

EP4 receptor stimulation down-regulates human eosinophil function

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Received: 3 May 2010/Revised: 7 February 2011/Accepted: 15 February 2011/Published online: 2 March 2011
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Abstract Accumulation of eosinophils in tissue is a hallmark of allergic inflammation. Here we observed that a selective agonist of the PGE₂ receptor EP4, ONO AE1-329, potently attenuated the chemotaxis of human peripheral blood eosinophils, upregulation of the adhesion molecule CD11b and the production of reactive oxygen species. These effects were accompanied by the inhibition of cytoskeletal rearrangement and Ca²⁺ mobilization. The involvement of the EP4 receptor was substantiated by a selective EP4 antagonist, which reversed the inhibitory effects of PGE₂ and the EP4 agonist. Selective kinase inhibitors revealed that the inhibitory effect of EP4 stimulation on eosinophil migration depended upon activation of PI 3-kinase and PKC, but not cAMP. Finally, we found that EP4 receptors are expressed by human eosinophils, and are also present on infiltrating leukocytes in inflamed

human nasal mucosa. These data indicate that EP4 agonists might be a novel therapeutic option in eosinophilic diseases.

Keywords Eosinophils · Prostaglandins · Receptors · Chemotaxis · Reactive oxygen species · Degranulation

Abbreviations

BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
COX	Cyclooxygenase
CREB	cAMP response element binding protein
EP	E-type prostanoid receptor
ERK	Extracellular signal-regulated kinase
FITC	Fluorescein isothiocyanate
IL	Interleukin
IP	I-type prostanoid receptor
LT	Leukotriene
PE	Phycoerythrin
PG	Prostaglandin
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
TP	Thromboxane-type prostanoid receptor

Electronic supplementary material The online version of this article (doi:10.1007/s00018-011-0642-5) contains supplementary material, which is available to authorized users.

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Introduction

Eosinophils play a major role in late-phase reactions by releasing bronchoconstrictor mediators such as leukotriene (LT) C₄ and other chemoattractants that cause further influx of inflammatory cells into the tissue, and immunoregulatory type-2 cytokines, interleukin (IL)-4, IL-5, IL-10,

and IL-13 [1]. Mucosal damage in chronic asthma is associated with cytotoxic mediators that are released by activated eosinophils, including matrix metalloproteases, major basic protein, eosinophil cationic protein, eosinophil peroxidase and eosinophil-derived neurotoxin, leading to airway remodeling and angiogenesis in chronically inflamed tissue [2, 3]. Importantly, it was shown that asthmatic patients who receive treatment based on eosinophil counts in sputum have significantly fewer severe asthma exacerbations than patients treated according to standard management therapy [4]. Therefore, eosinophils are currently considered a major therapeutic target in allergic diseases and asthma [5], eosinophilic esophagitis [6], colitis ulcerosa [7], or hypereosinophilic syndrome [8].

PGE₂ is the predominant cyclooxygenase (COX) product of airway macrophages, epithelial cells, and smooth muscle cells, and is regarded as a potent inflammatory mediator due to its effects on vasodilation, vascular permeability, and nociception. However, the role of PGE₂ in allergic inflammation is less clear. In the asthmatic lung, PGE₂ affects both airway smooth muscle and the inflammatory process: PGE₂ causes bronchial relaxation [9] and inhibits allergen-induced bronchoconstriction [10], but it may also provoke bronchoconstrictor responses and cough in some individuals [11, 12] because of activation of C-fibers and reflex cholinergic pathways [13]. In rats and humans, PGE₂ reduces allergen-induced airway eosinophilia [14, 15], attenuates anaphylactic mediator release from guinea-pig perfused lungs [16], abrogates eosinophil accumulation after passive cutaneous anaphylaxis in guinea pigs [17], and protects against bleomycin-induced pulmonary fibrosis in mice [18]. Conversely, eosinophil influx is exaggerated in COX-1 or COX-2 knockout mice [19, 20] and also in mice treated with selective COX-1 or COX-2 inhibitors [21]. At the cellular level, PGE₂ has been found to attenuate immunoglobulin-dependent degranulation and LTC₄ biosynthesis of eosinophils [22], and agonist-induced CD11b upregulation and L-selectin shedding in eosinophils and neutrophils [23]. In contrast, PGE₂ is anti-apoptotic for eosinophils [24], while its analogue misoprostol inhibits eosinophil survival *in vitro* [25].

The biological effects of PGE₂ are mediated through four different G protein-coupled heptahelical receptors, termed EP1, EP2, EP3, and EP4 [26]. Each of these receptors has a distinct pharmacological signature based on its selectivity towards synthetic PGE₂ analogs and intracellular signal transduction. Stimulation of the EP1 receptor results in G_q-mediated activation of phospholipase C and phosphatidylinositol hydrolysis, elevation of the intracellular Ca²⁺ level, and causes the activation of protein kinase C (PKC) [27]. The EP3 receptor exists as a number of splice variants displaying various degrees of

constitutive activity. EP3 signals through activation of a G_i protein to inhibit adenylyl cyclase leading to reduction of intracellular cyclic adenosine monophosphate (cAMP) generation and elevation of intracellular-free Ca²⁺ levels. However, isoforms of EP3 also have the capacity to enhance cAMP formation by coupling to G_s protein [28]. In contrast, stimulation of EP2 and EP4 receptors usually increases intracellular cAMP levels and activates protein kinase A (PKA) through G_s protein. Interestingly, EP2 receptor stimulation can also trigger Ca²⁺ currents in a cAMP-dependent/PKA-independent manner [29].

We recently described that stimulation of EP2 receptors attenuates eosinophil trafficking [30], and we also obtained some preliminary information on a potential role of EP4 receptors in the regulation of eosinophil function. Therefore, in this study we investigated the expression, function, and signaling of EP4 receptors in eosinophils in detail and suggest that EP4 receptors are even more substantially involved in the regulation of eosinophil effector functions as compared to EP2 receptors. EP4 agonists might hence be novel therapeutic options for the treatment of eosinophilic diseases.

Materials and methods

Chemicals

All laboratory reagents were from Sigma (Vienna, Austria), unless specified. Assay buffer used in all experiments was made from Dulbecco's modified phosphate-buffered saline (PBS; with 0.9 mM Ca²⁺ and 0.5 mM Mg²⁺; Invitrogen, Vienna, Austria), 0.1% bovine serum albumin (BSA); 10 mM HEPES and 10 mM glucose, pH 7.4. CellFix and FACS-Flow were from Becton-Dickinson (Vienna, Austria). Human eotaxin and IL-8 were from Peprotech (London, UK). PGD₂, PGE₂, the EP2 receptor agonist butaprost, the EP1/EP3 receptor agonist sulprostone, the EP4 receptor antagonist GW627368X and the EP1/I-type prostanoid (IP) receptor agonist iloprost were from Cayman Chemicals (Ann Arbor, MI, USA). Polyclonal rabbit anti-human EP4 receptor antibodies directed against the C terminus of the receptor were provided by Sigma and Cayman. Goat antibody against eosinophil peroxidase was from Santa Cruz Biotechnology (Heidelberg, Germany). Mouse anti-eosinophil peroxidase antibody was supplied by Becton-Dickinson. Donkey anti-mouse Alexa Fluor 555 and donkey anti-rabbit Alexa Fluor 488 antibodies were obtained from Invitrogen. Rabbit and goat control IgG were from Linaris (Wertheim-Bettingen, Germany). The adenylyl cyclase inhibitor SQ22536, the PI3K inhibitor LY294002 and the PKC inhibitor chelerythrine were supplied from Biomol (Hamburg, Germany). The EP4 agonist

ONO AE1-329 (2-[3-[(1R,2S,3R)-3-hydroxy-2-[(E,3S)-3-hydroxy-5-[2-(methoxymethyl)phenyl]pent-1-enyl]-5-oxocyclopentyl]sulfanylpropylsulfanyl]acetic acid) and the EP4 antagonist ONO AE3-208 (2-[[2-[2-(2-methylnaphthalen-1-yl)propanoylamino]phenyl]methyl]benzoic acid) were a kind gift from ONO Pharmaceutical (Osaka, Japan). ONO AE1-329 has been shown in competitive radioligand binding assays to selectively bind to EP4 receptors ($K_i = 9.7$ nM) relative to the EP1, EP2, and EP3 receptors ($K_i = > 10,000$, $> 2,000$ and $> 1,000$ nM, respectively) [31]. GW627368X (4-(4,9-dioxy-1,3-dihydro-1-oxo-2H-benz[f]isoindol-2-yl)-*N*-(phenyl sulfonyl)-benzeneacetamide) exhibits a K_i value of 100 nM towards EP4 and 158 nM to thromboxane-type prostanoid (TP) receptors, with K_i values above 10,000 nM for all other prostanoid receptors [32]. The K_i values of ONO AE3-208 are 1.3, 30, 790, 2,400 nM for EP4, EP3, F-type prostanoid (FP) receptor, and TP, respectively, and more than 10,000 nM for other prostanoid receptors [33].

Preparation of human leukocytes

This study was approved by the Ethics Committee of the Medical University of Graz. Prior to blood sampling from healthy non-atopic volunteers, all donors signed an informed-consent form. Platelet-rich plasma was removed by centrifugation of citrated whole blood. Erythrocytes were removed by dextran sedimentation. High-density polymorphonuclear leukocytes (PMNL, containing neutrophils and eosinophils) were isolated by Histopaque gradient centrifugation. Any erythrocyte contamination of the PMNL pellet was removed by hypotonic shock lysis as described previously [34]. Purified eosinophil preparations were obtained by negative magnetic selection using antibody cocktails (CD2, CD14, CD16, CD19, CD56, and glycophorin A) and colloidal magnetic particles from StemCell Technologies (Vancouver, Canada). Resulting purity and viability was typically $>97\%$.

Chemotaxis

Migration of eosinophils was determined in 48-well microBoyden chemotaxis chambers. Purified eosinophils were resuspended in assay buffer at 2×10^6 cells/ml and 50 μ l of the cell-suspension were loaded into the top wells of the chamber which were separated from the bottom wells by a 5- μ m pore-size polyvinylpyrrolidone-free polycarbonate filter. Thirty microliters of assay buffer or agonists were placed into the bottom wells of the chamber. Baseline migration was determined in wells containing only assay buffer. The chamber was incubated at 37°C for 1 h in a humidified incubator. The membrane was subsequently removed and migrated cells were enumerated by a

FACSCalibur flow cytometer (Becton-Dickinson, Mountain View, CA, USA) [35].

Leukocyte shape change assay

Preparations of polymorphonuclear leukocytes (PMNL; containing eosinophils and neutrophils) or purified eosinophils were resuspended in assay buffer and aliquots of the cell-suspension were mixed with agonists at a final volume of 100 μ l and stimulated for 4 min at 37°C. Cells were transferred to ice and 250 μ l of ice-cold fixative solution was added to terminate the reaction. Changes in the cell shape were estimated immediately by the increase of forward scatter using a FACSCalibur flow cytometer (Becton-Dickinson, Mountain View, CA, USA). Eosinophils were distinguished from neutrophils according to granularity (side scatter) and by their autofluorescence.

Respiratory burst

Purified eosinophils (5×10^5 cells/ml) were stimulated with agonists in the presence of 1 μ M dihydrorhodamine-123 for 20 min at 37°C and then fixed with 150 μ l of ice-cold 2.5% Cellfix. Respiratory burst of eosinophils was immediately quantified by flow cytometry as the increase of fluorescence in the FL-1 channel due to the oxidation by reactive oxygen species of the non-fluorescent dye dihydrorhodamine-123 into fluorescent rhodamine-123 [36]. Responses were expressed as percent changes from a control sample incubated with buffer alone.

Upregulation of eosinophil CD11b expression

Polymorphonuclear leukocyte preparations were incubated with agonists for 30 min at 37°C and then stained with anti-CD11b (FITC) and anti-CD16 (PE-Cy5) antibodies. CD11b expression on CD16-negative eosinophils was quantified by flow cytometry and expressed as percent of the maximal control response (i.e., in the absence of a prostanoid).

Calcium ion flux

Intracellular Ca^{2+} levels in eosinophils were analyzed by flow cytometry as described previously [37]. Polymorphonuclear leukocyte preparations were treated with 2 μ M of the acetoxymethyl ester of Fluo-3 in the presence of 0.02% pluronic F-127 for 60 min at room temperature before being washed with PBS without Ca^{2+} and Mg^{2+} . Cells were then stained with anti-CD16 (PE) and resuspended in assay buffer at 3×10^6 cells/ml. Changes in intracellular Ca^{2+} levels were detected by flow cytometry as the increase of the fluorescence of the Ca^{2+} sensitive dye

Fluo-3 in the FL1-channel. Eosinophils were identified as CD16-negative cells.

Flow cytometric analysis of EP receptor expression

Expression of EP1, EP2, EP3, and EP4 receptors on human peripheral blood eosinophils was quantified by indirect immunofluorescence flow cytometry. As the EP antibodies had been raised against the intracellular C terminus of the EP receptors, aliquots of isolated eosinophils were first permeabilized with Fix&Perm solution (ADG Bio Research; Kaumberg, Austria) for 15 min at room temperature. Samples were then treated with the following reagents for 30 min each on ice with appropriate washing steps in between: Ultra V Block (Labvision, Westinghouse, CA, USA) to block Fc receptors; 20 µg/ml polyclonal EP1, EP2, EP3 or EP4 antibody or 20 µg/ml polyclonal rabbit isotype control antibody; and 4 µg/ml anti-rabbit IgG secondary antibody conjugated with Alexa Fluor-488. After adding the fixative solution the cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Western blot of EP4 receptors

Purified eosinophils were lysed in a buffer containing 50 mM Tris-HCl, 25 mM KCl, 5 mM MgCl₂, and 0.2% Nonidet P-40 supplemented with protease inhibitors (Roche; Basel, Switzerland) and centrifuged at 10,000 rpm for 10 min at 4°C. Bradford protein assay (Bio-Rad; Vienna, Austria) was performed to determine the protein content in the supernatants. Protein samples (50 µg) were separated by SDS-polyacrylamide gel electrophoresis on a gradient gel (4–20%) and protein bands were blotted onto polyvinylidene fluoride (Bio-Rad) membrane. Target proteins were immunochemically detected using a polyclonal rabbit anti-human EP4 antibody (20 µg/ml). Bands were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG (4 µg/ml; Pierce, Rockford, IL, USA) and Amersham ECL Plus detection reagents (GE Healthcare; Vienna, Austria).

Immunohistochemistry of EP4 receptors

Paraffin blocks of nasal polyps that had been previously classified by a pathologist to contain eosinophil infiltrates were obtained from the Tissue Bank of the Medical University of Graz as approved by the local ethics committee. Five-micrometer sections were deparaffinized, antigen retrieval was performed in DakoCytomation Target Retrieval Solution (Glostrup, Denmark) for 10 min at 120°C, and sections were blocked with 1% BSA and 0.05% Triton X-100 in PBS for 30 min at room temperature. The samples were incubated overnight with a rabbit anti-human EP4

antibody (1.7 µg/ml, Sigma) or rabbit control IgG antibody. After washing, the bound antibody was detected using the Liquid DAB + Substrate Chromogen System (Dako Cytomation). Staining of eosinophil peroxidase-positive cells was performed with a polyclonal goat antibody against eosinophil peroxidase (20 µg/ml) or control antibody for 30 min at room temperature. After washing, the bound antibody was then visualized with the EnVision Permanent Red (Dako Cytomation). Slides were finally counterstained with Mayer's hematoxylin. Sections were visually examined with a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) and a Zeiss Plan-Neofluar ×20/05 lens. Photographs were taken with a CoolSNAP camera (1392 × 1040 pixels; Photometrics, Tucson, AZ, USA) and a RGB Tunable Micro*color filter (CRI, Woburn, MA, USA). Further processing of images was performed with MCID-M2 software (Imaging Research Inc., Brock University, St. Catharines, Ontario, Canada) for additional white balance, contrast, and brightness adjustments.

Double immunofluorescence stainings of eosinophil peroxidase and EP4 were performed as described [38] with incubation times of 10 and 50 min, respectively, using the following antibodies: mouse-anti-human eosinophil peroxidase antibody; rabbit anti-human EP4 antibody (12.5 µg/ml, Sigma); and donkey anti-mouse Alexa Fluor 555 or donkey anti-rabbit Alexa Fluor 488 (both 0.5 µg/ml). Images were taken with a confocal laser scanning microscope (Leica TCS SP2; Leica, Bensheim, Germany) using Leica Confocal Software version 2.61 Build 1537.

Statistical analyses

Data are shown as mean ± SEM for *n* observations. Comparisons of groups were performed using one-way ANOVA or two-way ANOVA for repeated measurements followed by Holm-Sidak post-hoc test to determine the levels of significance for each group. Probability values of *p* < 0.05 were considered as statistically significant.

Results

Involvement of EP4 receptors in the PGE₂-induced attenuation of eosinophil migration

We showed recently that PGE₂ and the EP2 agonist butaprost attenuate the migratory responsiveness of human eosinophil granulocytes [30]. Here we investigated the potential role of EP4 receptors in eosinophil function. For that purpose, we pretreated purified human eosinophils with the EP4 receptor antagonists GW627368X (1 or 10 µM) or ONO AE3-208 (100 nM) or their vehicle for 15 min at 37°C, and then mixed them with various concentrations of

PGE₂ (3–100 nM). Migration towards eotaxin (1 nM) was determined thereafter. PGE₂ led to a decrease of eosinophil migration in a concentration-dependent manner; at the highest concentration of PGE₂ (100 nM) migration was reduced by more than 70%. The inhibitory effect of PGE₂ was markedly attenuated by the selective EP4 receptor antagonists GW627368X (Fig. 1a) or ONO AE3-208 ($n = 4$, data not shown). In agreement with these findings, we also observed that the EP4-selective agonist ONO AE1-329 (3–100 nM) mimicked the effect of PGE₂ at inhibiting eosinophil migration towards eotaxin (1 nM) and PGD₂ (30 nM) with the same efficacy and potency as PGE₂ (Fig. 2a, b).

The expression of EP4 receptors on human eosinophils was investigated by indirect flow-cytometric immunostaining and Western blot. Eosinophils showed high positive staining for EP4 receptors in flow cytometry (Fig. 1b). The specificity of the EP4 staining was confirmed by applying the appropriate isotype control antibody, which gave considerably lower staining than the EP4 receptor antibody (Fig. 1b). EP4 receptors were also detected on neutrophils. EP4 expression was confirmed by Western-blot analysis

using the same EP4 antibody in three eosinophil samples from different donors and one neutrophil preparation (Fig. 1c). The EP4 receptor has previously been described both as a 65- or a 52-kDa protein [39, 40]. Our data show that it is the 65-kDa variant that is expressed by eosinophils. Moreover, we were able to confirm the expression of the other EP receptor isoforms (EP1, EP2, EP3) on circulating eosinophils by using indirect flow-cytometric staining (Suppl. Fig. S1). These data demonstrated that EP4 receptors on eosinophils negatively control locomotion.

EP4 receptors attenuate the migration of eosinophils via PI3K and PKC

We next investigated the potential pathways by which EP4 receptors control eosinophil chemotaxis. Elevated concentrations of intracellular cAMP are supposed to confer attenuation of eosinophil migration and EP4 receptors have been shown to stimulate adenylyl cyclase [26]. Purified eosinophils were pretreated with the adenylyl cyclase inhibitor SQ22536 (10 μ M) or the appropriate vehicle for 30 min at 37°C before they were mixed with varying

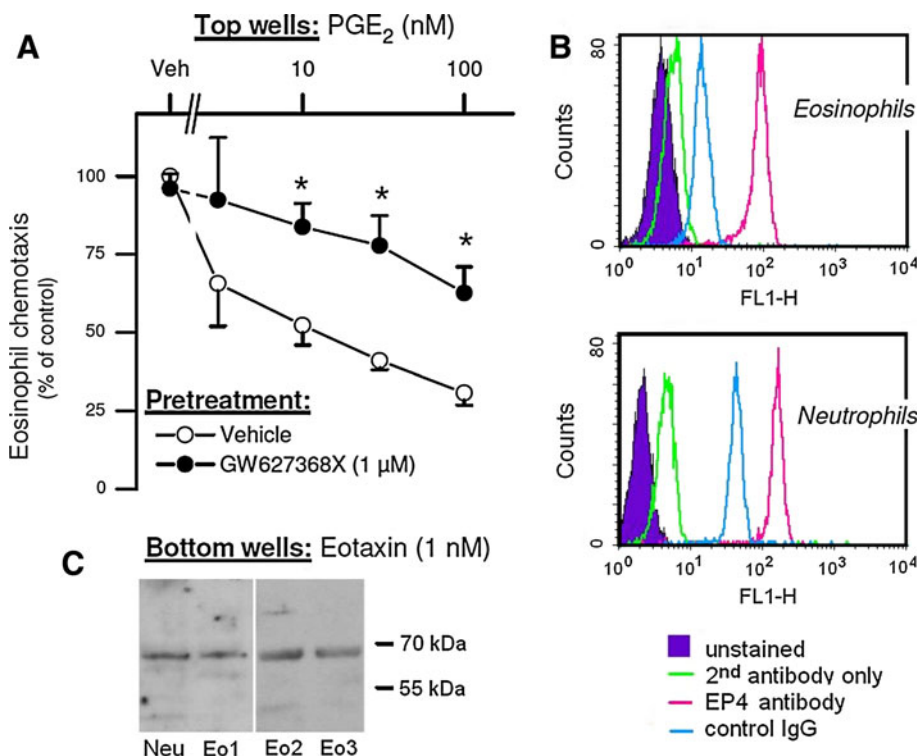


Fig. 1 EP4 receptors are expressed by human eosinophils and mediate the inhibitory effect of PGE₂ on migration. **a** Purified eosinophils were pretreated with the EP4 receptor antagonist GW627368X or vehicle, mixed with PGE₂ and loaded into the top wells of a microBoyden chamber. Cells were allowed to migrate towards eotaxin in the bottom wells. Responses were expressed as percent of the control response, i.e., to eotaxin only. **b** EP 4 receptor

expression on purified eosinophils or neutrophils was determined with indirect flow cytometric staining. The histograms show flow cytometric analyses representative of three experiments with different donors. **c** Western blot showing EP4 expression in one neutrophil sample (*Neu*) and three different eosinophil preparations (*Eo1–3*). Data are shown as mean \pm SEM, $n = 5$. * $p < 0.05$ versus vehicle

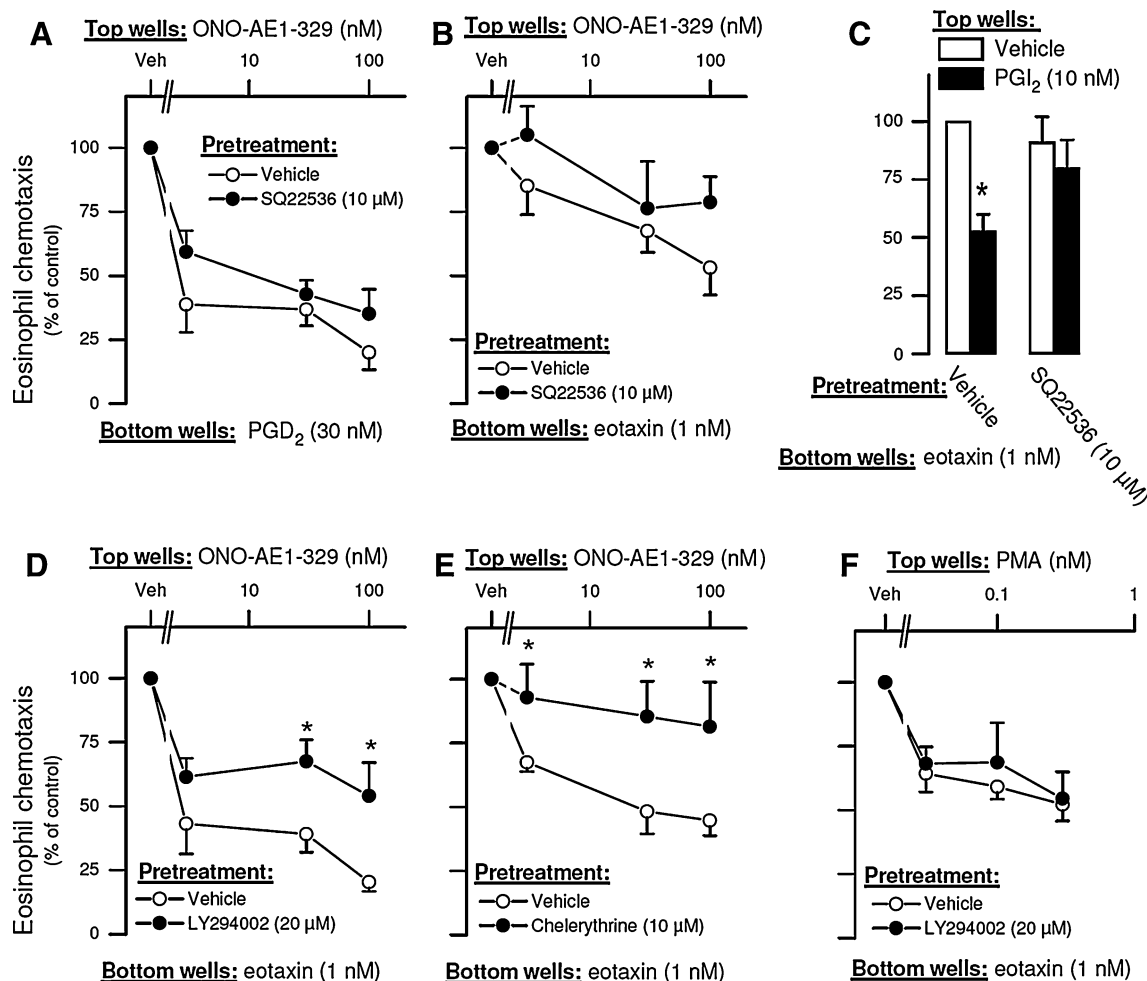


Fig. 2 The EP4 receptor mediates the attenuation of eosinophil migration via PI3K and PKC but not via PKA. **a–c** Purified eosinophils were pretreated with vehicle or the adenylyl cyclase inhibitor SQ22536, mixed with varying concentrations of the EP4 receptor agonist ONO AE1-329 or PGI₂, and migration towards PGD₂ or eotaxin was determined. **d, f** Purified eosinophils were pretreated with the PI3K inhibitor LY294002 or its vehicle, mixed with varying concentrations of the EP4 receptor agonist ONO AE1-329 or the PKC

activator agent PMA, and migration towards eotaxin was determined. **e** Purified eosinophils were pretreated with the PKC inhibitor chelerythrine or vehicle, mixed with varying concentrations of the EP4 receptor agonist ONO AE1-329, and migration towards eotaxin was determined. Responses were expressed as percent of the control response, e.g., eotaxin alone. Data are shown as mean \pm SEM.; $n = 4, 5$. * $p < 0.05$ versus vehicle

concentrations of the EP4 receptor agonist ONO AE1-329 and were allowed to migrate towards PGD₂ (30 nM) or eotaxin (1 nM), respectively. Unexpectedly, inhibition of adenylyl cyclase could not prevent the inhibitory effect of the EP4 receptor agonist ONO AE1-329 (Fig. 2a, b). In contrast, the same concentration of SQ22536 effectively reversed the effect of PGI₂ (100 nM) of attenuating eosinophil migration towards eotaxin (1 nM, Fig. 2c).

PI3K and PKC are involved in the regulation of eosinophil responses to chemoattractants [41, 42], and we have found that the PGE₂-induced inhibition of eosinophil chemotaxis was prevented by inhibitors of PI3K and PKC [30]. Therefore, we wondered whether PI3K and PKC might be linked to EP4 activation and mediated the attenuation of

eosinophil chemotaxis. To this end, cells were pretreated with the PI3K inhibitor LY294002 (20 μM), the PKC inhibitor chelerythrine (10 μM) or the respective vehicle for 20 min at 37°C. Next, the cells were mixed with varying concentrations of ONO AE1-329 and were then allowed to migrate towards eotaxin (1 nM). The inhibitory effect of the EP4 receptor agonist on eosinophil migration was significantly reduced by the PI3K inhibitor and was almost completely prevented by pretreatment with the PKC inhibitor (Fig. 2d, e). Direct activation of PKC with phorbol 12-myristate 13-acetate (PMA) also resulted in a decrease of eosinophil migration towards eotaxin but this response was not affected by pretreatment of the cells with the PI3K inhibitor LY294002 (Fig. 2f). These data suggested that

both PI3K and PKC, but not the adenylyl cyclase/cAMP pathway, are involved in the signaling of the EP4-mediated attenuation of eosinophil migration.

PGE₂ inhibits Ca²⁺ flux and shape change of eosinophils via EP4 receptors

It is known that chemoattractants like eotaxin or PGD₂ induce Ca²⁺ mobilization in eosinophil granulocytes, a response that is essential for effector functions such as respiratory burst, degranulation, and mediator release [43]. Therefore, we investigated the influence of PGE₂ on the chemoattractant-induced Ca²⁺ mobilization. Eosinophil Ca²⁺ flux, as induced by eotaxin (1 nM) or PGD₂ (3 nM), was attenuated in a concentration-dependent manner by PGE₂ (3–100 nM; Fig. 3a). Similarly, the EP4 agonist ONO AE1-329 (3–100 nM) led to a decrease in Ca²⁺ influx in eosinophils (Fig. 3a). To elucidate the role of EP receptor subtypes we used the EP2 receptor agonist butaprost, the EP1/EP3 receptor agonist sulprostone, the EP4 agonist ONO AE1-329 and the IP/EP1 receptor agonist iloprost. Out of these compounds only PGE₂ and ONO AE1-329 showed a significant attenuation of Ca²⁺ flux induced by eotaxin or PGD₂ in eosinophils (Fig. 3b).

We further investigated the influence of PGE₂ on the Ca²⁺ mobilization induced by higher concentrations of eotaxin. Stimulation with eotaxin (1, 3 and 10 nM) concentration-dependently increased the Ca²⁺ flux in eosinophils. These Ca²⁺ responses were attenuated over the entire range of eotaxin concentrations by PGE₂, and the extent of inhibition likewise depended on the concentration of PGE₂ (3–300 nM; Fig. 4a). To further substantiate the role of EP4 receptors in the PGE₂-mediated inhibition of Ca²⁺ mobilization, we pretreated the cells with the EP4 receptor antagonist ONO AE3-208 (100 nM) for 15 min at 37°C before the effects of PGE₂ and the EP4 receptor agonist ONO AE1-329 (30 nM each) on the eotaxin-induced Ca²⁺ flux were determined. In fact, ONO AE3-208 reversed the attenuation both by PGE₂ and ONO AE1-329 of the eotaxin-induced Ca²⁺ mobilization (Fig. 4b, c). Similar observations were made with the alternative EP4 antagonist GW627368X (data not shown, *n* = 5).

Besides eliciting an increase in free intracellular Ca²⁺, stimulation with chemoattractants results in rapid reorganization of the cytoskeleton and shape change of granulocytes, which can be detected by flow cytometry [44]. These responses are important prerequisites for granulocyte adhesion, polarization, locomotion, and degranulation. To elucidate the role of PGE₂ in chemoattractant-induced shape change responses, we mixed eosinophils with various concentrations of PGE₂ or its vehicle and induced eosinophil shape change with PGD₂ (0.06–20 nM). Similar to our results regarding migration and Ca²⁺ mobilization, we found

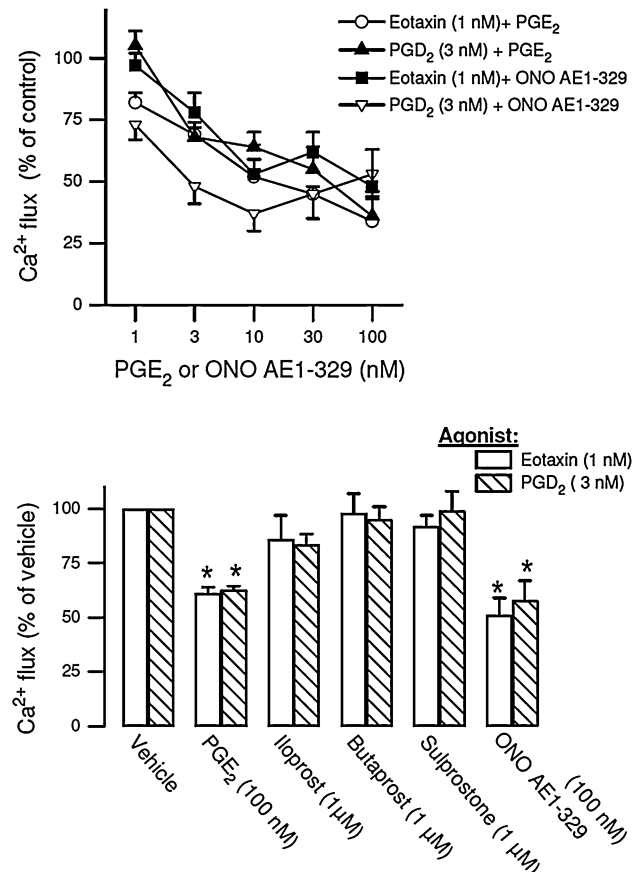
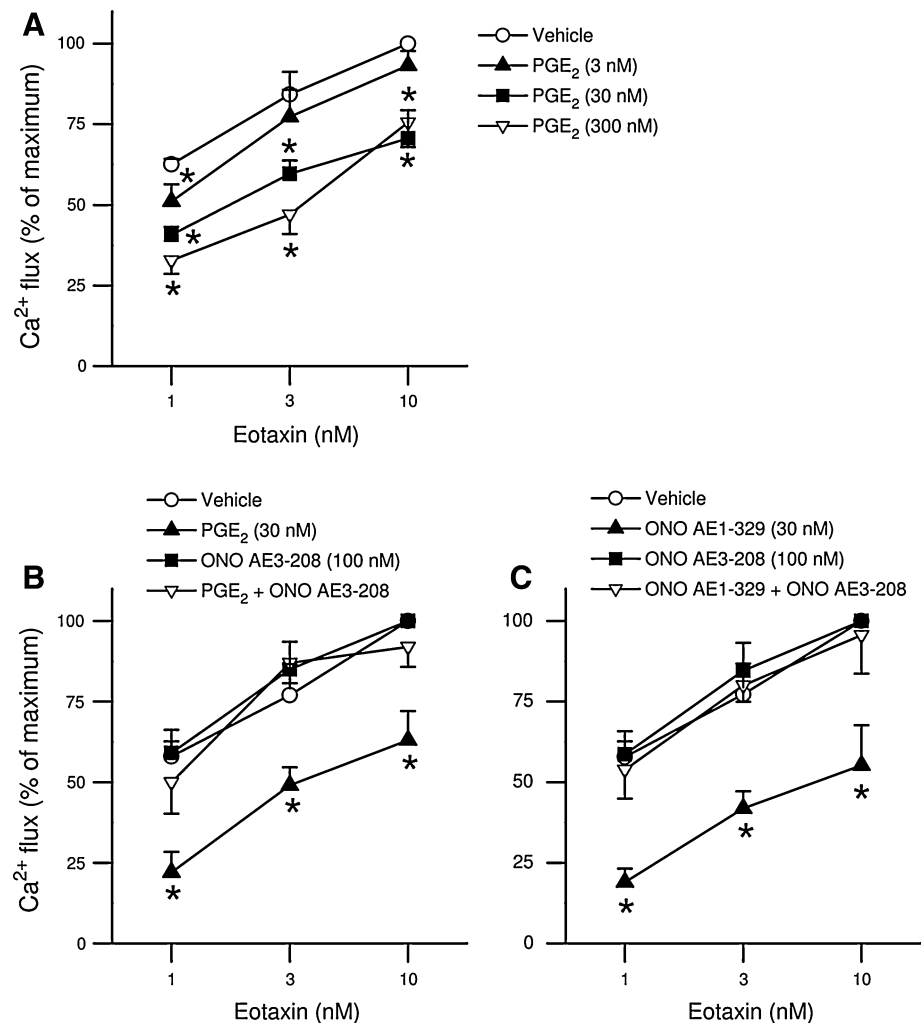


Fig. 3 PGE₂ attenuates the chemoattractant-induced Ca²⁺ flux in eosinophils. **a** Polymorphonuclear leukocyte preparations were mixed with vehicle, PGE₂, or the EP4 agonist ONO AE1-329, and Ca²⁺ flux was induced with eotaxin or PGD₂ in eosinophils. **b** Polymorphonuclear leukocyte preparations were mixed with vehicle, PGE₂, the IP/EP1 receptor agonist iloprost, the EP2 receptor agonist butaprost, the EP1/EP3 receptor agonist sulprostone, or the EP4 agonist ONO AE1-329, and Ca²⁺ flux was elicited with eotaxin or PGD₂. Ca²⁺ flux was recorded with flow cytometry as increase in fluorescence in FL-1. Eosinophils were distinguished from neutrophils as CD16-negative cells. Responses were expressed as percent of the control response, i.e., in the absence of a prostanoid. Data are shown as mean ± SEM.; *n* = 4–8. **p* < 0.05 versus vehicle

that PGE₂ inhibited eosinophil shape change in a concentration-dependent fashion with maximum attenuation at a concentration of 100 nM PGE₂ (Fig. 5a). In further experiments we investigated the involvement of the different EP receptor subtypes by pretreating the cells with the EP2 receptor agonist butaprost, the EP1/EP3 receptor agonist sulprostone and the IP/EP1 receptor agonist iloprost. Unlike PGE₂ none of these EP receptor agonists was able to inhibit PGD₂ induced eosinophil shape change (Fig. 5b), which suggested the involvement of the EP4 receptor in this PGE₂-induced effect. In fact, pretreatment of eosinophils with the EP4 receptor antagonist ONO AE3-208 (100 nM) or GW627368X (10 μM) before being mixed with PGE₂ (30 nM) or the EP4 receptor agonist ONO AE1-329 (30 nM)

Fig. 4 EP4 receptors mediate the PGE₂-induced inhibition of Ca²⁺ flux in eosinophils. **a** Polymorphonuclear leukocyte preparations were mixed with vehicle, or different concentrations of PGE₂, and Ca²⁺ flux was elicited with eotaxin. **b, c** Polymorphonuclear leukocyte preparations were pretreated with the EP4 antagonist ONO AE3-208 or vehicle, then mixed with vehicle, PGE₂ or the EP4 agonist ONO AE1-329, and Ca²⁺ flux was induced with eotaxin. Ca²⁺ flux was recorded by flow cytometry as increases in fluorescence in FL-1. Eosinophils were distinguished from neutrophils as CD16-negative cells. Responses were expressed as percent of the maximum control response, i.e., in the absence of a prostanoid. Data are shown as mean ± SEM.; n = 6–12. *p < 0.05 versus vehicle



almost completely reversed the inhibitory effects of PGE₂ and ONO AE1-329 on shape change responses to eotaxin (Fig. 5c, d). These data demonstrated that it is the EP4 receptor subtype that exclusively mediates the PGE₂-induced attenuation of eosinophil Ca²⁺ mobilization and shape change.

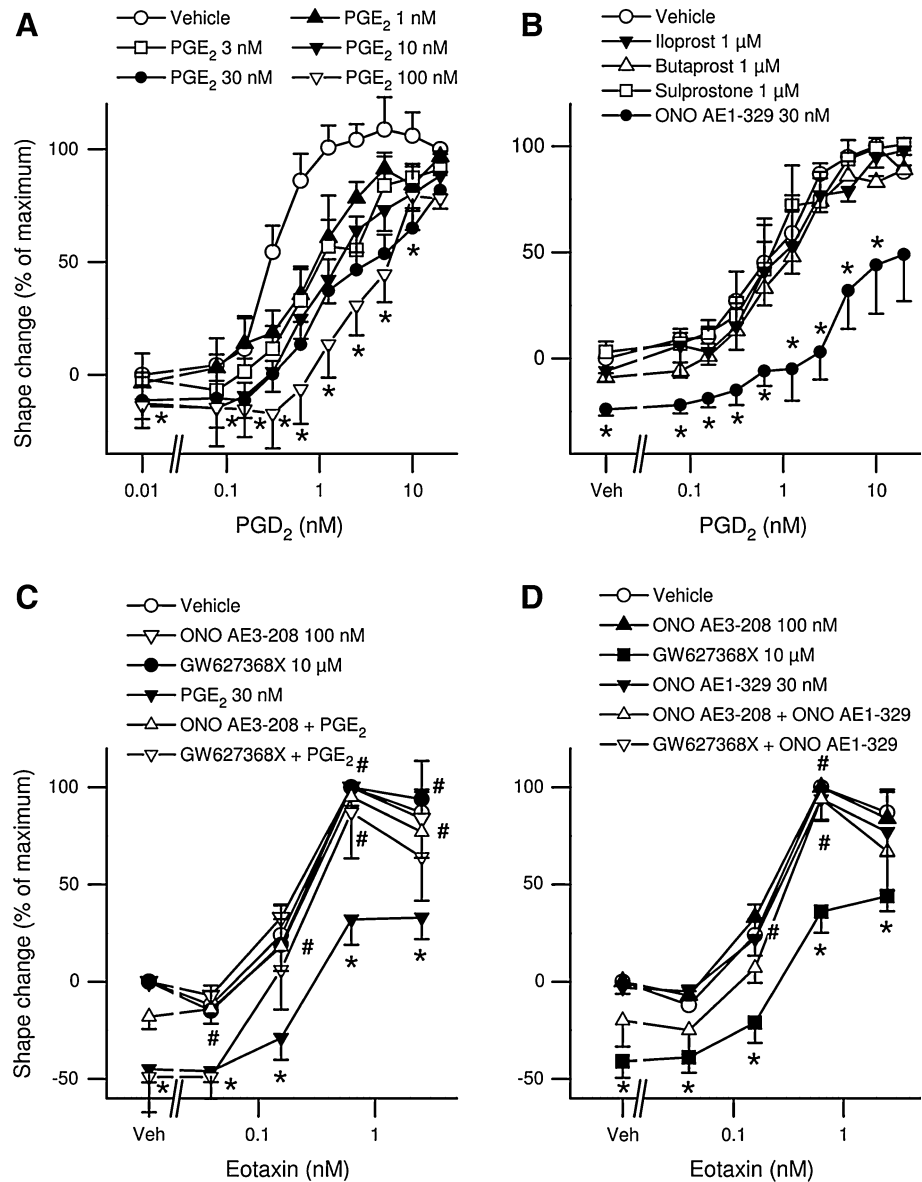
Further on, we determined the role of neutrophil granulocytes in the EP4-mediated effects of PGE₂ on eosinophil effector functions. Therefore, eosinophil shape change responses with regard to the EP4-mediated effects were compared for eosinophils contained within PMNL preparations and purified eosinophils. Shape change was induced by varying concentrations of eotaxin in the presence of PGE₂ (30 nM), the EP4 receptor agonist ONO AE1-329 (30 nM), the EP2 receptor agonist butaprost (1 μM) or the respective vehicle. As expected, the presence of neutrophil granulocytes had no effect on the EP4-mediated attenuation of eosinophil shape change (Suppl. Fig. S2A and B). Corresponding to these observations, we were able to show that neither shape change nor Ca²⁺ mobilization as induced by IL-8 was affected by PGE₂ in neutrophils (Suppl. Fig. S2C and D).

PGE₂ modulates eosinophil effector functions via EP4 receptors

Next we investigated the effect of PGE₂ on eosinophil effector functions such as respiratory burst and upregulation of adhesion molecules. Purified eosinophils were mixed with varying concentrations of PGE₂, the EP4 receptor agonist ONO AE1-329 (0.03–30 nM each), the EP2 receptor agonist butaprost (30 nM) or vehicle and were then stimulated with eotaxin (0.5–50 nM). Respiratory burst was quantified by flow cytometry. PGE₂ very potently attenuated the eotaxin-induced respiratory burst, at concentrations as low as 0.3 nM, while the EP2 receptor agonist butaprost did not mimic this PGE₂ effect at a concentration of 30 nM (Fig. 6a). With a similar potency and efficacy as PGE₂, the EP4 receptor agonist ONO AE1-329 afforded inhibition of eosinophil respiratory burst (Fig. 6b). In further experiments, we investigated whether pretreatment with the EP4 receptor antagonists ONO AE3-208 (100 nM) or GW627368X (10 μM) had an influence on the PGE₂-induced attenuation of the respiratory burst in

Fig. 5 PGE₂ attenuates the chemoattractant-induced shape change of eosinophils via EP4 receptors.

a, b Polymorphonuclear leukocyte preparations were mixed with vehicle, PGE₂, the IP/EP1 receptor agonist iloprost, the EP2 receptor agonist butaprost, the EP1/EP3 receptor agonist sulprostone or the EP4 agonist ONO AE1-329, and shape change was induced with PGD₂. **c, d** Polymorphonuclear leukocyte preparations were pretreated with the EP4 antagonists ONO AE3-208, GW627368X or vehicle, then mixed with PGE₂, ONO AE1-329 or its solvent and stimulated with eotaxin. Shape change was determined with flow cytometry as increase in forward scatter. Eosinophils were distinguished from neutrophils as cells with higher auto-fluorescence. Responses were expressed as percent of the maximum control response, i.e., in the absence of a prostanoid. Data are shown as mean ± SEM.; *n* = 3–4. **p* < 0.05 versus vehicle. #*p* < 0.05 versus PGE₂



eosinophil granulocytes. Both EP4 receptor antagonists, ONO AE3-208 and GW627368X, almost completely reversed the inhibitory effect of PGE₂ (Fig. 6c) and also that of the EP4 receptor agonist ONO AE1-329 (Fig. 6d).

Since the adhesion molecule CD11b is an important modulator of leukocyte responses such as migration and mediator release [45, 46], we also investigated the effect of PGE₂ on the chemoattractant-induced upregulation of CD11b on eosinophils. To this end, cells were mixed with different concentrations of PGE₂ (10–100 nM), the EP4 receptor agonist ONO AE1-329 (30 nM) or vehicle and were then stimulated with PGD₂ (5–500 nM) or eotaxin (0.3–10 nM). Cell surface expression of CD11b on eosinophils was upregulated by PGD₂ and eotaxin approximately 1.5-fold, and both PGE₂ and ONO AE1-329 led to a decrease of this response (Fig. 7a, b). These data indicate

that PGE₂ modulates eosinophil effector functions via the EP4 receptor.

EP4 receptors are expressed by infiltrating eosinophils in inflamed tissue

To investigate whether infiltrating cells in allergic airway inflammation express EP4 receptors, immunohistochemistry of human nasal polyps and allergic rhinosinusitis was performed. Epithelial cells and infiltrating leukocytes stained positive with the EP4 antibody (Fig. 8a) while sections incubated with the isotype-matched control antibody showed no staining (Fig. 8c), hence demonstrating the specificity of the EP4 antibody. The infiltrating leukocytes were also predominantly positive for eosinophil peroxidase (Fig. 8b). Double immunofluorescence staining

Fig. 6 PGE₂ prevents eosinophil respiratory burst via EP4 receptors. Purified eosinophils were mixed with PGE₂, the EP2 receptor agonist butaprost (a), the EP4 receptor agonist ONO AE1-329 (b), or vehicle before respiratory burst was induced with eotaxin. c, d Eosinophils were pretreated with the EP4 antagonists ONO AE3-208, GW627368X or vehicle, then mixed with PGE₂, ONO AE1-329 or its solvent, and stimulated with eotaxin. Respiratory burst was determined by flow cytometry as increase of fluorescence in FL-1. Responses were expressed as percent of baseline fluorescence. Data are shown as mean ± SEM, *n* = 3–15. **p* < 0.05 versus ONO AE3-208. #*p* < 0.05 versus GW627368X

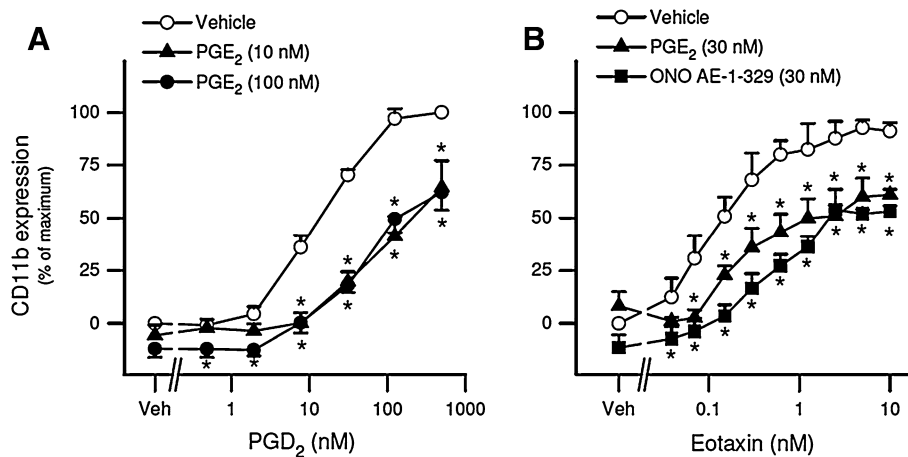
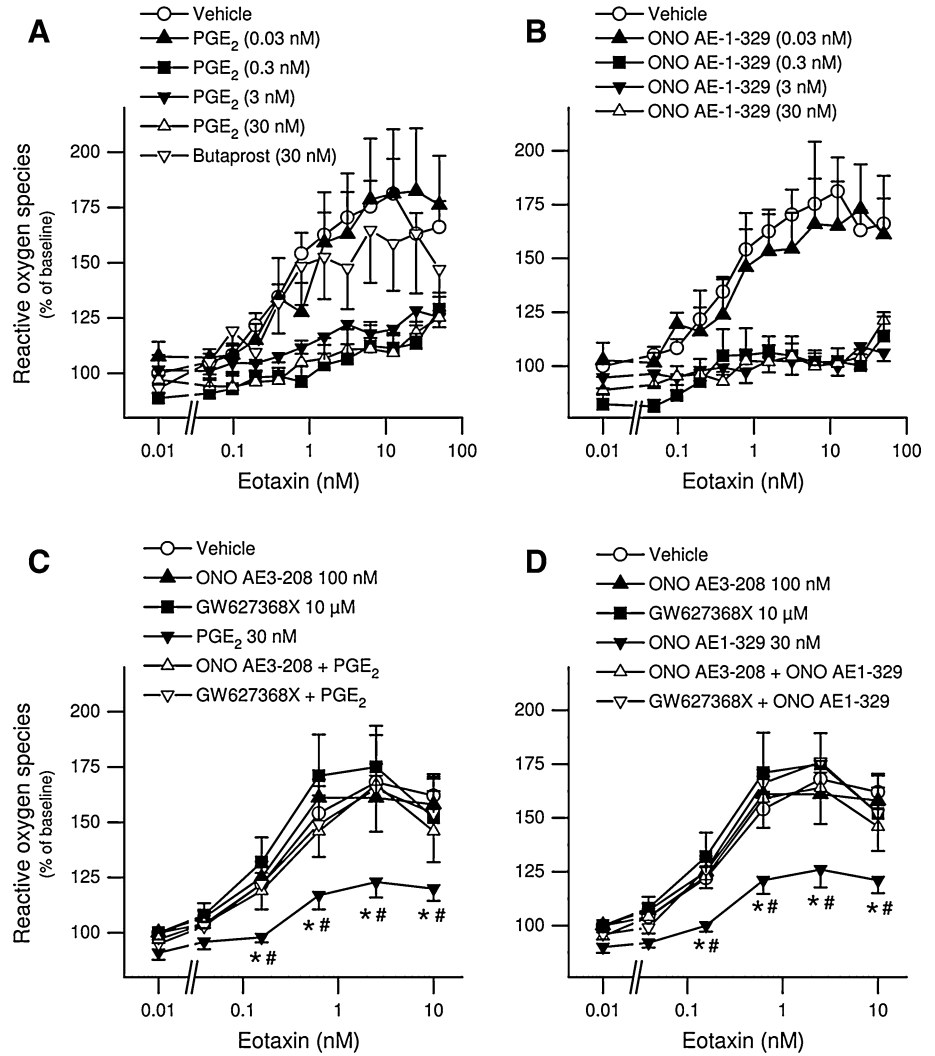


Fig. 7 Chemoattractant-induced upregulation of CD11b is modulated by PGE₂ and EP4 receptors. Polymorphonuclear leukocyte preparations were mixed with vehicle, PGE₂, or the EP4 agonist ONO AE1-329, and upregulation of the adhesion molecule CD11b was then stimulated with PGD₂ (a) or eotaxin (b). Cell surface expression of

CD11b was measured with flow cytometry by direct immunofluorescence staining. Eosinophils were distinguished from neutrophils as CD16-negative cells. Responses were expressed as percent of the maximum control response, i.e., in the absence of a prostanoid. Data are shown as mean ± SEM; *n* = 5–6. **p* < 0.05 versus vehicle

finally indicated the presence of EP4 receptors in eosinophil peroxidase-positive cells in the tissue (Fig. 8d).

Discussion

We have recently shown that PGE₂ attenuates the migration of human eosinophil granulocytes and that the EP2 receptor is involved in that effect [30]. In the current study, we report that the EP4 receptor is even more substantially involved in the regulation of eosinophil effector functions, since we demonstrate for the first time that the selective EP4 agonist

ONO-AE1-329 not only abrogates eosinophil migration but also abolishes the production of reactive oxygen species, Ca²⁺ responses and upregulation of adhesion molecules on eosinophils. Moreover, we show that eosinophils express EP4 receptors in peripheral blood and that infiltrating leukocytes are EP4-positive in inflamed tissue. Therefore, our data suggest that pharmacological activation of EP4 receptors using subtype-selective agonists may afford attenuation of pathogenic eosinophil functions that are substantially linked to allergen-induced airway inflammation and tissue damage [47, 48].

Our data presented in the current study and recently presented data [30] suggest that both the EP2 and EP4 receptor are mediating the inhibitory effect of PGE₂. Interestingly, blockade of either receptor is sufficient to largely abolish the inhibitory effect of PGE₂ on the migration of eosinophils. Since the EP2 antagonist AH6809 and the EP4 antagonist GW627368X have been shown to display very low affinity for EP4 and EP2 receptors, respectively [32, 49], one possible explanation for this unexpected observation might be the cooperative signaling of these two receptors in eosinophils, e.g., by forming EP2/EP4 heteromers. It was reported recently that the chemokine receptors CCR2, CCR5, and CXCR4 form heteromeric complexes, and specific antagonists of one receptor can lead to functional inhibition of the other heteromerization partner *in vitro* and *in vivo* [50]. The exact mechanism behind the cooperative signaling of EP2 and EP4 receptors awaits further studies. Importantly, the selective EP4 agonist ONO AE1-329 fully mimicked the inhibitory effect of PGE₂ on eosinophils. In agreement with these functional responses, we could show by Western blot and flow cytometric immunostaining that human peripheral blood eosinophils express EP4 receptors. Interestingly, we found that peripheral blood eosinophils also express EP1 and EP3 receptors, as suggested previously for eosinophils recovered from sputum [51]; the role of these receptors in eosinophil function is still unclear.

EP4 receptors were also detectable on infiltrating leukocytes, presumably eosinophils, in inflamed human nasal mucosa, and also in murine lungs after allergen exposure (data not shown). In contrast, EP4 receptor expression was confined to the epithelium in the lungs of control animals. These data suggest that the amelioration of airway eosinophilia as observed after administration of PGE₂ in humans and in experimental models [14, 15] might be mediated by EP4 receptors. Therefore, our data might provide the rationale for the evaluation of systemically available EP4 agonists in models of inflammation.

Stimulation of EP4 receptors usually increases intracellular cAMP levels. Interestingly, it seems that the inhibitory effect of EP4 receptor activation does not depend on activation of the adenylyl cyclase/PKA

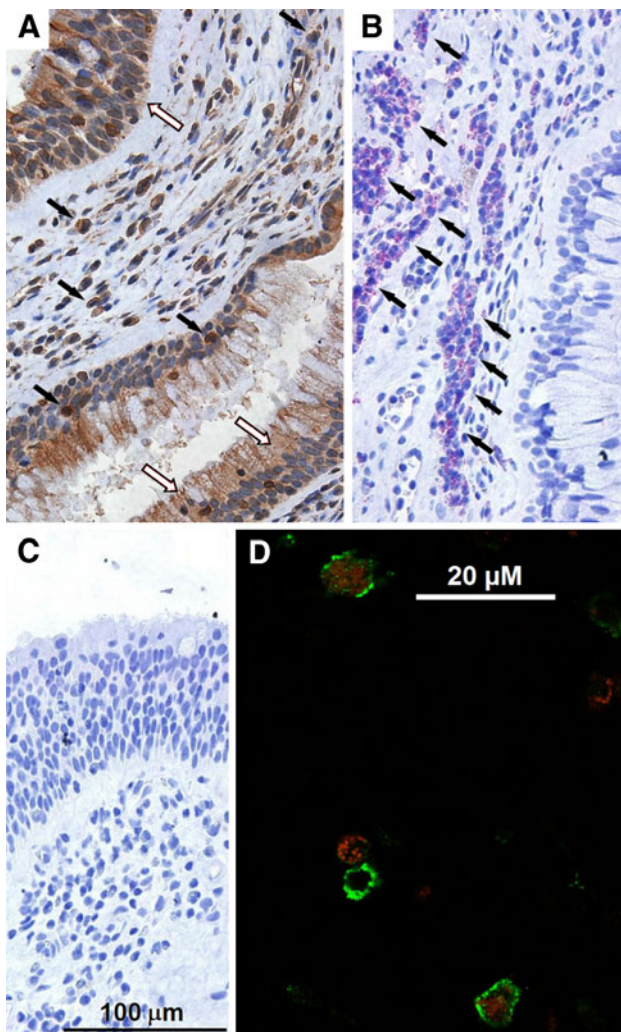


Fig. 8 Infiltrating inflammatory cells in human nasal polyps express EP4 receptors. **a** EP4 receptors (stained *brown*) were observed by immunohistochemistry in nasal epithelial cells (indicated by *white arrows*) and infiltrating leukocytes (*black arrows*). **b** Infiltrating leukocytes were also positive for eosinophil peroxidase. **c** No staining was observed with the control antibody. **d** Double immunofluorescence staining revealed specific co-expression of EP4 (*green*) and eosinophil peroxidase (*red*) in the majority of eosinophils. Images are representative of three polyps from different patients

pathway, as the adenylyl cyclase inhibitor SQ22536 did not reverse the inhibitory effect of ONO AE1-329 on eosinophil migration. The effectiveness of SQ22536 as an adenylyl cyclase inhibitor was demonstrated by the fact that SQ22536 prevented the attenuation of eosinophil migration induced by PGI₂, a known stimulator of adenylyl cyclase [52]. Stimulation of EP4 has been shown to cause phosphorylation of extracellular signal-regulated kinases (ERKs) through a PI3K-dependent mechanism [53]. Additionally, the existence of an alternative EP2/EP4 signaling pathway, linked to PKC activation has been postulated [54]. Therefore, we investigated the effect of the PI3K inhibitor LY294002 and the selective PKC inhibitor chelerythrine on eosinophil migration in the presence of the EP4 receptor agonist ONO AE1-329. In fact, inhibition of PI3K prevented the inhibition of eosinophil migration induced by ONO AE1-329. Moreover, exposure of eosinophils to the selective PKC inhibitor chelerythrine prior to stimulation of the EP4 receptor with ONO AE1-329 resulted in almost complete reversal of the attenuation of eosinophil chemotaxis. Since these data implicated PKC as a negative regulator of eosinophil migration, we investigated the effect of the PKC activator PMA on eosinophil migration. As expected, PMA very potently mimicked the inhibitory effect of PGE₂ and the EP4 receptor agonist on eosinophil migration. The inhibitory effect of PMA, however, could not be prevented by the PI3K inhibitor LY294002. Therefore, our data indicate that both PI3K and PKC are involved in the attenuation of eosinophil migration upon EP4 receptor activation and confirm previous data that modulation of PI3K mediates the inhibitory effect of PGE₂ in neutrophils [55].

Chemoattractants like PGD₂ or eotaxin are known to elevate intracellular Ca²⁺ concentrations in eosinophils, which is an essential requirement for effector functions like degranulation and respiratory burst [43]. Hence, we investigated the influence of PGE₂ on the Ca²⁺ mobilization induced by these chemoattractants. Our data show that PGE₂ inhibits chemoattractant-induced Ca²⁺ influx in eosinophils, and what is more, that this effect is mediated via EP4 receptors. This conclusion was based on the observations that the selective EP4 agonist ONO AE1-329 likewise inhibited eosinophil Ca²⁺ responses and that this effect was reversed in the presence of the selective EP4 receptor antagonist ONO AE3-208. In contrast, the PGE₂-induced attenuation of Ca²⁺ flux in eosinophils was not mimicked by the IP/EP1 receptor agonist iloprost, the EP2 receptor agonist butaprost, or the EP1/EP3 receptor agonist sulprostone. Another important prerequisite for chemoattractant-induced eosinophil migration, oxidative burst, and degranulation is the rapid reorganization of the cytoskeleton, which results in a shape change of the cells [43]. Similar to our observation with Ca²⁺ mobilization we

found that PGE₂ inhibits shape change of eosinophils solely by EP4 receptors, since inhibition of shape change could also be evoked with ONO AE1-329 but not with iloprost, butaprost, or sulprostone. This notion was supported by the fact that inhibition of eosinophil shape change induced by PGE₂ or ONO AE1-329 was reversed by the EP4 receptor antagonists ONO AE3-208 and GW627368X in a similar fashion.

Interestingly, neither chelerythrine nor LY294002 was able to reverse the inhibitory effect of the EP4 agonist on Ca²⁺ flux and shape change (unpublished observation), suggesting that the roles of PI3K and PKC are largely restricted to EP4-mediated inhibition of chemotaxis. This notion complements our finding that both EP2 and EP4 activation curtails eosinophil chemotaxis, while only EP4 controls shape change, Ca²⁺ flux, and production of reactive oxygen species. Therefore, a detailed analysis of EP4-related signaling needs to be carried out for each eosinophil function separately in future studies.

Since Ca²⁺ mobilization and shape change are important requirements for eosinophil adhesion, respiratory burst, and mediator release, we investigated the consequences of reduced Ca²⁺ responses by PGE₂ with regard to these eosinophil effector functions. Indeed we found that the eotaxin- and PGD₂-induced stimulation of CD11b expression was negatively modulated in the presence of PGE₂ in eosinophils, and that this effect could be mimicked by the EP4 receptor agonist ONO AE1-329. In contrast to other adhesion molecules, cell surface expression of CD11b is rapidly increased on leukocytes after stimulation [56]. The β₂-integrin CD11b/CD18 (also referred to as CR3, Mac-1, or α_mβ₂) is an important complement receptor that binds multiple ligands, including C3bi, ICAM-1, fibrinogen, and β-glucan. Apart from adhesion, CD11b/CD18 is an important modulator of further leukocyte responses, including migration [45], respiratory burst [57], degranulation [58], and apoptosis [59]. CD11b expression on circulating eosinophils is significantly elevated in various allergic disorders, including atopic dermatitis and bronchial asthma [60, 61]. Therefore, upregulation of surface CD11b closely reflects eosinophil activation and might be a prerequisite of leukocyte recruitment to sites of inflammation, by mediating the release of eosinophils from bone marrow [62] and their migration [63]. These observations suggest that the reduced CD11b response in the presence of PGE₂ might have a profound impact on eosinophil functions, such as migration to sites of allergic reactions, degranulation, and oxidative burst. In agreement with this notion, we were able to show that PGE₂ and the EP4 receptor agonist ONO AE1-329 attenuate the formation of reactive oxygen species with remarkably high potency, while the EP2 agonist butaprost had no effect. Therefore, it appears that the PGE₂-induced attenuation of eosinophil oxidative burst is also mediated

via the EP4 receptor rather than the EP2 receptor. This notion was further asserted by the EP4 receptor antagonist ONO AE3-208 and GW627368X, which reversed the inhibition of reactive oxygen species production by PGE₂ and ONO AE1-329.

In conclusion, the present study shows that eosinophils express EP4 receptors and activation of EP4 receptors negatively modulates eosinophil migration and respiratory burst. Furthermore, we were able to show that PI3K and PKC are involved in the inhibitory effect of EP4 receptors. In previous studies it was shown that PGE₂ has bronchoprotective and anti-inflammatory properties but the usefulness of PGE₂ as a therapeutic agent is limited because of its various side-effects such as acute bronchoconstriction, retrosternal soreness, transient cough, and flu-like symptoms [10, 11, 14]. While the latter effects of PGE₂ are thought to arise from EP1 or EP3 receptor stimulation, selective EP4 agonists might have more favorable pharmacological profiles. Therefore, EP4 agonists might be a useful novel approach in the treatment of eosinophilic diseases as they not only attenuate chemoattractant-induced eosinophil migration and degranulation, as EP2 receptor agonists do, but also abolish other eosinophil effector functions such as upregulation of adhesion molecules or production of reactive oxygen species.

Acknowledgments V. K. and S. P. were funded by the Ph. D. Program Molecular Medicine of the Medical University of Graz. This work was supported by the Jubiläumsfonds of the Austrian National Bank (Grants 11967 and 13487), the Austrian Science Fund FWF (Grants P19424-B05, P22521-B18 and P21004-B02), and the Franz Lanyar Foundation (Grants 315, 316, and 343). A. H. has received research support and consultancy fees from AstraZeneca and Almirall.

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