophils to fibronectin, as induced by eotaxin. Morphologic studies suggested that PGI₂ did not affect the eotaxin-induced flattening and polarization of eosinophils. Similarly, eotaxin-induced actin-dependent rearrangement of the cytoskeleton, as investigated with the flow cytometric shape-change assay, was not altered by PGI₂ (data not shown). In contrast, F-actin staining of adherent eosinophils showed that the eotaxin-stimulated formation of F-actin-dense protrusions, most likely podosomes, was specifically inhibited by PGI₂. Podosomes are

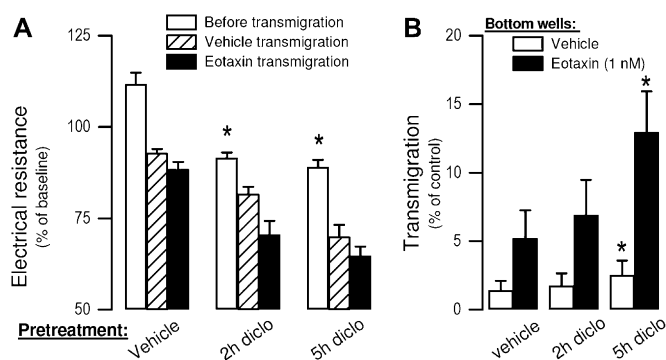


FIG 4. Inhibition of prostaglandin synthesis disturbs the barrier function of endothelial monolayers and enhances the transmigration of eosinophils. Endothelial cells that had been cultured in Transwell inserts until confluence were pretreated with vehicle or the COX inhibitor diclofenac (*diclo*; 10 μ mol/L) for 2 or 5 hours. Thereafter, eosinophils were added to the top wells and were allowed to transmigrate for 4 hours. **A**, Transendothelial electrical resistance was measured before and after migration and was expressed as a percentage of baseline value (ie, before diclofenac was added). **B**, Eosinophils that had migrated to the bottom wells were enumerated by means of flow cytometry, and data were expressed as a percentage of cells that had migrated toward eotaxin through cell-free filters. $n = 3-6$. * $P < .05$, diclofenac versus vehicle of diclofenac.

transient assemblies of cytoskeletal and membrane proteins that are believed to be important for cell movement and proteolysis of vascular cell adhesion molecule 1 and matrix proteins.^{30,31} Interestingly, airway eosinophils purified from antigen-challenged subjects demonstrated enhanced podosome formation and upregulation of the adhesion molecule Mac-1 (CD11b/CD18) and were more adhesive and migratory than unchallenged eosinophils.^{28,32} We found that the rapid eotaxin-induced upregulation of the adhesion molecule CD11b was attenuated by PGI₂, and this effect was mimicked by the adenylyl cyclase activator forskolin. Moreover, activation of the CD11/CD18 complex was likewise prevented by PGI₂. Apart from adhesion, CD11b/CD18 is an important modulator of leukocyte responses, including migration,³³ phagocytosis,³⁴ mediator release,³⁵ and apoptosis.³⁶ Therefore it is conceivable that the PGI₂-induced reduction of CD11b expression and activation might have profound effects on eosinophil accumulation and function at sites of inflammation.

To investigate the role of endogenous PGI₂, we used human pulmonary microvascular endothelial cells as a physiologic source of PGI₂ and studied the interaction of eosinophils and the endothelium. In fact, inhibition of PGI₂ biosynthesis in endothelial cells by using a COX inhibitor, which almost completely abrogated PGI₂ biosynthesis, or an IP antagonist markedly enhanced eosinophil adhesion to the endothelium on the one hand and augmented the transendothelial migration of eosinophils on the other hand. These observations demonstrated that endothelium-derived PGI₂ was able to control the migratory capacity of eosinophils. PGI₂ did not attenuate eosinophil migration by reducing eosinophil viability because a 3-hour incubation of eosinophils with PGI₂ or the IP agonist iloprost (30-100 nmol/L) did not alter the binding of annexin V, a measure of apoptosis, or the uptake of propidium iodide, which is indicative of necrosis (unpublished observation). Because endothelial cells also express IP receptors³⁷ and an IP agonist was found to enhance endothelial barrier formation,²⁷ we tested the hypothesis that inhibition of endogenous PGI₂ biosynthesis might enhance eosinophil transmigration also by compromising the barrier function of the

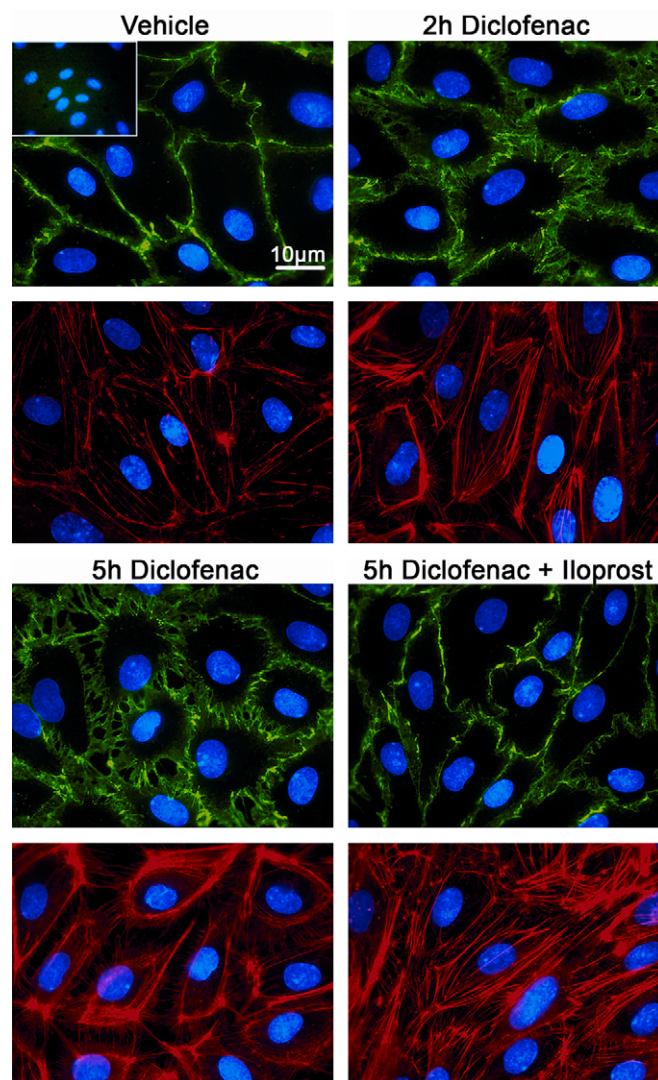


FIG 5. Fluorescence staining of VE-cadherin and F-actin reveals that inhibition of prostaglandin biosynthesis disintegrates the texture of endothelial monolayers. Endothelial cells that had been cultured until confluence were pretreated with vehicle, the COX inhibitor diclofenac (10 μ mol/L), or diclofenac plus iloprost (100 nmol/L) and were then stained with control antibody (*small insert*), anti-VE-cadherin antibody (green), or phalloidin-Texas Red and the nuclear stain DAPI (blue). The stainings shown are representative of 3 independent experiments.

endothelium. Incubation of endothelial monolayers with the COX inhibitor in fact reduced the transendothelial electrical resistance of the monolayers. This effect corresponded with disruption of endothelial adherent junctions after COX inhibition, as revealed by immunostaining of VE-cadherin, which was accompanied by actin rearrangement and formation of stress fibers. These morphologic alterations could be largely prevented by treatment of the monolayers with the IP agonist iloprost, which unequivocally showed that the lack of PGI₂ leads to enhanced permeability of the endothelial monolayer.

Cell migration is a complex process that includes actin rearrangement, polarization, modulation of adhesion molecules, adhesion, and detachment from substrate in a highly coordinated manner. Previous studies have shown that PGI₂ analogues are capable of attenuating the endothelial interaction of neutrophils and

monocytic cells.^{13,38,39} In this study we could demonstrate that endothelium-derived PGI₂ is an important modulator of eosinophil-endothelial interaction and might have a bearing on eosinophil accumulation at sites of allergic reaction. Our data might hence explain previous findings that deletion of IP receptors in mice augments the eosinophilic infiltrate in allergic responses of the lung and skin and enhances airway remodeling.^{8,9} Moreover, eosinophil influx has been shown to be exaggerated in COX-1 or COX-2 knockout mice^{40,41} and also in mice treated with selective COX-1 or COX-2 inhibitors,⁴² which might be consistent with the loss of the inhibitory action of PGI₂ on transendothelial migration of eosinophils. Endothelial dysfunction has been described in patients with bronchial asthma,⁴³ and plasma levels of 6-keto-PGF_{1α}, the stable metabolite of PGI₂, are significantly lower during symptomatic periods in asthmatic patients than in healthy subjects.⁴⁴ Interestingly, the plasma levels of 6-keto-PGF_{1α} after allergen challenge were inversely correlated with the frequency of asthmatic late-phase responses,⁴⁵ which are usually accompanied by increased eosinophil infiltration of the bronchi. Therefore clinical data suggest that attenuated release of PGI₂ caused by endothelial dysfunction or, alternatively, treatment with COX inhibitors might favor allergic late-phase responses. In fact, COX inhibitors precipitate asthma attacks in approximately 10% of asthmatic patients, which is referred to as aspirin-sensitive asthma, and eosinophils are consistently found in nasal and bronchial secretions, as well as in bronchial biopsy specimens of patients afflicted with aspirin-induced asthma.⁴⁶ The loss of the anti-inflammatory effect of PGI₂ after intake of COX inhibitors might be counterbalanced in patients with aspirin-tolerant asthma by means of concomitant abrogation of proinflammatory prostaglandins, such as PGD₂ or PGH₂, which are potent chemoattractants for eosinophils.^{47,48}

In conclusion, endothelial release of PGI₂ might be an important defense mechanism against inappropriate eosinophil infiltration and might limit allergic responses by means of a dual action: (1) inhibition of eosinophil responsiveness to chemoattractants in terms of adhesion and migration and (2) strengthening of the barrier function of the endothelium against infiltrating leukocytes. Therefore IP agonists might be a useful therapeutic option for otherwise inadequately controlled inflammation in patients with eosinophilic diseases by blunting their extravasation into tissue.

Clinical implications: The ability of PGI₂ to inhibit eosinophil trafficking suggests that PGI₂ analogues might provide a potential therapeutic approach to control eosinophilic inflammatory conditions, such as bronchial asthma.

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