Human Mast Cells Express Multiple EP Receptors for Prostaglandin E₂ That Differentially Modulate Activation Responses

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Abbreviations used in this paper:
AERD, aspirin-exacerbated respiratory disease; AHR, airway hyperresponsiveness;
cAMP, cyclic adenosine monophosphate; COX, cyclooxygenase; CREM, cyclic AMP response element modulator; cysLT, cysteinyl leukotriene; EPAC, exchange protein activated by cAMP; EPR, early phase response; ERK, extracellular signal-regulated kinase; EP receptor, receptor for PGE₂; FcεRI, high-affinity Fc receptor for

**Key Words:** Mast cells/basophils, human, cell activation
Abstract

Prostaglandin (PG) E₂ blocks mast cell (MC)-dependent allergic responses in humans but activates MCs in vitro. We assessed the functions of the EP receptors for PGE₂ on cultured human MCs (hMCs). hMCs expressed the EP₃, EP₂ and EP₄ receptors. PGE₂ stimulated the accumulation of cyclic adenosine monophosphate (cAMP), and suppressed both FcɛRI-mediated eicosanoid production and necrosis factor-α (TNF-α) generation. PGE₂ also caused phosphorylation of extracellular signal regulated kinase (ERK), exocytosis, and production of prostaglandin (PG)D₂, as well as leukotriene (LT)C₄ when protein kinase A (PKA) was inhibited. An EP₃ receptor-selective agonist, AE-248, mimicked PGE₂-mediated ERK phosphorylation, exocytosis, and eicosanoid formation. Selective agonists of both EP₂ and EP₄ receptors (AE1-259-01 and AE-329, respectively) stimulated cAMP accumulation. No selective agonist, alone or in combination, was as effective as PGE₂. AE-248, AE1-259-01, and AE-329 all inhibited FcɛRI-mediated TNF-α generation, while AE1-259-01 blocked eicosanoid production. PGE₂ caused the expression of inducible cAMP early repressor (ICER) by a pathway involving PKA and ERK. Thus while PGE₂ activates MCs through EP₃ receptors, it also counteracts FcɛRI-mediated eicosanoid production through EP₂ receptors and PKA, and blocks cytokine transcription. These functions explain the potency of PGE₂ as a suppressor of early- and late-phase allergic responses.
Introduction

Mast cells (MCs) initiate inflammatory responses to infectious organisms and allergens. In allergic diseases, MCs are activated by cross-linkage of their high-affinity Fc receptors for immunoglobulin (Ig)E (FcεRI), releasing pre-formed proteases and biogenic amines, and generating leukotriene C₄ (LTC₄), the parent molecule of the cysteiny1 leukotrienes (cysLTs), and prostaglandin (PG) D₂ (1-3). Aspirin-exacerbated respiratory disease (AERD) is characterized by nasal polyposis, asthma, and MC activation in response to challenge with nonselective inhibitors of cyclooxygenase (COX) (4,5). MC activation follows allergen challenge in the lungs or nose of susceptible individuals, inducing tissue swelling, bronchconstriction, and vascular leakage (early-phase response, EPR) (6). In vitro, FcεRI-induced MC activation also initiates transcription through the actions of nuclear factor κB (NF-κB), nuclear factor of activated T cells (NF-AT) and activator protein-1 (AP-1) transcription factors, resulting in sustained cytokine and chemokine generation (7). MC activation recruits leukocytes to allergen-challenged tissues, resulting in sustained swelling and inflammation (late-phase response, LPR) in a significant proportion of susceptible individuals (8). Mediator generation by MCs stimulated through toll-like receptors (TLRs) also play an important role in protective innate immunity (9-11). Thus whether activated through stimulation of FcεRI, idiosyncratic mechanisms, or pattern recognition receptors, MCs provide eicosanoids and inducible cytokines for inflammatory responses in vivo, benefiting the host in protective immunity, but also potentially inducing exacerbations of allergic diseases.
PGE₂, a functionally versatile eicosanoid, acts at four divergent G protein-coupled receptors (GPCRs), termed the EP₁, EP₂, EP₃, and EP₄ receptors (reviewed in 12). PGE₂ can amplify inflammatory gene expression and promote tissue pathology in colon cancer (13) and in mouse models of arthritis (14). In contrast, PGE₂ strongly suppresses allergic respiratory mucosal inflammation. Inhalation of PGE₂ before allergen challenge prevents both EPR and LPR in asthmatic subjects (15), and decreases the levels of PGD₂ that are detected in the bronchoalveolar lavage fluid after allergen challenge (16). In AERD, PGE₂ inhalation prevents bronchconstriction induced by challenge with nonselective COX inhibitors and abrogates the characteristic rise in urinary levels of LTE₄, the end-product of the cysLTs (5,17,18). Collectively, these observations suggest that PGE₂ inhibits MC activation in the respiratory tract. Although PGE₂ reportedly suppresses mediator release by some MC subtypes in vitro by raising intracellular levels of cyclic adenosine monophosphate (cAMP) (19,20), it also enhances mediator release from mouse MCs (21-23). The mechanisms and EP receptor subtypes responsible for PGE₂-mediated inhibition of MC activation are incompletely understood.

We explored the mechanisms and receptors by which PGE₂ modulates activation responses of cord blood-derived human MCs (hMCs). hMCs express EP₂, EP₃, and EP₄ receptors, and respond to PGE₂ and receptor-selective analogues with anticipated biochemical signatures and signaling events. In contrast to the results of earlier studies of human lung MCs, PGE₂ does not suppress FcεRI-dependent exocytosis by hMCs. Rather, PGE₂ causes exocytosis on its own when hMCs are primed with IL-4, a function reflecting EP₃ receptors. Moreover, EP₃ receptor-dependent stimulation of hMCs
induces phosphorylation of extracellular signal-regulated kinases (ERK) 1 and 2 and PGD$_2$ generation, and also causes the production of LTC$_4$ when protein kinase A (PKA) is inhibited. However, PGE$_2$ interferes with both LTC$_4$ and PGD$_2$ generation by hMCs stimulated by Fc$\varepsilon$RI cross-linkage, an effect mediated by EP$_2$ receptors, and strikingly inhibits Fc$\varepsilon$RI-mediated production of tumor necrosis factor (TNF)-$\alpha$. The latter event reflects complementary signaling through PKA and ERK-mediated signaling pathways, in turn reflecting combinatorial contributions from EP receptors leading to the expression of the transcriptional repressor protein inducible cAMP early repressor (ICER) (24,25). Thus while EP$_3$ receptor-dependent signaling causes or potentiates MC mediator release, EP$_2$ receptors inhibit Fc$\varepsilon$RI-initiated mediator generation and may limit tissue pathology in the LPR and other circumstances where MC activation induces inflammation.
Materials and Methods

Reagents

PGE₂, PGD₂, LTD₄, the EP₂/EP₃ dual receptor antagonist AH6809 (EC₅₀ = 50 µM) and the EP₂ receptor selective agonist butaprost (Ki ~ 10 µM) were purchased from Cayman Chemical Co (Ann Arbor, MI). EP₁, EP₂, EP₃, and EP₄ receptor-selective agonists (DI-004, AE1-259-01, AE-248, and AE-329, respectively) and an EP₄ receptor-selective antagonist (AE-208) were obtained from Ono Pharmaceuticals (Osaka, Japan). These agonists are potent (Ki ~ 3 nM, 150 nM, 7.5 nM and 10 nM for AE1-259-01, DI-004, AE-248, and AE-329, respectively) and reportedly show little to no cross-reactivity on cloned EP receptors even at doses 1000 times their Ki values (26,27). In primary cells, agonist doses between 0.1 and 10 µM are reportedly sufficient to elicit maximal functional and signaling responses using these agonists (28,29). Each selective reagent was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO). Pertussis toxin (PTX) was purchased from Sigma. The selective agonist of exchange protein activated by cAMP (Epac), 8-pCPT-2’-O-Me-cAMP, and an inhibitor of glycogen synthase kinase 3 (GSK-3) (GSK-3 inhibitor II) were purchased from Calbiochem. A second GSK-3 inhibitor, SB216763, was obtained from Sigma. Inhibitors of PKA (H89, Sigma) and MEK/ERK (UO126, Promega) were used at their recommended concentrations (10 µM and 5 µg/ml, respectively).
Derivation and priming of hMCs

hMCs were derived from cord blood mononuclear cells cultured in the presence of SCF (100 ng/ml), IL-6 (50 ng/ml), and IL-10 (10 ng/ml) (all from R&D, Minneapolis, MN), as previously described (30), and studied when they reached > 95% purity based on staining with toluidine blue (6-9 wk). For FcεRI-dependent exocytosis, cytokine generation, and eicosanoid production, hMCs were transferred to new medium containing SCF and IL-4 (10 ng/ml, R&D) for 5 d before activation (31,32). Because IL-4 did not alter the expression level of any EP receptor and did not change calcium fluxes in response to PGE₂, signaling studies were performed with unprimed hMCs. Each donor’s cells were used for one experiment per assay.

Flow cytometry

Expression of EP receptor proteins was assessed on fixed, permeabilized hMCs as described (33). Polyclonal Abs against each EP receptor (Cayman) and a monoclonal Ab against Kit (Pharmingen) were used at 2 µg/sample. The EP₃ receptor Ab was raised against a peptide sequence (amino acids 308-327) common to all isoforms of this receptor.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from hMCs at 6-8 wk of culture with TRI Reagent (Molecular Research, Cincinnati). RNA samples were primed with oligo(dT) and reverse transcribed using an RT kit (Clontech). Primer sequences for the amplification of the EP₁, EP₂, and EP₄ receptors (34) are as follows, reading from the 5’ to the 3’
direction: EP₁, sense ATCTGCTGGAGCCAATGCTGGTGT, antisense TCGTTGGGCCTCTGGTTGTGCTT; EP₂, sense CCTGGCCGTGCTGCCTGTCATCTAT, antisense CCATGGACACCCCTTTCCCTCTCCT; EP₄, sense TTTGCAGGCCATCCGAATTGCTTCT, antisense CCTGCCTCAAGGCCATTTTTCACTGG. Because the human EP₃ receptor gene gives rise to several splice variants (35,36), we designed primers for the selective amplification of each. Splice variants I-IV were amplified using a common sense strand primer (CTGAACCAGATCTTGGATCC) with the following isoform-specific antisense primers: EP₃-I, TCACCATCATAAGCTTATAC; EP₃-II, TACGAATGGCAGACTCAACA; EP₃-III and EP₃-IV TCATGGAGCTTCCAGTGATG. Specific primer pairs were used to amplify EP₃-V (sense CAGAGGTTTCCCAGAGAGGACGTGG, antisense TCCTGGACCTGCCTCCATGCATGACAAA) and EP₃-VI (sense GAGATGGGGCCTGATGGAAG, antisense TCATGGAGCTTCCAGTGATG). Primers for human glyceraldehyde -3- phosphate dehydrogenase (Clontech) were run as positive controls. PCR was performed in a Perkin-Elmer Thermal Cycler with 0.4 units of Taq polymerase (Perkin-Elmer) for 35 cycles (94°C x 1 min, 94°C x 30 sec, 56°C x 2 min, and 72°C x 4 min). Genomic DNA and non-reverse-transcribed RNA were used as positive and negative control templates, respectively. The PCR products were resolved on ethidium bromide-stained 1.5% agarose gels. The analyses for each receptor were repeated 8 times with RNA harvested from the cells of different donors.
**Calcium mobilization**

Changes in the concentration of cytosolic free Ca^{++} were assessed by two methods. First, a fluorescence imaging plate reader (FLIPR)-based calcium imaging assay was used to determine the optimal dosing range for PGE_{2}-mediated calcium flux. hMCs (8-wk-old) were washed into Hank's balanced salt solution (HBSS) containing 1% bovine serum albumin (BSA), 20 mM HEPES, and 2.5 mM probenecid at a density of 1.3 x 10^6 cells/ml and loaded with 2 \mu M Fluo-4 (InVitrogen) according to the manufacturer's protocol. The plates were then placed into a FLIPR™ (Molecular Devices) to monitor cell fluorescence (\lambda_{ex}=488 nm, \lambda_{EM}=540 nm) before and after the addition of various agonists. Triplicate samples of cells were stimulated with PGE_{2} (0.01-10 \mu M) or similar concentrations of PGD_{2} or LTD_{4} as negative and positive controls. The susceptibility of PGE_{2}-mediated calcium flux to interference by PTX (4-12 h treatment with 100 ng/ml) and the ability of EP receptor-selective agonists to elicit this response were tested with Fura-2 AM (Molecular Probes, Inc., Eugene, OR)-loaded hMCs using a fluorescence spectrophotometer (Hitachi F-4500) using excitation at 340 and 380 nm to monitor cytosolic free Ca^{++} as previously described (30).

**Cell activation for mediator secretion**

IL-4-primed hMCs were passively sensitized with human myeloma IgE (Chemicon, 2 \mu g/ml) overnight on the fourth day of priming. The cells were washed and resuspended in fresh medium at a concentration of 1 x 10^6 cells/ml, except for the samples used for analysis of exocytosis, which were suspended at a 10 x 10^6/ml. For
studies of exocytosis, samples of $2 \times 10^6$ hMCs were challenged with anti-IgE (Calbiochem, 1 $\mu$g/ml), or medium alone. In some experiments, PGE$_2$, AE-248, AE-329, AE1-259-01, DMSO, or butaprost were added just before activation. The cells were maintained in the presence of SCF (100 ng/ml) throughout to promote optimal viability. Activation was stopped on ice. Supernatants were separated from the pellets by centrifugation at 200 x g in an Eppendorf™ microcentrifuge at 4°C. The content of $\beta$-hexosaminidase ($\beta$-hex) was measured by spectrophotometric analysis as described previously (37), and percent release values were calculated. Data were expressed as mean release from duplicate readings.

For eicosanoid production, triplicate samples of $1 \times 10^5$ cells were stimulated with various doses of PGE$_2$ or its mimics (0.01-10 $\mu$M) or DMSO (1:1000), with and without anti-IgE (1 $\mu$g/ml) in the wells of 96-well flat bottom plates. Supernatants were collected at 30 min and stored at -20°C until further analysis with specific ELISAs to detect cysLTs (LTC$_4$, LTD$_4$, and LTE$_4$, Cayman) and PGD$_2$ (Amersham). In some experiments, the selective inhibitor of PKA, H89 (10 $\mu$M), was added to the cells 30 min before activation. The assay for PGD$_2$ did not detect PGE$_2$ at concentrations as high as 10 $\mu$M. For cytokine generation, sensitized, primed hMCs were challenged with Staphylococcus aureus peptidoglycan (PGN) (10 $\mu$g/ml, Sigma), anti-IgE (1 $\mu$g/ml), or medium alone in the presence or absence of agonists or controls as described for eicosanoids. Supernatants were collected at 6 h and frozen at -70°C until further analysis with ELISAs for TNF-$\alpha$ and IL-5 (both from eBiosiences). The data for each experiment were tabulated as the mean values from triplicate samples.
**cAMP measurements**

Triplicate samples of 2 x 10^5 hMCs were stimulated with various agonists or antagonists for 10 min as described previously (25). cAMP was measured with a commercial Biotrack™ cAMP ELISA kit (Amersham). The mean cAMP values for triplicate samples of cells stimulated with each agonist were compared with values from unstimulated cells, and the data expressed as absolute values.

**SDS-PAGE immunoblotting**

Samples of 2 x 10^5 hMCs were stimulated for various intervals with each agonist. Reactions were stopped on ice and the cells were lysed in a buffer containing 1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 5 μg/ml leupeptin, and 1 μg/ml pepstatin in 10 mM Tris (pH 8.0). Western blotting was performed as previously described (25). Dilutions (1:2000) of primary antibodies specific for the active, phosphorylated forms of ERK-1/ERK-2, c-Jun NH₂-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) were used. The same blots were stripped and probed again with rabbit polyclonal antibodies that detect total ERK-1/ERK-2, p38, and JNK at dilutions of 1:2,000. All anti-MAPK antibodies were purchased from Cell Signaling Technologies. ICER was detected with a rabbit polyclonal antiserum that recognizes all forms of cAMP response element modulator (CREM), including ICER (provided by Dr. Carlos Molina, University of New Jersey Medical School) at a concentration of 1:5000 (38).
Real-Time PCR

Samples of 5-10 x 10^6 primed, sensitized hMCs were stimulated for 2 h with anti-IgE or medium alone, all in the presence of SCF at a constant concentration of 100 ng/ml, with or without PGE2. The expression of TNF-α mRNA was determined with real-time PCR performed on ABI PRISM 7700™ Sequence Detection System (Applied Biosystems, Foster City, CA) as previously described (25). Primers and probes for the amplification of TNF-α and human β2 microglobulin and the corresponding VIC™ dye were purchased from Applied Biosystems.

Statistics

Data are expressed as mean ± SEM from at least three experiments except where otherwise indicated. Because quantities of eicosanoids and cytokines generated by hMCs from different donors varied widely, the data for some analyses were converted to percentages of the control (the samples not treated with PGE2 or EP receptor-selective agonists). Differences between treatment groups were determined with the student’s T test, with P < 0.05 considered significant. A Q test was used to eliminate outlying experiments.
Results

Expression and function of EP receptors

We used RT-PCR to determine the profile of EP receptor mRNAs expressed by hMCs. Bands corresponding to the predicted sizes of the EP2 and EP4 receptors were detected in mRNA samples from all 8 donors tested (as shown for one donor, Fig. 1A). All 8 donors showed both the EP3-II splice variant and at least one Gi-linked EP3 variant (5 showed EP3-I, 4 showed the EP3-IV, 4 showed EP3-VI, and none showed EP3-V transcript). EP1 receptor transcripts were detected in the RNA from the cells of only one donor (not shown). All of the primer sets yielded bands of the expected sizes from the positive control DNA template, and no products were detected from the negative control template (Fig. 1). The EP2, EP3, and EP4 receptor proteins were all detected by flow cytometry (n = 3, as shown for one experiment, Fig. 1B), while the EP1 receptor protein was not detected in the cells from any of these donors. IL-4 priming of the hMCs did not alter the profile or the level of EP receptor mRNA or proteins expressed (not shown). Virtually all of the cells showed high level cytofluorographic expression of Kit (Fig. 1B), confirming the identity of the EP receptor-positive cells as hMCs.

In a FLIPR assay, PGE2 (0.1-10 µM), but not PGD2, induced a calcium flux (an expected signature of both EP1 and EP3 receptors) (Fig. 2A). Subsequently, fluorescence spectrophotometry was used to measure calcium flux in hMCs following treatment with or without PTX. The cells from 8 donors were tested in this assay, and 3 exhibited calcium flux to PGE2 (1-10 µM); this flux was completely blocked by treatment of the cells with PTX (Fig. 2B). LTD4-mediated calcium flux occurred in every donor and
was unaffected by PTX. Of the receptor-selective reagents, only the EP₃ receptor-selective agonist AE-248 (10 µM) stimulated calcium flux, and was much weaker than PGE₂ at the same concentration (n = 2, data not shown). Priming of the cells with IL-4 did not change the strength of PGE₂-induced calcium flux.

Stimulation of hMCs with PGE₂ (1 µM) rapidly (5 min) induced phosphorylation of ERK-2 and ERK-1, with ERK-2 being expressed more strongly (Fig. 2C). ERK was also phosphorylated in response to stimulation of the hMCs with the EP₃ receptor-selective agonist AE-248 at 1 µM, but not with the EP₁, EP₂ and EP₄ receptor-selective agonists at the same concentration (n = 4, as shown for a single experiment, Fig. 2C). ERK phosphorylation in response to stimulation with both PGE₂ (Fig. 2C) and AE-248 (not shown) was completely blocked by pre-treatment of the cells with PTX or with the MEK inhibitor UO126 (5 µg/ml), but was unaffected by treatment with H89 (10 µM, n = 3, not shown). p38 and JNK were constitutively phosphorylated and not affected by PGE₂ or its analogues.

Stimulation of the cells with PGE₂ dose-dependently induced the accumulation of cAMP for every donor tested (n = 4, as shown for one representative experiment, Fig. 3A). The EP₂ receptor-selective agonist AE1-259-01 modestly stimulated cAMP accumulation at concentrations of 0.1, 1, and 10 µM (n = 5, Fig. 3B). A second EP₂ receptor agonist, butaprost, weakly stimulated cAMP accumulation, reaching significance at 10 µM (210 ± 6 vs. 158 ± 10 fmol for buffer treated cells, P = 0.03, n = 3, data not shown). The EP₄ receptor-selective agonist AE-329 also induced cAMP
accumulation at 1 and 10 µM (Fig. 3B). cAMP values in cell samples stimulated with AE-248 at 10 µM tended to be higher than controls, but did not reach significance (P = 0.08 relative to unstimulated controls, n = 5). The EP₁ receptor-selective agonist D1-004 was inactive (n = 3, not shown). Even at the highest doses tested, none of the selective agonists stimulated as much cAMP accumulation as PGE₂, and various combinations of the selective agonists did not additively stimulate cAMP over a wide concentration range (0.01-1 µM, n = 3, not shown). The EP₄ receptor-selective antagonist AE-208 (10 µM) abrogated cAMP accumulation in response to 10 µM of AE-329, but its effect on cAMP accumulation occurring in response to PGE₂ (1 µM) was not significant (P = 0.054, n = 4, Fig. 3C). The EP₂/EP₃ receptor selective antagonist AH6809 (50 µM) blocked cAMP accumulation in response to 1 and 10 µM PGE₂ by 69 ± 6 and 42 ± 16%, respectively, but failed to block cAMP accumulation in response to AE-329 (mean ± ½ range, n = 2, data not shown). PTX pre-treatment failed to alter cAMP accumulation in response to any agonist (not shown).

Effects of PGE₂ on exocytosis and eicosanoid production

PGE₂ at doses as high as 10 µM failed to attenuate FcγRI-dependent or PGN-dependent exocytosis of β-hexosaminidase by IL-4-primed hMCs and tended to induce some exocytosis by itself under these conditions (Fig. 4A). To determine whether this unanticipated response depended on IL-4, we tested PGE₂-mediated exocytosis in both IL-4-primed and unprimed cells. PGE₂ induced exocytosis only in the IL-4-primed cells, and this response was blocked by pre-treatment of the cells with PTX (Fig. 4B). Stimulation of the primed hMCs with the EP₃ receptor-selective agonist AE-248 caused
a small amount of exocytosis (4.3 ± 1% net release, n = 3, not shown), whereas none of the other agonists induced this response.

Primed hMCs generated both LTC₄ (as determined by the detection of cysLTs) and PGD₂ when stimulated by FcεRI cross-linkage (34). Treatment of the cells with PGE₂ inhibited the production of both PGD₂ and LTC₄ in a concentration-dependent manner (n = 2, as shown for one dose-response experiment, Figure 5A). PGE₂-induced suppression of both eicosanoids was reversed completely by treatment with AH6809, but not with AE-208 (n = 1, not shown). Of the selective agonists, only AE1-259-01 (Fig. 5B) tended to block LTC₄ and PGD₂ production (n = 5, Fig. 5B). The inhibition of LTC₄ generation, but not that of PGD₂ production, was reversed by the PKA inhibitor H89 (n = 2, data not shown). When used by itself, PGE₂ (0.1-10 μM) induced the production of PGD₂ (15, 18, and 22 ng/10⁶ hMCs in response to 0.1, 1 and 10 μM PGE₂ in a dose-response experiment, data not shown). Both PGE₂ and AE-248 (0.1-10 μM each) also induced LTC₄ production by the primed hMCs when the cells were pre-treated with H89 (as shown for the 10 μM concentration, Fig. 5C). PGE₂-mediated eicosanoid generation was completely blocked by pre-treatment of the cells with PTX (n = 2, not shown). The selective agonists of the EP₁, EP₂, and EP₄ receptors failed to induce eicosanoid generation by themselves, or when used in combination with H89, even at the highest doses tested (10 μM, n = 3, data not shown).
**Cytokine Generation and ICER induction**

Primed hMCs generated IL-5 and TNF-α when stimulated with either anti-IgE or PGN. PGE₂ interfered with the production of each cytokine in a dose-dependent manner (as shown in a representative experiment, Fig. 6A), in response to both anti-IgE and to PGN (Fig. 6B). AH6809 (50 µM) and AE-208 (10 µM) reversed the inhibitory effect of PGE₂ on TNF-α generation by 24 and 50%, respectively (n = 1, not shown). AE1-259-01 inhibited TNF-α generation by hMCs in a dose-dependent manner, being statistically significant at 10 µM (n = 3, Fig. 6C). Butaprost was also active for this function at 10 µM (n = 2, not shown). Both AE-248 and AE-329 also inhibited TNF-α production by hMCs (Fig. 6C). H89 failed to reverse the inhibitory effect of any agonist on cytokine generation (n = 3, not shown). PGE₂ at 10 µM strongly inhibited the FcεRI-induced steady-state expression of TNF-α mRNA, as determined by real-time PCR (n = 2); this effect was reproduced by both AE1-259-01 and AE-329 (as shown for one experiment, Fig. 6D). Pre-treatment of the cells with the selective Epac agonist 8-pCPT-2’-O-Me-cAMP (10 µM) modestly inhibited FcεRI-mediated TNF-α generation (43% inhibition), but had no effect on IL-5 production, and two inhibitors of GSK-3 (GSK-3 inhibitor II (20 µM) and SB 216763 (20 µM) had no effect on TNFα generation (14 and 12% inhibition, respectively, not shown).

At 0.1-10 µM, PGE₂ dose-dependently induced ICER expression (Fig. 7A), peaking at 2-3 h after stimulation (Fig. 1B). This effect was partly mimicked by stimulation of the cells with forskolin at 200 µM (Fig. 7D). AE1-259-01 and AE-329 both also induced ICER expression at 1 µM (Fig. 7B, 7D), while the EP₃ receptor-selective
agonist AE-248 did not strongly induce ICER expression. Pre-treatment of the cells with either UO126 or H89 (5 μg/ml and 10 μM) tended to attenuate the PGE₂-induced expression of ICER (P = 0.08 and 0.06 relative to PGE₂ alone, n = 4, Fig. 7C, as shown for one experiment, Fig. 7C). Combined treatment with H89 and UO126 totally abrogated ICER induction, and pretreatment with either AH6809 or AE-208 modestly reduced ICER induction (n = 2, 7D, as shown for one experiment, Fig. 7C). H89, but not UO126, attenuated ICER induction by AE1-259-01 (n = 3, not shown). Treatment of the cells with 8-pCPT-2'-O-Me-cAMP did not induce ICER expression (n = 2, not shown).
Discussion

Both COX-1 and COX-2-dependent synthetic pathways mediate PGE$_2$ production in models of experimentally induced allergic pulmonary disease in mice (39,40). The abrogation of functional COX-1 or COX-2 activity through genetic (39) or pharmacologic (40) approaches amplifies mucosal inflammation, eosinophilia, and airway hyperresponsiveness in such models by enhancing cytokine generation by T helper cells (41). Because MCs are an apparent target of the protective effects of PGE$_2$ against provocative challenges in humans with asthma (15,16) and AERD (17), we used well-characterized non-transformed hMCs derived in vitro to better define the EP receptors contributing to the effects of PGE$_2$ on mediator release, and to determine whether PGE$_2$ might also activate hMCs in certain contexts.

EP receptors induce differential signaling events through specific patterns of G protein utilization. Heterologously expressed EP$_2$ and EP$_4$ receptors both use Gs proteins to stimulate adenylyl cyclase and induce accumulation of cAMP (42), with EP$_2$ receptors being more potent. We detected both of these receptors at the protein and mRNA levels (Fig. 1), and showed that both were functional based on the cAMP accumulation induced by the selective agonists AE1-259-01 and AE-329 (Fig. 3). The complete blockade of AE-329-induced cAMP accumulation by the EP$_4$ receptor antagonist AE-208 (Fig. 3C) confirmed the specificity of this agonist at 10 µM, and the functionality of the receptor. However, the failure of AE-208 to significantly suppress PGE$_2$-induced cAMP stimulation suggests a more dominant role for EP$_2$ receptors in this response, also reflected by inhibition by AH6809. Despite reported nanomolar
range potency on EP transfectants (26,27), none of the selective agonists, alone or in various combinations, approached the efficacy of PGE$_2$ itself. We speculate that primary cells that express comparatively low levels of individual EP receptor proteins (Fig. 1) may require the assembly of ligand-induced, hetero-oligomeric complexes between different EP receptors to amplify PGE$_2$-dependent signaling events, as reported for other co-expressed GPCRs (43). Such complexes may not be efficiently induced by the selective agonists. We also cannot exclude the potential existence of previously unrecognized EP receptors among the orphan GPCRs.

Splice variants of the human EP$_3$ receptor mRNA produce multiple isoforms with identical ligand binding properties but different C-termini that confer the ability to either stimulate PTX-sensitive Gi proteins (all isoforms) or Gs proteins (EP$_3$-II and EP$_3$-IV isoforms) (35,36), mediating opposing effects on adenyly cyclase/cAMP (44,45). The consistent expression of the EP$_3$-II message with a combination of Gi-linked variants suggests that the EP$_3$ receptor protein expressed by hMCs (Fig. 1B) reflects more than one isoform. The modest increment in cAMP that was induced by AE-248 (Fig. 3) suggests the function of the EP$_3$-II receptor, although this effect did not reach statistical significance and only occurred at a dose potentially capable of crossover effects (27). Since cAMP elevation attenuates calcium signaling (45), concomitant Gs-induced signaling from EP$_3$-II or other receptors may explain why EP$_3$ receptor-dependent calcium flux was observed in only 3 of the 8 donors tested (Fig. 2). Nonetheless, PTX-sensitive ERK phosphorylation in response to PGE$_2$ and AE-248 was observed in all
donors tested (Fig. 2), reflecting a robust signal through the Gi-linked EP₃ receptor variants responsible for the secretory events observed (Fig. 4, 5).

For mediator release, we primed hMCs with IL-4 since this cytokine augments both exocytosis and eicosanoid generation (32,47). While earlier studies reported that PGE₂ (by raising cAMP levels) inhibited FcεRI-dependent exocytosis and eicosanoid release from MCs (20,21), other studies reported that PGE₂ potentiates histamine release (22) and IL-6 production (23) by mBMMCs through effects at the Gi-linked EP₃ receptor. Despite raising cAMP, PGE₂ did not inhibit FcεRI-dependent exocytosis, instead inducing some exocytosis by itself (Fig. 4A). This response required IL-4, was blocked by PTX pre-treatment (Fig. 4B), and was partly mimicked only by AE-248. IL-4 may thus alter the coupling of Gi-linked EP₃ receptor signaling to exocytosis, presumably distal to phospholipase C, inositol phosphate production, protein kinase C activation, and calcium flux since priming was not required for the latter event (Fig. 1). IL-4, which is abundant in allergic inflammation, could promote MC activation responses to PGE₂ in tissues where MCs express EP₃ receptors.

FcεRI-dependent MC activation simultaneously induces pre-formed mediator release (histamine and proteases) and eicosanoid generation (LTC₄ and PGD₂) de novo. Eicosanoid generation requires ERK-dependent phosphorylation of cytosolic PLA₂ (48), providing arachidonic acid to both the COX-PGD synthase and 5-lipoxygenase-LTC₄ synthase (5-LO/LTC₄S) pathways. Because PGE₂ blocks the production of both PGD₂ and cysLTs in response to provocative challenges in vivo, it
was not surprising that pre-treatment of the cells with PGE$_2$ (or with the EP$_2$ receptor-selective antagonist AE1-259-01) attenuated FcεRI-mediated generation of both PGD$_2$ and cysLTs (Fig. 5A, 5B). Unexpectedly, hMCs stimulated with PGE$_2$ also generated PGD$_2$, but not LTC$_4$ (reflected by the cysLT-specific ELISA). cAMP-dependent PKA phosphorylates 5-LO, preventing its translocation to the nuclear envelope to synthesize the LT precursor LTA$_4$ (49). This likely explains why cysLT generation occurred in response to PGE$_2$ when PKA was inhibited by H89 (Fig. 5C). This response was also elicited, albeit weakly, by AE-248. Combined with the abrogation of PGE$_2$-induced exocytosis and eicosanoid generation by pre-treatment of the cells with PTX, these findings firmly implicate EP$_3$ receptors in these effector responses. The clearly opposing cellular and signaling responses to EP$_2$ and EP$_3$ receptor agonists strongly supports the selectivity of these reagents for their intended receptors at the doses used. It appears likely that EP$_2$ and EP$_3$ receptors counterbalance one another's effects on cellular activation responses when co-expressed. EP$_2$ receptor-selective stimulation was recently reported to induce the production of vascular endothelial growth factor by hMCs (50). Thus EP$_2$ receptor signaling in allergic disease may attenuate effector eicosanoid formation while simultaneously promoting tissue remodeling to facilitate the resolution phase of mucosal inflammation.

TNF-α localizes to MCs in airway biopsy specimens from patients with severe asthma who respond therapeutically to TNF-α-selective antagonists (51) and promotes granulocyte influx in MC-dependent innate immune responses in mice (11). In our previous studies, adenine nucleotides suppressed TNF-α generation by hMCs (25),
initiating a cAMP-dependent, PKA-independent signaling cascade resulting in the expression of ICER, a transcriptional repressor protein that is the product of an alternate promoter in the gene encoding cAMP response element modulator (38). ICER prevents binding of NF-AT and AP-1 transcription factors to the promoter regions of the genes encoding TNF-α and other cytokines (52). The marked suppression of TNF-α protein and mRNA expression by PGE₂ (Fig. 6) suggested a process involving ICER. Indeed, PGE₂ strongly induced ICER expression (Fig. 7), as did the adenylyl cyclase activator forskolin. Importantly, this response likely involved input from more than one EP receptor, since both AE1-259-01 and AE-329 both induced ICER on their own at 1 µM (Fig. 7B, 7D). Moreover, H89 (10 µM) only partly blocked ICER induction, but completely blocked it when used in combination with UO126 (Fig. 7C, 7D). Thus PKA (reflecting the EP₂ and/or EP₄ receptors) and ERK (implicating EP₃ receptors, Fig. 2) cooperate to induce ICER. Since the Epac agonist and GSK-3 antagonists did not block cytokine generation or induce ICER expression, the effect of PGE₂ in our study was not a consequence of the recently reported Epac/GSK-3 transcriptional repressor pathway (53). Thus PGE₂ suppresses cytokine generation by cell types with different EP receptor expression profiles (34, 52-55) through multiple pathways. This versatility may ensure that PGE₂, when not suppressed by COX inhibitors, tempers cytokine generation and consequent inflammation in the hours after MC activation occurs in allergic disease.

Our data reveal that PGE₂ can either induce (via EP₃ receptors) or suppress (via EP₂ receptors) mediator release from MCs that induce the EPR. EP₂ receptor-dependent signaling is an attractive candidate to explain PGE₂-dependent suppression
of eicosanoid generation in allergen or aspirin challenge. Moreover, the suppressive effect of PGE$_2$ on allergen-induced LPRs (15) could in part reflect transcriptional repression through cooperative signaling from multiple EP receptors. Finally, EP$_3$ receptor-dependent MC activation is reported to occur in the skin in a mouse model of cutaneous inflammation (21), and this may reflect both the release of histamine and PGD$_2$. It seems likely that the pro- and anti-MC activating effects of PGE$_2$ reported for respectively for skin (21) and lung (15) reflect regional differences between MCs EP receptor expression and function.
References


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Figures and Legends

**Figure 1. Profile of EP receptors expressed by hMCs.** A. Total RNA was extracted from 8 wk-old hMCs derived in the presence of SCF, IL-6, and IL-10 and samples were subjected to RT-PCR with primers specific for each indicated receptor. Positive control (from genomic DNA) and negative control (from non-reverse-transcribed RNA) are
displayed. B. 8-wk-old hMCs were fixed, permeabilized, and stained with affinity-purified polyclonal antibodies specific for the indicated receptor proteins. IL-4 priming did not change the levels of expression of any EP receptor (not shown). Bold tracings = EP receptors; light tracings = control IgG. MFI = mean fluorescence intensity. RT-PCR data in A and flow cytometry in B are from experiments representative of 8 and 3, respectively, each using the cells of a different donor.
**Figure 2. Functional signatures of the EP3 receptor.** A. FLIPR assay revealing dose-dependent calcium fluxes by hMCs stimulated with the indicated concentrations of PGE₂, PGD₂, or LTD₄. Each tracing is representative of triplicate samples, all of which showed virtually identical responses. B. Effect of overnight treatment with PTX on calcium flux by Fura-2-AM-loaded hMCs stimulated with PGE₂ (10 µM). Data are from one of three experiments in which a calcium response was elicited. C. SDS-PAGE immunoblot showing signals corresponding to phosphorylated and total ERK MAPK in samples of hMCs stimulated for 5 min with the indicated agonists (1 µM each) in the presence or absence (-) of the MEK inhibitor, UO126, or PTX. Data are from a single experiment representative of four performed, all of which yielded similar results.
Figure 3. Effect of stimulation of hMCs with PGE$_2$ and receptor-selective analogues on cAMP accumulation. A. Effect of PGE$_2$. Samples of $2 \times 10^5$ hMCs were stimulated for 10 min with the indicated concentrations of PGE$_2$. Values are mean ± SEM of triplicate samples from a single experiment. Similar results were obtained with hMCs from two additional donors. B. Effect of selective EP receptor agonists. Cells were stimulated for 10 min with the indicated concentrations of selective agonists of the EP$_1$ (D1-004), EP$_2$ (AE1-259-01), EP$_3$ (AE-248), and EP4 (AE-329) receptors, or with
DMSO alone. Values are expressed as absolute quantities of cAMP and are expressed as mean ± SEM for 5 experiments. C. Effect of the EP₄ receptor-selective antagonist AE-208 (10 μM) on cAMP accumulation in response to hMC stimulation with PGE₂ (1 μM) or AE-329 (10 μM). Values are expressed as percent of control (DMSO alone) and are mean ± SEM from four experiments. * denotes significance.
**Figure 4. Effect of PGE₂ on exocytosis of β-hex by hMCs.** A. hMCs were primed for 5 d with IL-4, sensitized with IgE overnight, and then stimulated with medium alone, anti-IgE, or staphylococcal PGN (50 µg/ml) for 30 min in the presence or absence of PGE₂ (10 µM). Values are expressed as percent of total cellular β-hex release and are the mean ± SEM from 5 experiments. B. Primed and unprimed hMCs were stimulated for 30 min with PGE₂ (10 µM) and compared for exocytosis. Some of the primed cells were treated overnight with PTX before activation. Values are mean ± SEM from 3 experiments. * denotes significance relative to unprimed cells.
A  FcεRI-induced eicosanoid production: PGE₂

Dose-response

![Graph showing dose-response for cysLTs and PGD₂](image)

Fcc-induced eicosanoid production

![Graph showing % of control for LTC₄ and PGD₂](image)

B  FcεRI-induced eicosanoid production: EP receptor-selective agonists

Dose-response

![Graph showing dose-response for PGD₂ and cysLTs](image)

C  Direct induction of eicosanoid generation

Dose-response

![Graph showing dose-response for PGD₂ and cysLTs](image)
Figure 5. Effect of PGE$_2$ and its analogues on Fc$_{ε}$RI-dependent and -independent eicosanoid generation. A. Dose-response (left) for the suppressive effect of PGE$_2$ on eicosanoid formation by IL-4-primed, sensitized hMCs stimulated for 30 min with anti-IgE. Data in second experiment were similar. Effect of 10 $\mu$M PGE$_2$ (right) or buffer control. Data are mean ± SEM over 5 experiments, expressed as percent of control (quantities generated in the presence of buffer alone). B. Dose-dependent effects of EP$_2$ receptor-selective antagonist AE1-259-01 and EP$_3$ receptor-selective agonist AE-248 on Fc$_{ε}$RI-mediated PGD$_2$ generation (top panel). Results from a second experiment were similar. Effect of 10 $\mu$M AE1-259, AE-248, and the EP$_4$ receptor-selective agonist AE-329 (10 $\mu$M each) on Fc$_{ε}$RI-mediated generation of LTC$_4$ (middle, as measured by an ELISA for cysLTs) and PGD$_2$ (bottom). Values are mean ± SEM from 4 experiments, normalized to percent of control (cells stimulated in the presence of DMSO). C. Induction of eicosanoid generation directly in response to PGE$_2$ and its analogues. Cells were stimulated for 30 min with PGE$_2$ or the indicated receptor-selective analogues, or with buffer control (DMSO), with or without pre-treatment with H89 for 30 min. Dose-dependent effect of AE-248 and AE1-259-01 on PGD$_2$ generation by primed hMCs without Fc$_{ε}$RI cross-linkage (top). Data in a second experiment were similar. The effects of 10 $\mu$M PGE$_2$ and selective agonists in the presence or absence of the PKA inhibitor H89 (10 $\mu$M) are displayed for cys-LTs (middle panel) and PGD$_2$ (bottom). Data in the latter two panels are mean ± SEM from 3 experiments.
Figure 6. Effect of PGE2 and receptor-selective analogues on cytokine generation by hMCs. A. Effect of various doses of PGE2 on the generation of TNF-α by IL-4-primed, sensitized hMCs stimulated for 6 h with anti-IgE or PGN (10 µg/ml) in the presence of PGE2. Values are the mean of triplicate samples from a single experiment. Similar data were obtained with the cells of a second donor. B. Effect of PGE2 (10 µM) on the production of TNF-α and IL-5 by IL-4-primed, sensitized hMCs stimulated with anti-IgE or PGN for 6 h. Values are expressed as percent of control and are the mean ± SEM from three separate experiments. C. Effect of receptor-selective agonists on TNF-α generation by hMCs stimulated with anti-IgE. Primed, sensitized hMCs were stimulated for 6 h in the presence of PGE2 (10 µM) or the indicated concentrations of the EP receptor-selective agonists. Values are expressed as percent of the DMSO
control and are mean ± SEM from 3 experiments. D. Real-time PCR showing effect of PGE₂ and receptor-selective agonists on the FcεRI-induced steady-state expression of TNF-α mRNA. Data are from a single experiment, and are similar to that obtained in a second experiment.
Figure 7. Expression of the transcriptional repressor ICER. A. hMCs were stimulated with PGE₂ at the indicated concentrations for 3 h. Lysates of the cells were resolved by SDS-PAGE, and immunoblotting was performed with a CREM-specific antiserum that recognizes ICER. B. Time-dependent induction of ICER expression in response to PGE₂ or EP receptor-selective analogues. Lysates were generated from hMCs stimulated with the indicated agonist (1 µM each) for 5 min-3 h. The samples were analyzed by Western blotting with a CREM-specific antiserum that recognizes ICER. ICER expression was normalized to actin levels. C. Inhibitors: The effect of different inhibitors on ICER expression was assessed. D. Densitometry: The ICER expression levels were quantified by densitometry and normalized to actin levels. The data are presented as the mean ± SD of three independent experiments.
were subjected to SDS-PAGE and immunoblotting with anti-CREM. The same blot was stripped and probed for β-actin. Data in A and B are from single experiments representative of three performed for each. C. Effects of H89 (10 µM), UO126 (5 µg/ml), AH6809 (50 µM) and AE-248 (10 µM) on the induced expression of ICER in response to stimulation with PGE₂ (1 µM) for 3 h. Results in a second experiment were similar. D. Quantitative densitometry showing induction of ICER following stimulation with the indicated agonists (1 µM) or their respective buffer controls for 3 h. Results are the mean ± SEM from a minimum of four separate experiments, except for the combined effect of H89 with UO126 (top), and the effects of the EP₂ and EP₄ receptor-selective antagonists AH6809 and AE-208, both of which represent mean ± ½ range from two of these experiments. * indicates P < 0.05 relative to control (buffer or DMSO alone).
Human mast cells express multiple EP receptors for prostaglandin E2 that differentially modulate activation responses

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