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In Vitro and *In Vivo* Validation of EP2-Receptor Agonism to Selectively Achieve Inhibition of Mast Cell Activity

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ABSTRACT

Purpose: Agonism of the prostaglandin E2 receptor, E-prostanoid receptor 2 (EP2), may represent an alternative protective mechanism in mast cell (MC)-mediated diseases. Previous studies have suggested that activation of the MC EP2 receptor prevents pathological changes in the murine models of allergic asthma. This work aimed to analytically validate the EP2 receptor on MCs as a therapeutic target.

Methods: Murine MC lines and primary cultures, and MCs bearing the human immunoglobulin E (IgE) receptor were subjected to IgE-mediated activation subsequent to incubation with selective EP2 agonists. Two molecularly unrelated agonists, butaprost and CP-533536, were tested either *in vitro* or in 2 *in vivo* models of allergy.

Results: The diverse range of MC populations was consistently inhibited through selective EP2 agonism in spite of exhibiting a heterogeneous phenotype. Such inhibition occurred in both mouse and human IgE (hIgE)-mediated activation. The use of molecularly unrelated selective EP2 agonists allowed for the confirmation of the specificity of this protective mechanism. This effect was further demonstrated in 2 *in vivo* murine models of allergy where MCs are a key to pathological changes: cutaneous anaphylaxis in a transgenic mouse model expressing the hIgE receptor and aeroallergen-induced murine model of asthma.

Conclusions: Selective EP2 agonism is a powerful pharmacological strategy to prevent MCs from being activated through IgE-mediated mechanisms and from causing deleterious effects. The MC EP2 receptor may be an effective pharmacological target in allergic and other MC-mediated conditions.

Keywords: allergy; anaphylaxis; asthma; IgE; inflammation; mast cell; prostaglandin E2; prostaglandin E receptor 2

INTRODUCTION

The incidence of allergic diseases and thus the economic burden they represent have grown steadily for more than 50 years. The increased incidence correlates with socioeconomic factors, but it is also driven by the inability to cure or properly control their progression.[1-3]

Corticosteroids remain the most effective treatment against allergic diseases, notably asthma.[4] However, the benefit-to-risk balance of these drugs is often unfavourable. Corticosteroid-like strategies, *i.e.*, management approaches based on the use of wide-spectrum endogenous compounds that may overcome corticosteroid limitations, such as prostaglandins (PGs) or PG analogues, could represent a valuable alternative.[5] Indeed, we have shown that activation of the PGE2 receptor E- prostanoid receptor 2 (EP2) subtype is likely to exert an array of functions. We have primarily studied its ability to minimize airway hyper-reactivity and inflammation in the murine models of allergic asthma.

Mast cells (MCs) play a key role in allergies as effector cells and likely also act as regulatory cells. MC-derived mediators trigger the early asthmatic response manifested by bronchospasm, mucus secretion, and mucosal edema. They may also contribute to inflammation[6,7] and thereby function in orchestrating the late asthmatic response.[8,9] Early restriction of MC activity by EP2-receptor activation may thus reduce the natural progression of allergic diseases. Whereas PGs (produced by COX activity) have been traditionally viewed as pathogenic in inflammatory diseases, some data suggest that PGE2 prevents airway inflammation and hyper-reactivity in allergic asthma models by inducing MC blockade.[10-14] Conversely, as per previous work, inhibition of COX-2, which decreases PGE2 levels, results in increased airway hyper-reactivity.[15-18] Accordingly, diminished PGE2 production has been demonstrated in airway cells isolated from asthmatic patients.[19,20] MCs express a range of EP receptors (EP1-EP4)[21] with a broad variety of functions.[22] The expression pattern of these receptors on MCs likely determines whether PGE2 elicits a stimulatory or an inhibitory signal.

Despite the importance of these findings, the above-mentioned studies were limited to specific MC populations and agonists. To ascertain the selective role of EP2 in preventing MC activity, we tested 2 structurally unrelated EP2 agonists for their ability to prevent MC degranulation. We conducted *in vitro* experiments in a wide range of phenotypically distinct murine and human MC populations as well as *in vivo* studies in a humanized model of anaphylaxis. This multifaceted experimental approach permitted a full preclinical validation of EP2 as potentially protective in immunoglobulin E (IgE) and MC-mediated conditions.

MATERIALS AND METHODS

Materials

Anti-2,4-dinitrophenol (DNP) mouse IgE was kindly provided by Dr. Juan Rivera from the National Institutes of Health (NIH; Bethesda, MD, USA). Biotinylated human IgE (hIgE) was obtained from ABBIOTEC (San Diego, CA, USA), and streptavidin (SA) was purchased from BD Biosciences (San Jose, CA, USA). Chimeric hIgE anti-4-nitrophenol (NP) was purchased from AbD Serotec (Kidlington, United Kingdom), and NP-bovine serum albumin (BSA) was obtained from Biosearch Technologies (Petaluma, CA, USA). PGE2 and the EP2-agonist butaprost were purchased from Cayman Chemical (Ann Arbor, MI, USA). The selective EP2 agonist, Pfizer's CP-533536 (Evatanepag), was synthetized by Galchimia (A Coruña, Spain) as described below. The EP2 antagonist AH6809, DNP-human serum albumin (DNP-HSA), phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), DNP, Evans blue, formamide, and methacholine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol was obtained from Panreac Applichem (Barcelona, Spain), and physiological saline solution (PS) was purchased from B. Braun (Melsungen, Germany).
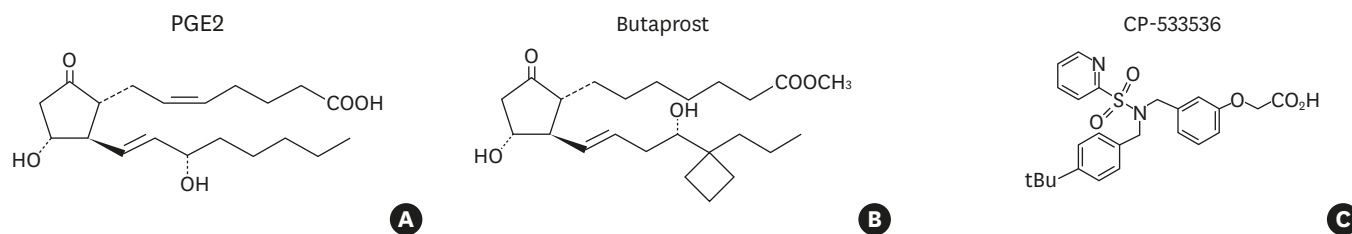
Fig. 1. Chemical structure of PGE2, butaprost, and CP-533536. Molecular structure of (A) the cyclic prostanoid PGE2, (B) the prostanoid EP2 agonist butaprost, and (C) the 3-pyridyl sulphonamide EP2 agonist CP-533536.
PGE2, prostaglandin E2; EP2, E prostanoid receptor 2.

Synthesis of the EP2 agonist CP-533536

Pfizer's CP-533536 is a 3-piridil sulphonamide with high *in vitro* potency and selectivity for the EP2 receptor.[23,24] **Fig. 1** shows the structural differences between the non-selective EP2 agonist PGE2 and the 2 highly selective albeit chemically distinct EP2 agonists: CP-533536 and butaprost. Butaprost is a PG-like lipid compound, while CP-533536 is molecularly unrelated to PG.

CP-533536 was synthesized by Galchimia. The chemical identity of Pfizer's compound was tested by nuclear magnetic resonance imaging and liquid chromatography-mass spectrometry. The purity was 98.32%. The sodium salt was crystallized to obtain an optimal solution.

Laboratory animals

All animal procedures were approved by the Ethics Committee for Animal Research (approval number CEAAH3770) of the Universitat Autònoma de Barcelona (Barcelona, Spain). These procedures were conducted in accordance with the Royal Decree 53/2013 on "Protection of animals used in research and for other scientific purposes" of Spanish Legislation and in accordance with directive 2010/63/EU of the European Parliament and the Council. Eight-week-old female BALB/c mice from Charles River Laboratories (Calco, Italy) were used for the *in vivo* studies as well as the *in vitro* experiments involving primary cultures of peritoneum-derived MC (PDMC) and lung MC (LMC).

Eight- to 12-week-old high-affinity IgE receptor (FcεRI)$^{-/-}$ human FcεRI (hFcεRI)$^+$ BALB/c mice[25] housed under a 12-hour light/dark cycle were used for passive cutaneous anaphylaxis (PCA) and for the obtention of primary cultures of humanized PDMCs. They were bred at the Animal Facility of the Universitat Autònoma de Barcelona from 2 breeding pairs kindly provided by Dr. Jean-Pierre Kinet from the Beth Israel Deaconess Medical Centre at Harvard University (Boston, MA, USA).

Murine and human MC populations

Five MC populations, either murine or human-derived, cultured in a 37°C incubator with 5% carbon dioxide (CO_2) in a humidified atmosphere, were used in this study. 1) PDMCs were harvested from mice after injection of 4-mL Roswell Park Memorial Institute (RPMI) medium into the peritoneal cavity. Cells were then cultured for 3 weeks in the presence of interleukin (IL)-3 (20 ng·mL^{-1}) and stem cell factor (SCF) (20 ng·mL^{-1}), in RPMI with 20% fetal bovine serum (FBS) to obtain an expanded population of PDMCs.[26] 2) Murine airway or LMCs were harvested from the lungs, which were minced into 3-mm pieces and submerged in IL-3 (20 ng·mL^{-1}), SCF (20 ng·mL^{-1}), and amphotericin B (250 ng·mL^{-1}) in RPMI medium with 20% FBS for 4 weeks to expand the MC population. 3) The Cl.MC/C57.1 (C57.1) MC line, kindly provided by Dr. Stephen J. Galli from Stanford University School Medical Centre (Stanford, CA, USA), was originally

derived from BALB/c mice.[27] C57.1 MCs were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (FCS), L-glutamine, and 2-mercaptoethanol. 4) Human leukemic MCs (LAD2), an MC line derived from a patient with untreated MC sarcoma,[28] were kindly provided by Dr. Arnold Kirshernbaum and Dr. Dean Metcalfe from the National Institute of Allergy and Infectious Diseases/NIH. LAD2 MCs were cultured in StemPro-34 serum-free medium supplemented with 2 mM L-glutamine, 100 U·mL⁻¹ penicillin, 50 µg·mL⁻¹ streptomycin, and 100 ng·mL⁻¹ human SCF. Half of the culture medium was replaced every 7 days. 5) The humanized rat basophilic leukemic cell line RS-ATL8[29] was kindly provided by Dr. Ryosuke Nakamura from National Institute of Health Sciences (Tokyo, Japan). RS-ATL8 cells were derived from rat basophils, but phenotypically replicate the features of MCs. RS-ATL8 cells were cultured weekly with Minimum Essential Medium medium, supplemented with 10% FCS (heat-inactivated at 56°C for 30 minutes), 1% penicillin, 1% streptomycin, 1.2 mg·mL⁻¹ geneticin, and 200 µg·mL⁻¹ hygromycin B. Geneticin and hygromycin B were used to maintain expression of hFcεRI genes and nuclear factor of activated T-cell luciferase. Before the *in vitro* assays, all MC types were maintained overnight in the absence of specific growth factors.

MC stimulation and release assay

Murine cells (C57.1, PDMC, and LMC) were sensitized with 1 µg·mL⁻¹ DNP-specific IgE for 2 hours in SCF- and IL-3-free media. After sensitization, cells were washed and resuspended in HEPES buffer (10 mM HEPES [pH 7.4], 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na_2HPO_4_7H₂O, 5.6 mM glucose, 1.8 mM $CaCl_2$_2H₂O, and 1.3 mM $MgSO_4$_7H₂O) with 0.04% BSA. Cells were seeded on a V-bottom 96-well plate, with 50,000 cells in a final volume of 100 µL, and treated with 10^{-5} M butaprost, 10^{-5} M PGE2, or vehicle (PBS with 0.1% DMSO) for 30 minutes and 10^{-5} M AH6809 (LMC) or vehicle (PS with 20% ethanol) for 1 hour at 37°C with 5% v/v CO₂. PDMCs from wild-type (WT) BALB/c were also treated with increasing concentrations of butaprost (10^{-6} M, $3 \cdot 10^{-6}$ M, and $3 \cdot 10^{-5}$ M). Cells were stimulated with 50 ng·mL⁻¹ DNP-HSA as an antigen (Ag) for 30 minutes. LAD2 MCs were sensitized with 100 ng·mL⁻¹ for 2 hours in SCF- and IL-3-free media. RS-ATL8 cells were sensitized with 500 ng·mL⁻¹ biotinylated hIgE for 16 hours. PDMCs from FcεRI⁻/⁻hFcεRI⁺ BALB/c mice were sensitized with 100 ng·mL⁻¹ chimeric hIgE anti-NP for 16 hours. After sensitization, cells were washed, resuspended with HEPES buffer with 0.04% BSA, and seeded on a V-bottom 96-well plate, with 150,000 cells in a final volume of 300 µL (LAD2) or 200,000 cells in a final volume of 320 µL (PDMCs). Two days before the release assay, 50,000 RS-ATL8 cells were cultured in a final volume of 100 µL to obtain 100,000 adhering cells. Cells were treated for 2 hours and 15 minutes at 37°C with 5% v/v CO₂ as follows: increasing concentrations of butaprost and CP-533536 (10^{-7} M, $3 \cdot 10^{-7}$ M, 10^{-6} M, $3 \cdot 10^{-6}$ M, 10^{-5} M, $3 \cdot 10^{-5}$ M, 10^{-4} M, or $3 \cdot 10^{-4}$ M) or vehicle (PBS with 0.1% DMSO) in LAD2; CP-533536 (10^{-12} M, 10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M, or 10^{-4} M) in RS-ATL8; and butaprost (10^{-6} M, $3 \cdot 10^{-6}$ M, or 10^{-5} M) in PDMCs from FcεRI⁻/⁻hFcεRI⁺ mice. Cells were challenged with 100 ng·mL⁻¹ SA (LAD2), 1,000 ng·mL⁻¹ SA (RS-ATL8), or 50 ng·mL⁻¹ NP-BSA (PDMC from FcεRI⁻/⁻hFcεRI⁺ mice) for 30 minutes at 37°C with 5% v/v CO₂. Degranulation was stopped by placing the cells in iced water, and the cell suspension was centrifuged for 10 minutes at 4°C at 1,500 rpm.

The enzymatic activity of the granule marker β-hexosaminidase released into the extracellular media was measured in the supernatants and pellets of activated cells as previously described.[30] The results are expressed as percentage of the total activity [% degranulation = 100 × (supernatant content)/(supernatant + lysate content)]. Percentage β-hexosaminidase inhibition by agonists was determined by subtracting the IgE+/Ag− release [% inhibition = 100 − (% release − % IgE+/Ag − release)/(% IgE+/Ag+ release − % IgE+/Ag − release) × 100].
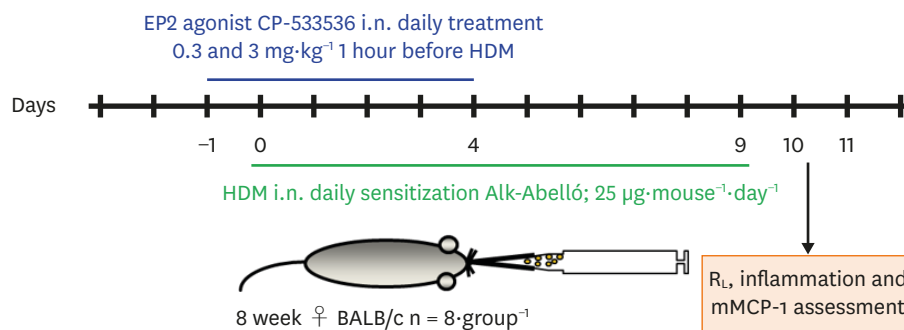
Fig. 2. HDM sensitization and selective EP2 agonist CP-533536 treatment protocol. Sensitization to HDM aeroallergens was conducted by nasal exposure to HDM extract (25 μg·mouse⁻¹·day⁻¹) for 10 consecutive days (days 0-9). The EP2 agonist CP-533536 (0.3 mg·kg⁻¹ or 3 mg·kg⁻¹) was administered i.n. 1 hour before exposure to HDM extract from the day before initiating sensitization (day −1) through day 4.
HDM, house dust mite; EP2, E prostanoid receptor 2; i.n., intranasally; mMCP-1, mouse mast cell protease-1; R_L, lung resistance.

Mice sensitization to house dust mite (HDM) aeroallergens

Sensitization to HDM allergens was performed according to Cates et al.[31] Briefly, BALB/c mice (Charles River Laboratories) were exposed to a purified HDM extract (Alk-Abelló, Madrid, Spain) with low lipopolysaccharide content (<0.5 EU·dose⁻¹). The aeroallergens were administered intranasally (i.n.) under light isoflurane anesthesia at a dose of 25 μg·mouse⁻¹ for 10 consecutive days (**Fig. 2**). Non-sensitized control animals received i.n. physiologic saline solution.

Treatment with the EP2 agonist CP-533536

Sensitized mice were treated with the EP2 agonist CP-533536 (**Fig. 2**). CP-533536 was administered i.n. (0.3 mg·kg⁻¹ or 3 mg·kg⁻¹) 1 hour before exposure to the HDM extract, starting 1 day before initiating sensitization (day −1) and continuing through the first 4 days of sensitization (day 4). HDM-sensitized untreated mice received i.n. PBS in 0.1% DMSO.

Assessment of airway hyper-responsiveness

Twenty-four hours after exposure to HDM, airway reactivity to increasing doses of methacholine was assessed. Lung resistance (R_L) was measured with the Buxco FinePointe plethysmograph system (Buxco, Troy, NY, USA) in mice anesthetized with ketamine and xylazine. The trachea was cannulated with an 18-gauge needle, and mice were ventilated with a pump ventilator (tidal volume, 12.5 mL·kg⁻¹; frequency, 120 breaths·min⁻¹; and positive end-respiratory pressure, 2.5- to 3.0-cm H_2O). Changes from baseline airway reactivity (R_L) were assessed after 1 minute of nebulized intratracheal methacholine.

Assessment of airway inflammation

HDM-induced airway inflammation in treated and untreated mice was assessed 24 hours after exposure to the aeroallergen by determining the differential cell count in fluid obtained by bronchoalveolar lavage (BAL). BAL was performed by slowly infusing 0.3-mL PBS 2 times, then recovering the fluid by gentle aspiration. The fluid was stained with Turk solution (0.01% crystal violet in 1% acetic acid). Total airway cellularity and differential cell count (Diff-Quik; Siemens Healthcare, Erlangen, Germany) were performed in BAL cytospins. The relative number and percentage of eosinophils, lymphocytes, macrophages, and neutrophils were determined.

Lung mouse MC protease-1 (mMCP-1) determination

To evaluate airway MC activity, the right lung lobes were collected for protein extraction. The lysis buffer (250 μL; mini complete tablet, Roche Diagnostics, Mannheim, Germany) was added to the tissue samples, and the resulting mixture was homogenized. The samples were centrifuged and the supernatants were collected to measure mMCP-1 levels using a sandwich enzyme-linked immunosorbent assay (ELISA-Ready-SET Go; eBioscience, San Diego, CA, USA). The results were normalized to the total lung protein concentration (Lowry, Folin & Ciocalteau's Phenol Reagent Working solution and Protein Standard Solution; Sigma-Aldrich) in each sample.

PCA

Humanized FcεRI BALB/c mice (FcεRI$^{-/-}$hFcεRI$^+$) were passively sensitized with 300-ng chimeric hIgE anti-NP in a 20-μL volume administered as an intradermal injection in the right ear. The contralateral ear was injected with 20-μL physiologic saline as a negative control. After 24 hours, mice were challenged intravenously with 200-μg Ag (NP-BSA) in PBS containing 1% Evans blue (100 μL). Mice undergoing PCA were treated twice with the EP2-agonist butaprost. First, 4, 6, or 12 μg of butaprost were pre-incubated together with chimeric hIgE anti-NP for 20 minutes, after which the mice were sensitized intradermally. Mice were then treated with an intravenous injection of butaprost in 100-μL physiologic saline or vehicle (100-μL PS with 0.1% DMSO) 1 hour before Ag injection. Mice were euthanized with CO_2 30 minutes after Ag injection. Their right and left ears were cut and minced, and the Evans blue dye was extracted by exposure to 700-μL formamide at 55°C for 2 hours. Evans blue absorbance in the extract was determined at 620 nm.

Data and statistical analysis

Data are represented as mean ± standard error of the mean. Statistical significance was determined using a 2-tailed Student's t test for mean-to-mean comparisons (MC β-hexosaminidase release, inflammation, mMCP-1, and PCA). R_L differences among treatments were compared using 2-way analysis of variance with Bonferroni *post hoc* tests. A P value of <0.05 was considered statistically significant.

RESULTS

Effect of EP2 modulation in phenotypically diverse murine MC populations

The consistency of the effect of selective and non-selective prostanoid EP2 agonists was assessed in murine MC populations expressing a diversity of phenotypic features. The preventive nature of EP2 on MC activity was further tested using an EP2 antagonist.

The inhibitory capacity of selective and non-selective EP2 receptor agonists was assessed in the murine MC line C57.1. **Fig. 3** depicts the effects of butaprost (selective EP2 agonist) and PGE2 (non-selective agonist) on FcεRI-induced MC degranulation upon IgE-DNP sensitization and immunological activation. Binding of DNP-specific IgE led to an approximately 45% baseline release of β-hexosaminidase. Upon stimulation with DNP-HSA, β-hexosaminidase release reached 65%. Selective activation of EP2 by the agonist butaprost significantly reduced FcεRI-mediated degranulation by 20.7% (DNP-HSA 25 ng·mL^{-1}) and 42.85% (DNP-HSA 50 ng·mL^{-1}). However, PGE2 only reduced β-hexosaminidase release by 22.5% upon 50 ng·mL^{-1} DNP-HSA stimulation. Therefore, the inhibitory effect on C57.1 murine MCs is higher with a selective EP2 agonist.
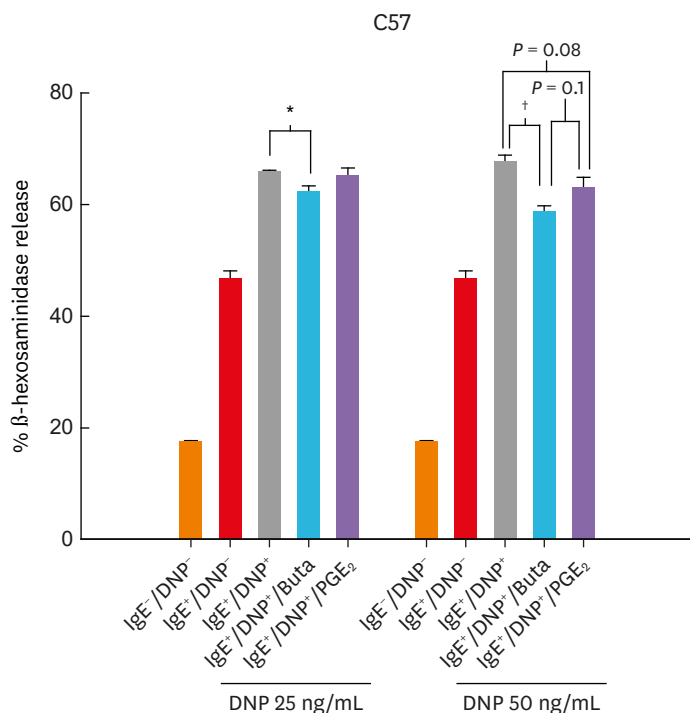
Fig. 3. Effect of the selective EP2 agonist Buta and the non-selective agonist PGE2 on MC degranulation in the murine MC line C57.1. Cells were sensitized with 1 μg·mL^{-1} DNP-specific IgE and treated with 10^{-5} M Buta, 10^{-5} M PGE2, or vehicle (phosphate buffered saline with 0.1% dimethyl sulfoxide) for 30 minutes and stimulated with 25 ng·mL^{-1} or 50 ng·L^{-1} antigen (DNP-HSA). The β-hexosaminidase released into the extracellular media after 30 minutes was measured using a colorimetric assay. The inhibitory effect was higher for Buta than PGE2 after 50 ng·mL^{-1} DNP-HSA challenge. Results are representative of 2 independent experiments performed in triplicate and reported as mean ± standard error of the mean.
MC, mast cell; DNP, anti-2,4-dinitrophenol; DNP-HSA, DNP-human serum albumin; PGE2, prostaglandin E2; EP2, E prostanoid receptor 2; IgE, immunoglobulin E; Buta, butaprost; C57.1, mouse C57.1 mast cell.
*$P < 0.05$; †$P < 0.001$.

The effects of selective and non-selective EP2 receptor agonists were also evaluated in primary cultures of 2 phenotypically distinct MCs populations of BALB/c mice: the peritoneum and lung. **Fig. 4** shows the effects induced by butaprost and PGE2 in both populations. After IgE-DNP sensitization and antigenic challenge, there was an approximately 40% MC degranulatory response. In PDMCs, EP2 agonism (10^{-5} M butaprost) inhibited β-hexosaminidase release by 3.75% (25 ng·mL^{-1} DNP-HSA) and 25% (50 ng·mL^{-1} DNP-HSA), whereas the non-selective agonist PGE2 increased β-hexosaminidase release by 15% (**Fig. 4A**). Similarly, selective activation of LMC by butaprost reduced FcεRI-induced MC degranulation by 40%-60% (**Fig. 4B**), whereas PGE2 induced a further stimulatory effect of 10%. Thus, in both PDMC and LMC populations, selective EP2 agonism partially prevented MC degranulation, whereas non-selective activation potentiated Ag's stimulatory effect.

To further confirm the preventive nature of EP2 activation on MCs, the non-selective EP2 receptor antagonist AH6809 was tested in the mouse LMC population after specific IgE-mediated activation, followed by DNP-HSA challenge (**Fig. 5**). The β-hexosaminidase release was approximately 33% with DNP-HSA challenge. The release significantly increased to 41% with the EP2 antagonist (10^{-5} M). Thus, EP2 antagonism significantly potentiated MC activity in LMC, contrary to the inhibitory effect observed with EP2 agonism.
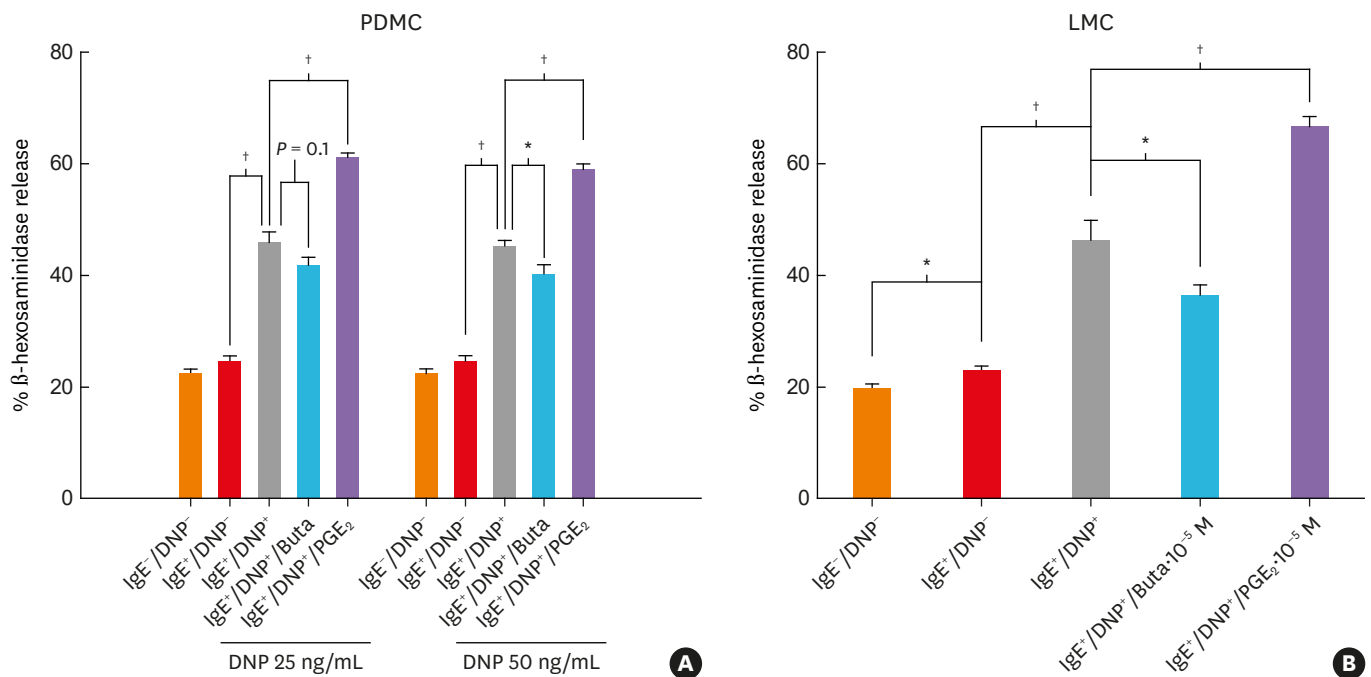
Fig. 4. Effect of the selective EP2 agonist Buta and the non-selective agonist PGE2 on MC degranulation in 2 primary cultures. (A) PDMCs and (B) LMC. Cells were sensitized with 1 μg·mL^{-1} DNP-specific IgE and treated with 10^{-5} M Buta, 10^{-5} M PGE2, or vehicle (phosphate buffered saline with 0.1% dimethyl sulfoxide) for 30 minutes, followed by stimulation with 25 ng·mL^{-1} or 50 ng·mL^{-1} DNP-HSA (PDMC) and 50 ng·mL^{-1} DNP-HSA (LMC). Then, β-hexosaminidase released into the extracellular media after 30 minutes was measured using a colorimetric assay. Buta inhibited MC degranulation in both PDMC and LMC. In contrast, PGE2 enhanced MC degranulation after DNP-HSA challenge. Results are representative of 2 independent experiments performed in triplicate and reported as mean ± standard error of the mean.
PDMC, peritoneum-derived mast cell; LMC, lung mast cells; MC, mast cell; DNP, anti-2,4-dinitrophenol; DNP-HSA, DNP-human serum albumin; Buta, butaprost; PGE2, prostaglandin E2; EP2, E prostanoid receptor 2; IgE, immunoglobulin E.
*P < 0.05; †P < 0.001.

Effect of the selective prostanoid EP2 agonist butaprost on hIgE-mediated MC stimulation *in vivo* and *in vitro*

The inhibitory potential exerted by EP2 activation was also investigated in a diverse range of MC populations expressing the hIgE receptor hFcεRI. Distinct intracellular signalling may lead to different reactivity upon EP2 stimulation. To determine the consistency of the preventive nature of butaprost activation, hIgE-dependent activity was studied *in vivo* and *in vitro*.

To determine the potential for EP2-induced signalling to directly interfere with hIgE receptor-mediated activation, primary MC cultures from FcεRI$^{-/-}$hFcεRI$^+$ and WT BALB/c mice were immunologically stimulated and incubated with butaprost. **Fig. 6** highlights the effects of increasing concentrations of butaprost in PDMCs from mice expressing hFcεRI and WT mice. Ag-triggered PDMCs induced an activity through hIgE-induced MC degranulation of approximately 30%, whereas stimulation of WT mice-derived PDMCs produced an approximately 65% release. The β-hexosaminidase release was inhibited in a dose-dependent manner by butaprost in both primary MC cultures (expressing either the human or the murine IgE receptor). Inhibition virtually reached baseline levels (IgE+/Ag−) at the maximum concentration (10^{-5} M) in both MC cultures. Therefore, both murine and hFcεRI-mediated signalling were fully prevented by EP2 activation.

A model of PCA in mice expressing the hIgE receptor (FcεRI$^{-/-}$hFcεRI$^+$), which is fully dependent on MC activity,[12] was used to confirm the inhibitory capacity of EP2 agonism *in vivo*. Sensitization with hIgE and Ag challenge induced a significant 4-fold increase in MC-
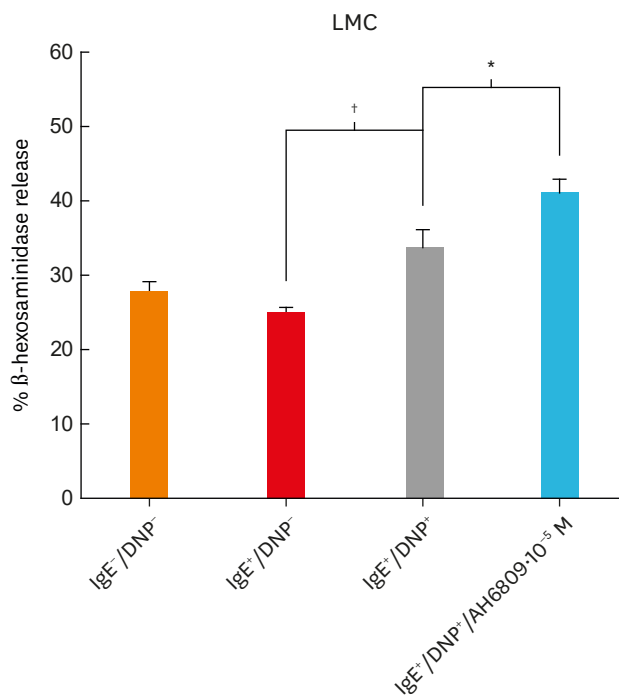
Fig. 5. Effect of the EP2 antagonist AH6809 on MC degranulation in the primary culture of LMC. Cells were sensitized with 1 μg·mL^{-1} DNP-specific IgE and treated with 10^{-5} M AH6809 or vehicle (saline with 20% ethanol) for 1 hour, followed by stimulation with 50 ng·mL^{-1} antigen (DNP-HSA). The β-hexosaminidase released into the extracellular media after 30 minutes was measured using a colorimetric assay. AH6809 enhanced MC degranulation after DNP-HSA challenge. Results are representative of 2 independent experiments performed in triplicate and reported as mean ± standard error of the mean.
LMC, lung mast cells; EP2, E prostanoid receptor 2; DNP, anti-2,4-dinitrophenol; DNP-HSA, DNP-human serum albumin; IgE, immunoglobulin E; AH6809, an EP1/EP2 antagonist; MC, mast cell.
*P < 0.05; †P < 0.01.

mediated vascular permeability as measured by extravasation of Evans blue dye (the right ear compared with the non-sensitized left ear) (**Fig. 7**). Mice treated with butaprost (4 μg; 23 hours and 1 hour before challenge) and subsequently challenged with NP-BSA via hIgE exhibited lower vascular permeability (decrease of approximately 45%). The inhibitory effect of butaprost upon hIgE-mediated stimulation *in vitro* was thus replicated *in vivo*.

In vivo and *in vitro* effects of the non-lipidic EP2 agonist CP-533536 on MC activation and airway sensitization

Confirmation of the anti-degranulation effect of EP2 agonism was demonstrated using the molecularly distinct, PG-unrelated, Pfizer's compound CP-533536. CP-533536's structure is based on a 3-pyridyl sulphonamide rather than a cyclic prostanoid like PGE2 or butaprost (see Methods). This comparative approach was meant to further establish the consistency of the protective nature of EP2 agonism.

The effect of 2 chemically distant EP2 agonists (butaprost and CP-533536) on MC activity was analyzed. **Fig. 8** shows an *in vitro* dose-response experiment performed with LAD2, a human MC line. Baseline β-hexosaminidase release was approximately 25%. After immunological sensitization with human biotinylated IgE and SA, degranulation was approximately 60%. The β-hexosaminidase release from LAD2 was inhibited in a dose-dependent manner by both of the selective EP2 agonists. Inhibition was higher with butaprost than with CP-533536 (50.5% vs. 37.2%).
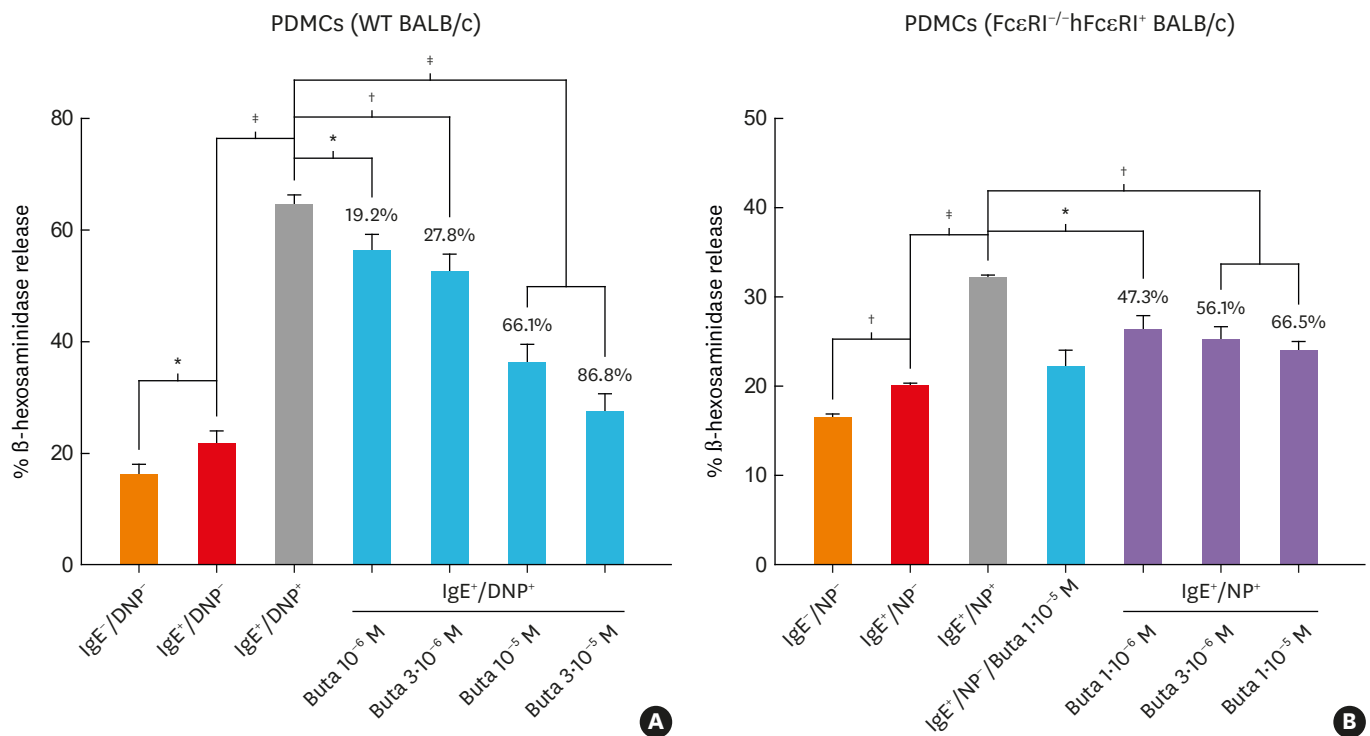
Fig. 6. Effect of the selective EP2 agonist Buta on MC degranulation in the primary culture of PDMCs of (A) FcεRI$^{-/-}$hFcεRI$^+$ mice and (B) WT BALB/c mice. Cells from FcεRI$^{-/-}$hFcεRI$^+$ mice were sensitized with 100 ng·mL^{-1} chimeric human IgE anti-NP, and cells from WT BALB/c mice were sensitized with 1 μg·mL^{-1} IgE-specific DNP. Cells were treated with 10^{-6} M, 3·10^{-6} M, 10^{-5} M, and 3·10^{-5} M Buta or vehicle (phosphate buffered saline with 0.1% dimethyl sulfoxide) for 30 minutes, followed by stimulation with 50 ng·mL^{-1} NP-bovine serum albumin (PDMC of FcεRI$^{-/-}$hFcεRI$^+$ mice) or 50 ng·mL^{-1} DNP-human serum albumin (PDMC of WT BALB/c mice). The β-hexosaminidase released into the extracellular media after 30 minutes was measured using a colorimetric assay. The percentages represent inhibition vs. immunologically stimulated MC in the absence of EP2 agonist. Buta inhibited FcεRI-induced MC degranulation in a dose-dependent manner in both sources of PDMC. Results are representative of 3 independent experiments performed in triplicate and reported as mean ± standard error of the mean.
PDMC, peritoneum-derived mast cell; Buta, butaprost; DNP, anti-2,4-dinitrophenol; IgE, immunoglobulin E; FcεRI, high affinity IgE receptor; hFcεRI, human high affinity IgE receptor; NP, nitrophenol; WT, wild-type; EP2, E prostanoid receptor 2; MC, mast cell.
*P < 0.05; †P < 0.01; ‡P < 0.001.

To further characterize CP-533536 effects on MCs, a full dose-response study was conducted using the rat basophilic leukemic humanized cell line RS-ATL8, which expresses hFcεRI. The β-hexosaminidase release from MCs after IgE stimulation and Ag challenge was approximately 23% (**Fig. 9**). Incubation with the non-prostanoid selective EP2 agonist CP-533536 inhibited hFcεRI-induced MC degranulation in a dose-dependent manner from 1.4% (10^{-12} M) to a maximum effect of 46% at 10^{-8} M. At 10^{-9} M, the effect of CP-533536 plateaued. CP-533536 therefore prevented hIgE-mediated MC degranulation in both LAD2 and RS-ATL8 cells.

To further characterize the potential MC inhibitory effect of the non-prostanoid EP2 agonist CP-533536, an *in vivo* study was performed in HDM-sensitized mice. HDM-sensitized BALB/c mice were exposed i.n. to CP-553536. Airway reactivity, inflammation, and LMC activity were assessed (**Fig. 2**).

Fig. 10A depicts airway reactivity to methacholine in mice sensitized to HDM aeroallergens for 10 days and exposed i.n. to the EP2 selective agonist CP-533536 from days −1 to 4 of sensitization. Mice sensitized and challenged with HDM exhibited a significant increase in airway reactivity to methacholine. Local administration of CP-533536 at 0.3 mg·kg^{-1} prevented aeroallergen-driven increased R$_L$, which was approximately half of the reactivity measured in non-treated mice. This effect, however, did not occur at the 3 mg·kg^{-1} dose.
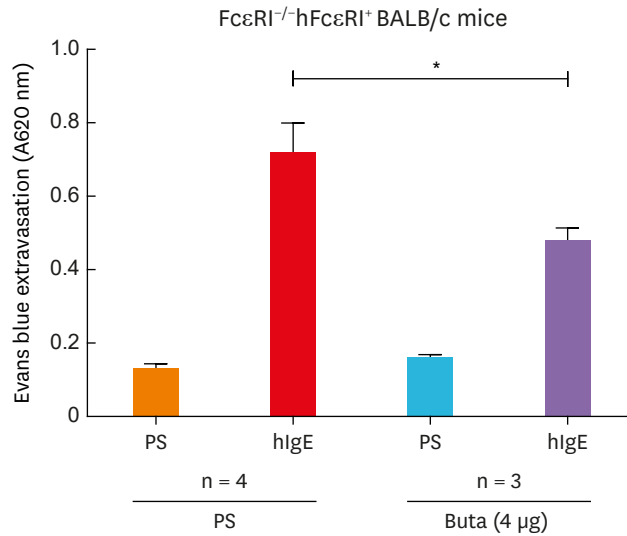
Fig. 7. Effect of the selective EP2 agonist Buta on MC degranulation in FcεRI$^{-/-}$hFcεRI$^+$ mice (n = 3–4/experimental group). An intravenous injection of Buta (4 μg) prior to Ag (NP-bovine serum albumin) challenge (200 μg) prevented skin hypersensitivity reaction induced by local sensitization (300-ng chimeric hIgE anti-NP/ear) and Ag activation of skin MCs as measured by extravasation of Evans blue into the ears (passive cutaneous anaphylaxis). Results represent mean ± standard error of the mean.
Buta, butaprost; FcεRI, high affinity IgE receptor, hFcεRI, human high affinity IgE receptor; hIgE, human immunoglobulin E; IgE, immunoglobulin E; NP, nitrophenol; PS, physiological saline solution; MC, mast cell; Ag, antigen.
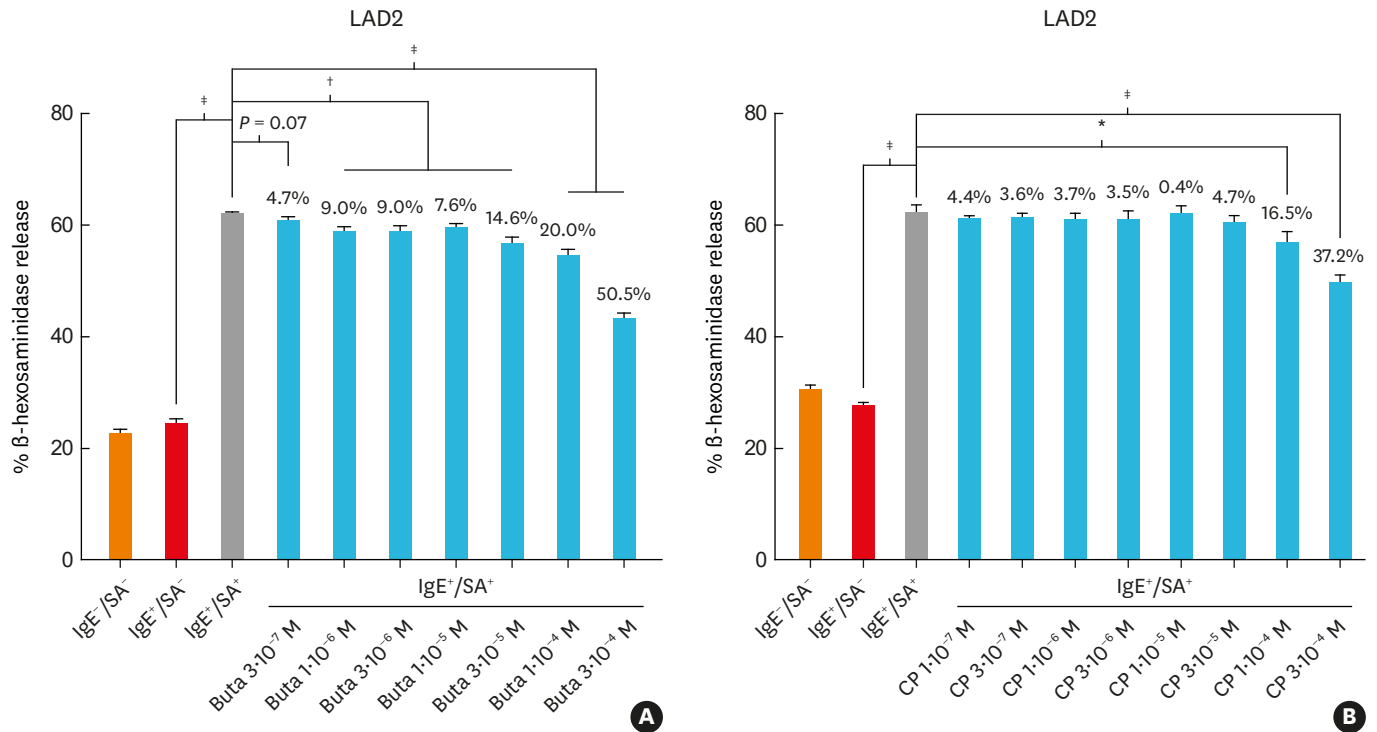*P < 0.05.

Fig. 8. Effect of the selective EP2 agonists Buta (A) and CP (B) on MC degranulation in the human MC line LAD2. Cells were sensitized with 100 ng·mL^{-1} human IgE. Cells were treated with 10^{-7} M, 3·10^{-7} M, 10^{-6} M, 3·10^{-6} M, 10^{-5} M, 3·10^{-5} M, 10^{-4} M, or 3·10^{-4} M Buta and CP or vehicle (phosphate buffered saline with 0.1% dimethyl sulfoxide) for 30 minutes, followed by stimulation with 100 ng·mL^{-1} antigen (SA). The β-hexosaminidase released into the extracellular media after 30 minutes was measured using a colorimetric assay. The percentages represent inhibition vs. immunologically stimulated MC in the absence of EP2 agonist. Buta and CP inhibited FcεRI-induced MC degranulation in a dose-dependent manner. Buta had a slightly greater effect than CP. Results are representative of 2 independent experiments performed in triplicate and reported as mean ± standard error of the mean.
Buta, butaprost; CP, CP-533536; LAD2, human leukemic mast cells; SA, streptavidin; FcεRI, high affinity IgE receptor; IgE, immunoglobulin E; EP2, E prostanoid receptor 2; MC, mast cell.
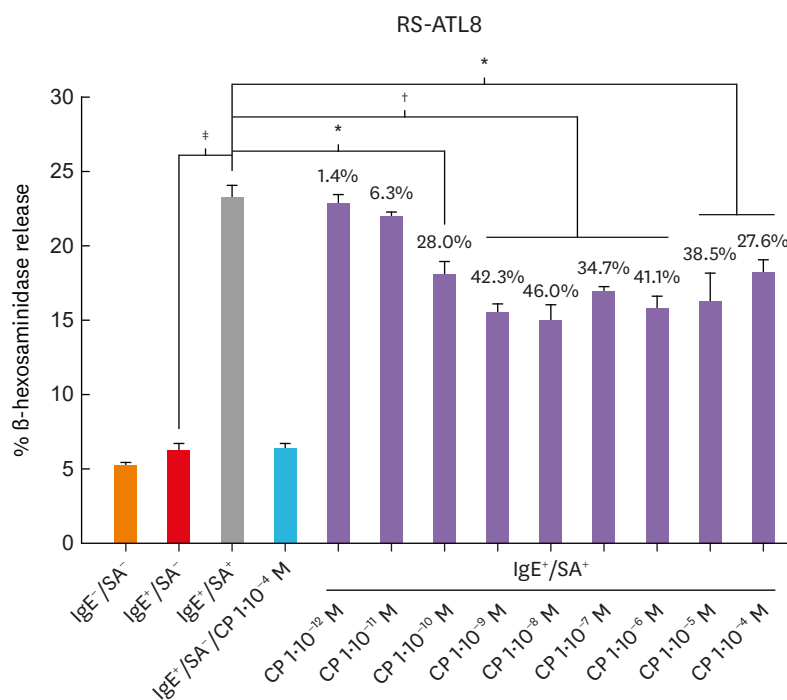*P < 0.05; †P < 0.01; ‡P < 0.001.

Fig. 9. Effect of the selective EP2 agonist CP on MC degranulation in the rat basophilic leukemia humanized cell line RS-ATL8. Cells were sensitized with 500 ng·mL^{-1} human IgE. Cells were treated with 10^{-12} M, 10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M, or 10^{-4} M CP or vehicle (PBS with 0.1% DMSO) for 30 minutes, followed by stimulation with 1,000 ng·mL^{-1} antigen (SA). The β-hexosaminidase released into the extracellular media after 30 minutes was measured using a colorimetric assay. The percentages represent inhibition vs. immunologically stimulated MC in the absence of EP2 agonist. CP inhibited FcεRI-induced MC degranulation in a dose-dependent manner from 10^{-12} M to 10^{-8} M. Results are representative of 2 independent experiments performed in triplicate and reported as mean ± standard error of the mean.
CP, CP-533536; IgE, immunoglobulin E; SA, streptavidin; FcεRI, high affinity IgE receptor; EP2, E prostanoid receptor 2; MC, mast cell.
*$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

Differential inflammatory cell recruitment in the airways was also assessed in mice exposed to CP-533536 (**Fig. 10B**). Strong eosinophilic recruitment was induced in HDM-sensitized mice. Differential airway inflammatory cell count was not altered by pre-treatment with CP-533536.

Finally, HDM-induced LMC activation was evaluated by measuring lung mMCP-1 concentrations. **Fig. 10C** shows mMCP-1 concentration normalized to total protein in lung extract homogenates. The mMCP-1 was overexpressed locally by a factor of 5.4 in HDM-exposed vs. non-exposed mice. Treatment with CP-533536 did not produce a statistically significant change in mMCP-1, but the 3 mg·kg^{-1} dose prevented the enhanced MC activity by approximately 48% ($P = 0.13$).
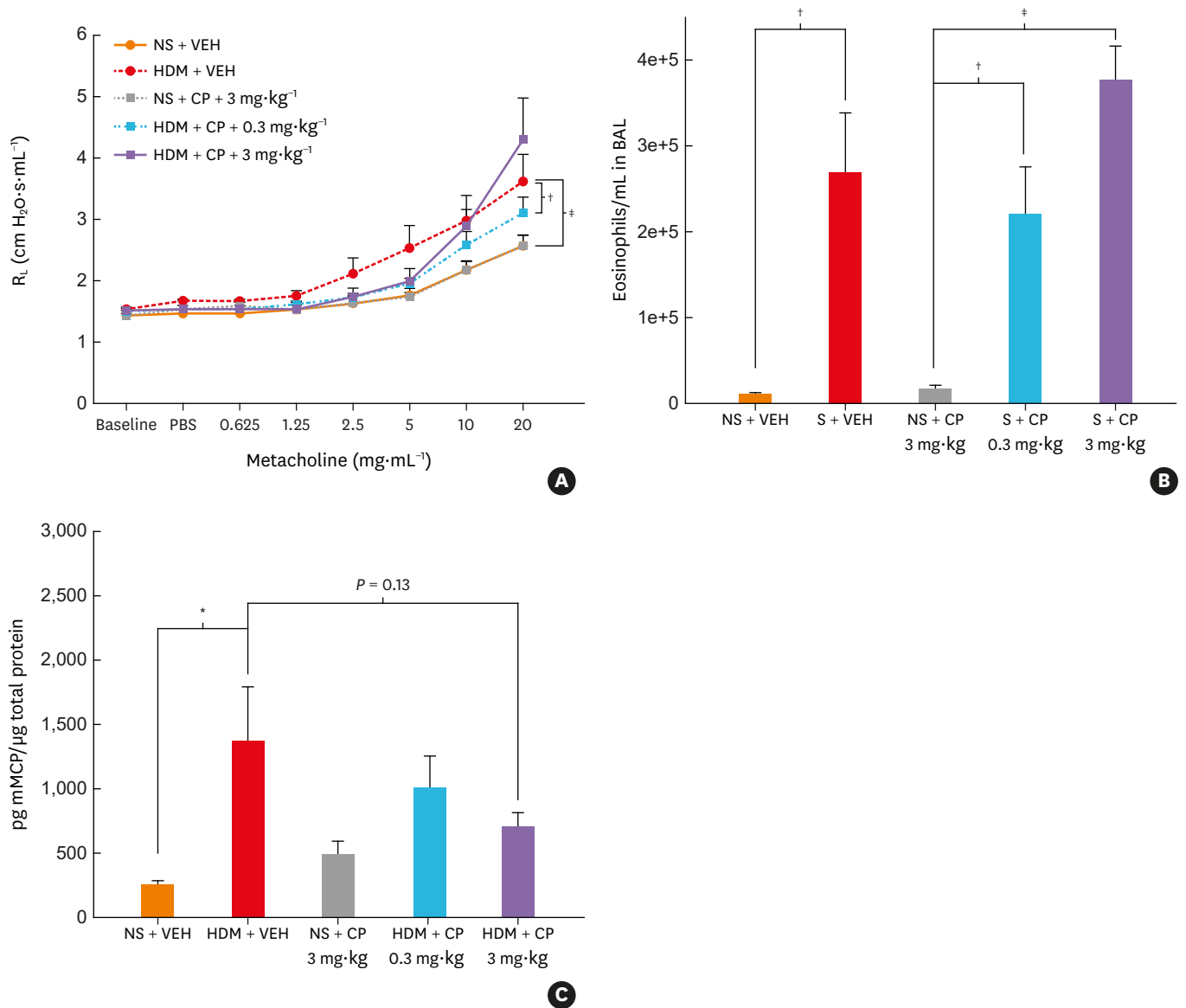
Fig. 10. Airway response to HDM exposure in BALB/c mice treated with the selective EP2 agonist CP (n = 8/experimental group). (A) The EP2 agonist CP, administered intranasally 1 hour before HDM sensitization from day −1 through day 4, reduced airway hyper-responsiveness as assessed by methacholine challenge in anesthetized and ventilated mice. (B) Eosinophil count from a differential inflammatory cell assessment per mL determined from counting at least 300 cells. (C) Airway mast cell activity in sensitized treated and untreated mice assessed by measuring production of mMCP-1 in lung extracts, normalized for the total protein concentration. CP produced no significant changes in lung eosinophil counts, whereas a trend toward decreased mMCP-1 was observed after 3 mg·kg⁻¹ CP treatment. Results represent mean ± standard error of the mean.
HDM, house dust mite; EP2, E prostanoid receptor 2; mMCP-1, mouse mast cell protease-1; CP, CP-533536; NS, non-sensitized; R_L, lung resistance, VEH, vehicle; PBS, phosphate buffered saline; BAL, bronchoalveolar lavage; S, sensitized.
*$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

DISCUSSION

Through a multifaceted *in vitro* and *in vivo* experimental approach, we have pre-clinically validated the association between selective activation of EP2 and decreased MC activity as a potential therapeutic mechanism in IgE-mediated disorders.

We evaluated the reactivity to PGE2 and/or EP2-selective activators in a heterogeneous range of MC populations. The murine C57.1 MC line, and mouse-derived primary cultures of PDMCs and LMCs have different tissue origins and are characterized as connective tissue or mucosal types of MC. Despite their phenotypic differences, specific activation of EP2 by butaprost significantly prevented MC degranulation in these populations. Use of the non-selective prostanoid PGE2 either further stimulated (PDMC and LMC) or reduced (C57.1) MC activity. The different behavior between the selective and non-selective stimulation may be attributed to different expression patterns of the 4 EP receptors on the surface of these cells as previously described.[12,32,33] Indeed, in cells with dominant EP2 expression (C57.1), PGE2 prevented FcεRI-induced degranulation, whereas in cells with dominant EP3 expression (PDMC), PGE2 enhanced MC activity.[12] Therefore, the response to PGE2, a non-selective agonist, varied from inhibitory to stimulatory, depending on the relative expression of EP receptors.

The observed EP2-mediated preventive effect on murine MC degranulation was applicable to MCs stimulated through hIgE whether transgenic or of human origin. Indeed, the human LAD2 MC line exhibited reduced activity when immunologically activated in the presence of butaprost as shown in other human MC populations.[6,12,34-36] To confirm that the molecular machinery triggered by hIgE coupled to hFcεRI may also be modulated by EP2 activation, we tested the effect of butaprost on primary MCs (PDMCs) from BALB/c mice expressing hFcεRI (FcεRI$^{-/-}$hFcεRI$^+$ mice). The selective EP2 agonist butaprost triggered dose-dependent inhibition of hFcεRI-induced degranulation. Together, these results confirm that selective engagement of EP2 in a wide diversity of MC populations dampened human or mouse IgE-mediated MC degranulation, regardless of the specific phenotypic features of the cell populations. These data thereby suggest that EP2-driven prevention of MC degranulation is a well-preserved feature applicable to MCs residing in different organs and tissues.

Despite evaluating heterogeneous MC populations, limiting EP2 stimulation to a single selective agonist (butaprost) may undermine the consistency of the conclusions. Indeed, butaprost is a classic prostanoid-like EP2 agonist, so that its use as the only tool for selective EP2 activation may bias data interpretation if there are unknown EP2-independent effects involved. To mitigate this potential risk, we also evaluated the effects of a chemically unrelated (non-prostanoid, non-lipidic) EP2 agonist: CP-533536.[24] CP-533536 induced an inhibitory effect *in vitro* in 2 different MC populations (LAD2 and RS-ATL8). Its effect on LAD2 cells was comparable to that of butaprost, preventing the release of MC mediators to a similar degree. CP-533536 also inhibited hIgE-mediated MC activity in RS-ATL8 cells, another transgenic population expressing the hIgE receptor. Convergence of the effects exerted by these 2 molecularly distinct EP2 activators emphasizes the likely benefits in the anaphylaxis of selective EP2 receptor agonism via MC blockade. As a final *in vitro* confirmatory approach, we evaluated the inhibitory potential of the MC-anaphylaxis axis using an EP2 antagonism strategy. Pre-incubating LMC with the EP2 antagonist AH6809 triggered a stimulatory effect upon Ag activation. This increase in MC degranulation was consistent with data from Kay *et al.*,[35] which indicated that AH6809 counteracted the effect of PGE2 as an inhibitor of histamine release in MCs. The role of EP2 as a modulator of agonist/antagonist balance is consistent with previous data showing that PGE2 was unable to reduce activity in MCs isolated from EP2-knock out mice.[12]

The demonstration of consistent outcomes on MC modulatory capacity using 2 unrelated EP2 agonists and an antagonist as well as 5 phenotypically distinct MC populations stimulated through either human or murine IgE, thereby confirming MC EP2 as a key pharmacological target for preventing MC activity.

We further sought to demonstrate that *in vitro* modulation of MC activity by EP2 affects Ag-induced reactions *in vivo*. We first tested the effect of the selective EP2-agonist butaprost in PCA, an *in vivo* MC-dependent model of allergy.[37] Previous observations in WT, MC-deficient (Wsh), and EP2-knockout mice showed that butaprost prevented ear swelling by an EP2-mediated mechanism.[12,13] The current study confirms the importance of EP2 as a protective molecule in PCA in a hIgE-mediated system. In this humanized PCA model, injecting specific hIgE and NP in BALB/c mice expressing hFcεRI led to MC-driven ear vascular permeability. When butaprost was administered to passively sensitized mice, the Ag-triggered ear reaction was significantly reduced. Thus, hIgE-dependent MC stimulation was suppressed *in vivo* through EP2 agonism.

The observed *in vivo* EP2-driven effect could presumably counteract diseases based on IgE-mediated MC overactivation or hyper-releasability. We thereby assessed LMC activity and airway responses in HDM-sensitized BALB/c mice, which exhibited increased airway MC activity according to mouse mMCP-1 protease determination. The selective EP2-agonist CP-533536 partially (albeit insignificantly) prevented the ability of airway MCs to release mMCP-1. The CP-533536 effect was less than that of butaprost[13] as anticipated by its more limited inhibitory effect *in vitro*. In parallel with preventing airway MC activity, CP-533536 also reduced HDM aeroallergen-induced increased R_L response to methacholine. This confirms earlier findings that butaprost suppressed airway hyper-reactivity and inflammation.[13] CP-533536 produced a nonsignificant reduction in eosinophilic inflammation, which may be explained by its relatively limited ability to reduce MC activity (compared to butaprost).

Adding to previous observations of the preventive benefits of butaprost, we have now confirmed through an array of experimental approaches that EP2 receptors on the MC surface may by themselves mediate the described benefits of PGE2 in sensitized mice. Our findings validate the relevance of selective activation of EP2 to MC-blockade as well as its potential applicability as a defensive strategy in asthma and other allergic or MC-mediated diseases.

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