Altered expression and signalling of EP_2 receptor in nasal polyps of AERD patients: role in inflammation and remodelling*

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Rhinology 54: 254-265, 2016
DOI:10.4193/Rhino15.207

***Received for publication:** July 17, 2015
Accepted: October 15, 2015

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Abstract

Background: Down-regulation of the E-prostanoid $(EP)_2$ receptor has been reported in aspirin exacerbated respiratory disease (AERD). We aimed to evaluate the expression and activation of EP receptors in AERD and their role in prostaglandin (PG) E_2 signalling.

Methods: Samples were obtained from nasal mucosa of control subjects (NM-C, n=7) and from nasal polyps of AERD patients (NP-AERD, n=7). Expression of $EP_{1\text{-}4}$ was assessed at baseline. Fibroblasts were stimulated with receptor agonists to measure cAMP levels, cell proliferation and granulocyte-macrophage colony-stimulating factor (GM-CSF) release.

Results: NM-C and NP-AERD samples and fibroblasts expressed EP_2, EP_3 and EP_4 at baseline. Lower expression of EP_2 and higher expression of EP_4 was observed in NP-AERD compared with NM-C. Stimulation with PGE_2 and butaprost caused a higher increase in cAMP in NM-C than in NP-AERD. On the contrary, CAY10598 produced a higher production of cAMP in NP-AERD compared with NM-C. The anti-proliferative effect of PGE_2 and butaprost was lower in NP-AERD than in NM-C fibroblasts. Similarly, the capacity of PGE_2 and butaprost to inhibit GM-CSF release was lower in NP-AERD than in NM-C.

Conclusions: The altered expression of EP_2 in AERD may contribute to reduce the capacity of PGE_2 to mediate anti-proliferative and anti-inflammatory effects.

Key words: aspirin-exacerbated respiratory disease, fibroblasts, nasal polyp, prostaglandin E_2, prostaglandin E_2 receptors

Introduction

Prostaglandin (PG) E_2 is a product of the cyclooxygenase (COX) pathway of arachidonic acid (AA) metabolism [(1)]. The action of PGE_2 is mediated by a group of four G-protein-coupled membrane receptors named E-prostanoid (EP): $EP_{1\text{-}4}$ [(2)]. The differential expression of these receptors could be responsible for the multiple effects of PGE_2 [(3)]. EP are classified according to their intracellular signalling and second messenger [(4)]. EP_1 receptor signals through Gαq, which increases Ca^{2+} levels [(5,6)]. EP_2 and EP_4 signal through Gαs to increase cyclic adenosine monophosphate (cAMP) levels through activation of adenyl cyclase (AC), while EP_3 primarily signals through Gαi to decrease cAMP levels [(7)]. Aspirin exacerbated respiratory disease (AERD) is characterized by hypersensitivity to aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), bronchial asthma, and chronic rhinosinusitis (CRS) with recurrent nasal polyps (NPs) [(8,9)]. Patho-

genic mechanisms of AERD appear to be closely linked to an imbalance in eicosanoid metabolism characterized by overactive 5-lipoxygenase (5-LO) and reduced COX pathway, resulting in increased cysteinyl leukotriene production and low PGE_2 release [10-12]. Moreover, alterations in EP receptors expression also seem to be involved in this disorder [13].

EP_2 down-regulation has been reported in both upper and lower airways of AERD patients [13]. All in all, the altered synthesis of PGE_2 and the lower expression of EP_2 receptor may contribute to reduce the protective capacity of PGE_2 that results in the marked inflammation and remodelling response usually found in AERD. Patients with AERD experience more exacerbations, worse quality of life and poor lung function, compared with non-AERD patients [10,14].

EP_2 and EP_4 receptors stimulation initiates the production of intracellular cAMP and activates protein kinase A (PKA), which regulates 5-LO by the suppression of its functions [15,16]. The repercussions of low EP_2 receptor on the downstream signalling pathway are still unclear. To assess the physiological activity of EP receptors, Corrigan et al. [17] used peripheral blood cells. However, these cells isolated from AERD patients did not show any difference in EP receptor expression compared to healthy controls and non-AERD asthma patients. In consequence, they could not be used to evaluate the impact of the decreased expression of EP_2. In contrast, fibroblasts derived from NPs of AERD patients consistently show alterations in the regulation of the COX pathway and EP_2 expression [18]. Based on these observations, we used cultured fibroblasts from nasal mucosa (NM) of control subjects (NM-C) and NPs from AERD patients (NP-AERD) to examine the expression of EP receptors in AERD, their impact in intracellular cAMP levels upon receptor activation, as well as the functional repercussion of potential EP alterations in chronic inflammatory and remodelling processes. Moreover, we used whole NM-C and NP-AERD tissue explants to confirm observations in cultured fibroblasts.

We hypothesized that abnormalities in EP expression in AERD modify its capacity to increase intracellular cAMP levels in response to PGE_2 and that these upstream alterations could be responsible for the exacerbated inflammation and remodelling response usually present in AERD. This study aimed to assess the expression of PGE_2 receptors as well as their capacity to mediate anti-proliferative and anti-inflammatory effects in AERD.

Materials and methods

Subjects

Control samples of human NM were obtained from 7 subjects without asthma undergoing nasal corrective surgery. All the control subjects had taken normal doses of aspirin or NSAIDs without adverse reactions. Human NP tissue was obtained from 7 subjects with AERD undergoing functional endoscopic sinus surgery. Study subjects were selected based on established criteria that include severe nasal polyposis, the demonstration of a reversible bronchial obstruction and a history of asthma attacks precipitated by NSAIDs [19]. AERD was confirmed by means of lysine-aspirin nasal challenge, as previously described [19]. None of the subjects received oral or intranasal corticosteroids for at least 2 weeks before the operation. All patients agreed to participate in the study, providing informed consent and the study was approved by the Ethics Committee of the Hospital Clínic de Barcelona (approval number: 2013/8533). Demographic and clinical characteristics of subjects are shown in Table 1.

Table 1. Demographic data and clinical characteristics of the study population.

Characteristics	NM-C	NP-AERD
Subjects, n	7	7
Age, years (mean ± SD)	51.3 ± 23.0	53.4 ± 17.5
Female sex, n	3	5
Moderate / severe asthma, n	0	5 / 2
Aspirin intolerance, n	0	7
Skin prick test positivity, n	1	2
Blood eosinophilia, cells/μL (mean ± SD)	340.0 ± 167.3	842.9 ± 457.7
Intranasal corticosteroid (budesonide equivalent), n (μg/day, mean ± SD)	0 (0)	7 (300 ± 100)
Inhaled corticosteroid (budesonide equivalent), n (μg/day, mean ± SD)	0 (0)	7 (891.4 ± 218.7)
Previous polypectomies, mean (range)	0 (0)	1.57 (1 – 3)

Tissue handling and cell culture

At the time of surgery, and for further whole tissue analysis, NM-C and NP-AERD specimens were snap-frozen in liquid nitrogen and stored at -80ºC.

To set fibroblast cell cultures, NM-C and NP-AERD fresh samples were cut into small fragments (approximately 3x3 mm) and placed in 6-well plates (NUNC, Wiesbaden, Germany) containing Dulbecco's modified Eagle's media (DMEM) (Lonza, Verviers, Belgium) supplemented with 10% foetal bovine serum (FBS), 100IU/mL penicillin, 100μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and 2μg/mL amphotericin B (Sigma, St. Louis, MO, USA). Culture plates were kept in a humidified atmosphere at 37ºC and 5% CO_2. When fibroblasts covered approximately 50% of the well surface through adhesion and migration on the plastic surface, tissue fragments were removed, and the first passage was performed. Fibroblasts were detached by adding 0.05% trypsin/0.02% EDTA (Invitrogen) for 5min and the reaction was stopped using 10% FBS-supplemented DMEM. Cells were then

centrifuged (400g) and seeded in 150cm^2 flasks (NUNC). The experiments were performed between passages 3 and 7. To perform culture characterization, fibroblasts were cultured in CultureSlides®. The same batch of FBS was used for the whole experimental period. Mycoplasma contamination was tested by PCR in the cultures and all were negative.

Culture characterization

Culture characterization was performed by immunofluorescence for vimentin (fibroblasts), cytokeratins (epithelial cells) and alpha smooth muscle actin (myofibroblasts) in CultureSlides® incubated with serum-free media (SFM) for 24h.

Study design

1) EP receptor expression

EP receptor expression was assessed in whole tissue and cultured fibroblasts isolated from NM-C and NP-AERD. Fibroblasts were plated on 90 cm^2-diameter and six-well culture dishes. When the cells reached 80% confluence, FBS-supplemented media was switched to SFM for 24h and the protein and RNA extraction protocols were performed. Moreover, the expression of EP receptors in whole tissue was assessed in samples frozen at the time of surgery.

2) Intracellular production of cAMP

To assess intracellular production of cAMP, cultured fibroblasts from NM-C and NP-AERD were plated into six-well plates in DMEM supplemented with 10% FBS at 2x10^5 cells/well and allowed to growth to subconfluence. The cells were then serum starved in DMEM for 24h at 37ºC. Cyclic nucleotide phosphodiesterases (PDEs) regulate the cellular levels of cAMP and participate in its degradation. In order to measure the cellular concentration of cAMP, cultured fibroblasts were treated with 3-Isobutyl-1-methylxanthine (IBMX) (13347, Cayman), a PDE inhibitor, for 1h at 1mM prior stimulation with different concentrations of PGE_2, or the specific EP_2 receptor agonist butaprost free-acid (13741, Cayman) and EP_4 receptor agonist CAY10598 (13281, Cayman) or forskolin (F3917, Sigma), an AC activator, for 15min at 37ºC. The concentration of intracellular cAMP produced through the activation of EP_3 receptor was evaluated by incubating the cells with both forskolin (20µM) and sulprostone (14765, Cayman), an EP_3 receptor agonist, at different concentrations for 15min.

3) Nasal fibroblast proliferation

The effect of PGE_2 and EP_2 receptor agonist on cell proliferation was assessed in NM-C and NP-AERD cultured fibroblasts. Briefly, fibroblasts (1.5x10^5 cells/plate) were plated on 60-mm petri dishes and allowed to recover overnight. Cells were deprived of serum for the next 24h and then switched to media with 2.5% FBS and treated with or without PGE_2 (10^{-7}M to 10^{-5}M) or butaprost (10^{-7}M to 10^{-5}M) for 18h. After that, cells were incubated for an additional 2h with 10µM 5-ethynyl-2'-deoxyuridine (EdU).

4) Granulocyte-macrophage colony-stimulating factor (GM-CSF) release

To measure GM-CSF release in cell culture supernatants, cultured fibroblasts from NM-C and NP-AERD were seeded in 24-well plates and allowed to attach for 24h. Cells were washed with phosphate buffered saline (PBS) and incubated with SFM prior to treatment. Fibroblasts were stimulated with different concentrations of PGE_2 or butaprost in DMEM containing 5% FBS for 24h.

Immunofluorescence

Cells were grown in 4-well CultureSlides® and fixed with cold 4% paraformaldehyde for 15min. Then, fibroblasts were permeabilized with 0.5% Triton X-100 for 30min and blocked with 1X PBS/1% bovine serum albumin (BSA) (Sigma, St Louis, MO, USA) for 1 h. The primary antibodies were against alpha-smooth muscle actin (M0851, DAKO, Glostrup, Denmark) at dilution 1:500, vimentin at 1:100 (V5255, Sigma, St Louis, MO, USA) or pan-cytokeratin at 1:200 (C2562, recognizing cytokeratins 1, 4, 5, 6, 8, 10, 13, 18 and 19, Sigma). The percentage of positive cells was quantified using fluorescence microscopy.

Protein extraction

Whole tissue: total protein extraction from whole tissue samples was carried out by adding an ice-cold lysis buffer containing Complete™ protease inhibitor cocktail tablet (Roche) in 0.05M Hepes buffer solution, 0.5% v/v Triton X-100, and 625mM phenylmethylsulfonyl fluoride directly to the specimen. Samples were kept in ice and sonicated twice for 15s in a sonifier (Branson, Danbury, CT, USA) to obtain a homogenized mixture. The lysates were centrifuged at 4ºC, 12,000g for 10min.

Fibroblasts: fibroblasts plated on 90cm^2-diameter culture dishes were washed twice with ice-cold PBS and lysed in ice-cold RIPA buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% SDS, 1% nonidet P-40 Igepal, 1µg/mL leupeptin, 1µg/mL aprotinin, 0.1mM Na_3VO_4, 1mM NaF, 1mM dithiothrieitol, 0.5mg/mL Pefabloc, and 5mg/mL sodium deoxycholate (Sigma, St Louis, MO, USA)). Samples were sonicated and centrifuged as previously described.

Western blot analysis

Twenty microgram of protein was denatured in a thermocycler (70ºC for 10min) in loading buffer (NuPAGE LDS sample buffer). The proteins were electrophoresed in 7% TRIS-Acetate gels, run at 125V for 90min on a Novex XCell II Mini-Cell (Invitrogen, San Diego, CA, USA) and finally transferred to nitrocellulose membranes. For EP protein expression analysis the non-specific binding sites were blocked using blocking buffer (5% non-fat dry milk and 0.1% Tween 20 in 10nmol/L PBS) for 1h at room temperature in an orbital shaker. Membranes were incubated overnight at 4ºC with 1:1000 dilution in blocking buffer of the primary antibodies against EP_1 (101740, Cayman chemicals, Mi-

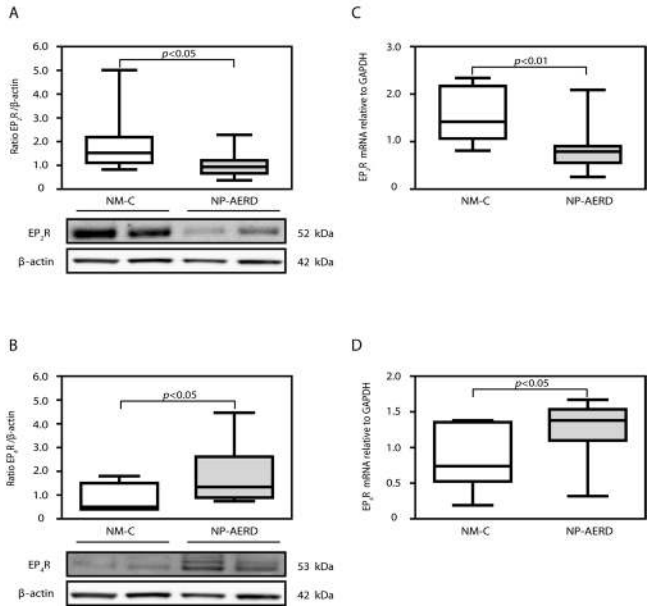

Figure 1. Basal expression of EP_2 and EP_4 receptors in nasal samples. Densitometric analysis and representative western blot of (A) EP_2 and (B) EP_4 protein expression normalized by β-actin in nasal samples from NM-C and NP-AERD (n=7 each). (C) EP_2 and (D) EP_4 mRNA expression in nasal samples from NM-C and NP-AERD (n=7 each) was analysed by quantitative real-time PCR and normalized to the GAPDH constitutive gene.

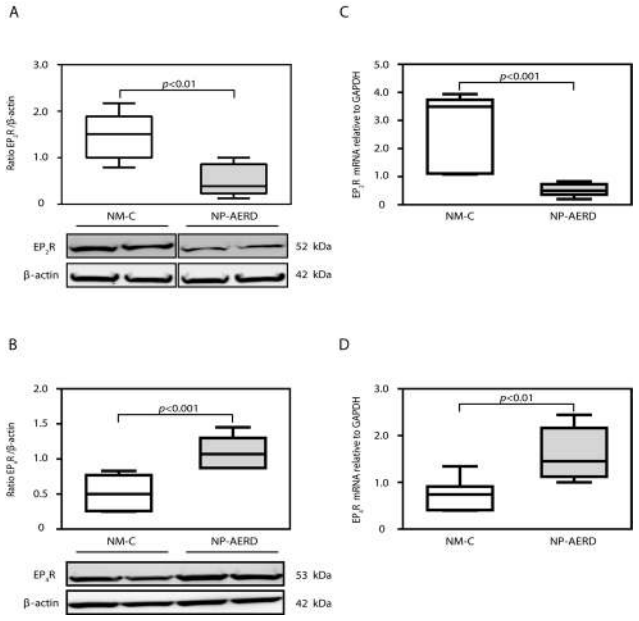

Figure 2. Basal expression of EP_2 and EP_4 receptors in nasal cultured fibroblasts. Quiescent fibroblasts from NM-C and NP-AERD (n=7 each) were incubated for 24h with culture media without FBS. Densitometric analysis and representative western blot of (A) EP_2 and (B) EP_4 protein expression normalized by β-actin. Quantitative real-time PCR analysis of (C) EP_2 and (D) EP_4 mRNA expression normalized to the GAPDH constitutive gene.

chigan, USA), EP_2 (101750, Cayman), EP_3 (101760, Cayman), EP_4 (45863; Abcam, Inc, Cambridge, MA, USA) and 1:10000 dilution for β-actin (A5316, Sigma). Therefore, blots were washed 4 times in 0.05% Tween 20 in 10nmol/L PBS and incubated with an appropriate horseradish peroxidase-labelled secondary antibody in blocking buffer, at room temperature for approximately 2h. After repeating the wash as previously, blots were incubated with an enhanced chemiluminescent substrate (Supersignal West Pico Chemiluminescent Substrate, Rockford, IL, USA) and light emissions were detected using a CCD Camera System LAS 4000 (Fujifilm, Tokyo, Japan). Band intensities were quantified with Fujifilm Image Gauge v.4.0 Software. For EP receptor expression, band intensities were normalized to the intensity of constitutive β-actin assessed in the same sample.

RNA extraction

Whole tissue: total RNA isolation from whole tissues was performed using TRIzol Reagent procedure (Invitrogen) according to the manufacturer's protocol with the exception of minor modifications. Tissue samples were homogenized using a power homogenizer and directly transferred into 1mL of TRIzol Reagent (Invitrogen). After incubation of the homogenized samples for 5min at room temperature, 0.2mL chloroform per mL of TRIzol was added to the mixture. The samples were mixed vigorously and centrifuged at 12,000g for 15min at 4°C to separate the aqueous and inorganic phases. The, aqueous, upper phase was recovered, mixed with 0.5mL of 100% isopropanol, incubated for 24h at -20°C and centrifuged at 12,000g for 10min at 4°C. The pellet was washed once with ice cold 75% ethanol, centrifuged again for 5min at 7,500g, air-dried and dissolved in 20μL RNase-free water. Total RNA was quantified with a spectrophotometer (NanoDrop 2000, Thermo Scientific, Milan, Italy) and stored at -80°C.

Fibroblasts: fibroblasts were plated in six-well culture plates until subconfluence and growth-arrested for 24h. Therefore, total RNA was extracted by using the RNeasy Mini kit (QIAGEN, Valencia, CA, USA), following the protocol suggested by the manufacturer.

Quantitative real-time PCR analysis

One microgram of total RNA was converted to cDNA by using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The mRNA expression of EP_1, EP_2, EP_3, EP_4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analysed by quantitative real-time PCR (7900HT Fast Real-Time PCR System, Applied Biosystems) using TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression Assays (PTGER1, Hs00168752_m1; PTGER2, Hs00168754_m1; PT-

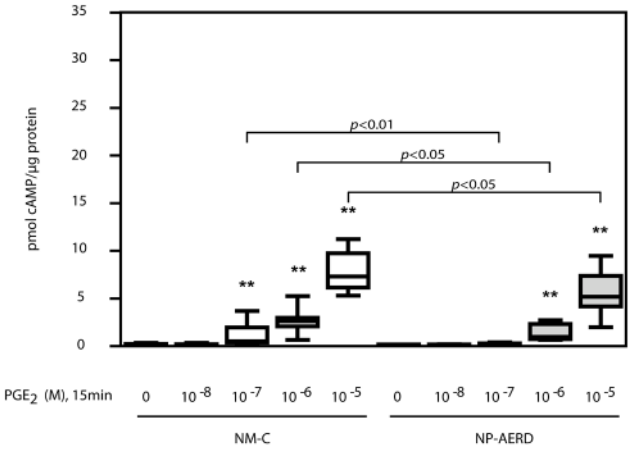

Figure 3. PGE_2 effect on intracellular cAMP levels in cultured fibroblasts. Fibroblasts from NM-C and AERD (n=7 each) were preincubated with 1mM IBMX for 1h and treated for 15min with PGE_2 from 10^{-8}M to 10^{-5}M. Intracellular cAMP levels were measured by ELISA. Results were normalized to the total protein concentration present in each sample. ** p<0.01 compared with respective control (IBMX, 1mM).

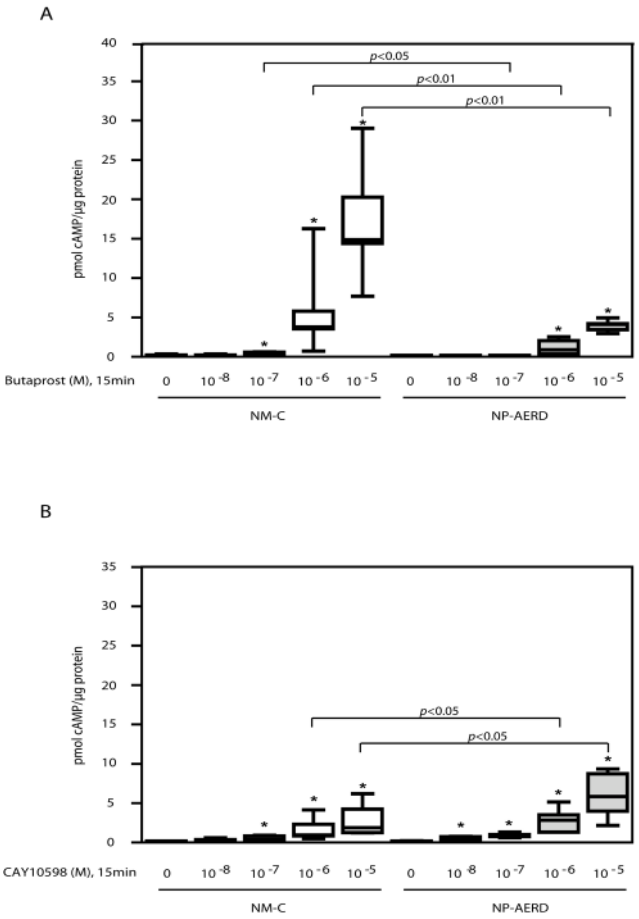

Figure 4. Butaprost and CAY10598 effect on intracellular cAMP levels in cultured fibroblasts. Fibroblasts from NM-C and NP-AERD (n=7 each) were preincubated with 1mM IBMX for 1h and treated for 15min with (A) butaprost (EP_2 selective agonist) and (B) CAY10598 (EP_4 selective agonist) from 10^{-8}M to 10^{-5}M. Intracellular cAMP levels were measured by ELISA. Results were normalized to the total protein concentration present in each sample. * p<0.05 compared with respective control (IBMX, 1mM).

GER3, Hs00168755_m1; PTGER4, Hs00168761_m1). EP receptors mRNA expression was normalized to the mRNA expression of the constitutive gene GAPDH (Hs02758991_g1) and data were calculated using the ΔΔCt method.

cAMP measurement

Intracellular cAMP levels were determined using a commercial enzyme-linked immunosorbent assay (ELISA) (Arbor Assays, Ann Arbor), according to the manufacturer's instructions. Briefly, to stop the reaction, media was aspirated and cells were washed with cold PBS. Then the fibroblasts were treated directly with the sample diluent provided for 10min at room temperature and scraped to detach them from the plate surface. The cell suspension was centrifuged at 10,000g at 4ºC for 15min and the supernatants were collected and frozen for later analysis. The assay range was 0.617 to 150pmol/mL. Total protein content in the cell lysates was used as loading control. The concentration of cAMP was expressed as pmol of cAMP/µg of total protein.

Fibroblast proliferation

Cell proliferation was determined by measuring the incorporation of the thymidine analog EdU, followed by immunodetection using a commercially available kit (Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit, #C10420, Life Technologies). Cells were fixed and permeabilized according to the manufacturer's instructions. Following washes with 1% BSA-PBS, fibroblasts were incubated with Click-iT reaction cocktail prepared from the Click-iT EdU Alexa Fluor 488 kit for 30min at room temperature and protected from the light. Samples were analysed by a BD FACSCanto™ II flow cytometer. Over 10,000 events were collected per sample. Proliferative cells were counted based on high fluorescence in the Alexa Fluor 488 channel.

Measurement of GM-CSF release

Human GM-CSF release in supernatants was quantified using a commercial ELISA DuoSet kit (R&D Systems, Abingdon, UK), according to the manufacturer's instructions. Samples were tested in duplicate and cytokine production (pg/mL) was corrected by cell number using the colorimetric Cell Proliferation Kit II (XTT, Roche Diagnostics GmbH, Mannheim, Germany). The detection threshold was 15.6 pg/mL.

Statistical analysis

All statistical analyses were performed with GraphPad Prism

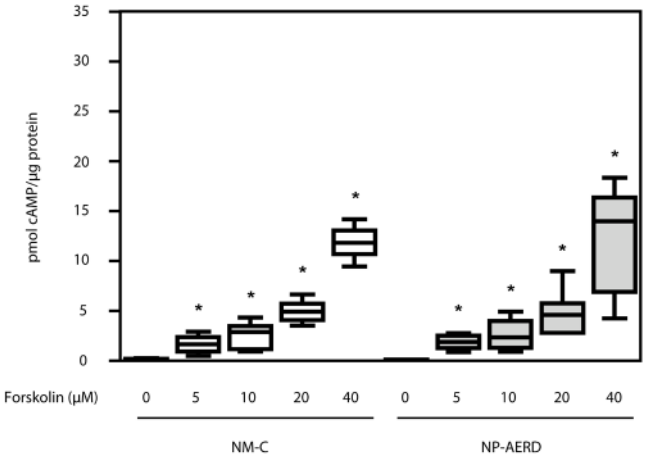

Figure 5. Forskolin effect on intracellular cAMP levels in cultured fibroblasts. Fibroblasts from NM-C and NP-AERD (n=7 each) were preincubated with 1mM IBMX for 1h and treated for 15min with forskolin from 5 to 40µM. Intracellular cAMP levels were measured by ELISA. Results were normalized to the total protein concentration present in each sample. * p<0.05 compared with respective control (IBMX, 1mM).

software (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as median and 25th to 75th percentile. The non-parametric statistical Mann-Whitney U-test was used for NM-C and NP-AERD group comparisons and the Wilcoxon test was used for paired comparisons. Statistical significance was established as p values below 0.05.

Results

Culture characterization

All cultured cells were vimentin positive and cytokeratin-negative, confirming the fibroblast phenotype and the total absence of epithelial cells. The myofibroblast percentage in fibroblast cultures was very low and did not differ between NM-C and NP-AERD.

EP receptor expression in nasal samples

The baseline protein and mRNA expression of EP receptors was assessed by western blot and quantitative real-time PCR techniques, respectively. The expression of EP_1 protein and mRNA was undetectable in nasal samples from both NM-C and NP-AERD, while EP_2, EP_3, and EP_4 protein and mRNA were detected in both samples. Protein (Figure 1A) and mRNA (Figure 1C) expression of EP_2 was significantly lower in NP-AERD when compared with NM-C samples. No differences were found in protein and mRNA levels of EP_3 between NM-C and NP-AERD (Additional file 1A and 1B). On the other hand, protein (Figure 1B) and mRNA (Figure 1D) expression of EP_4 was higher in NP-AERD when compared with NM-C.

EP receptor expression in nasal cultured fibroblasts

We also measured the baseline expression of EP receptors in nasal fibroblasts cultured from NM-C and NP-AERD. After 24h in SFM, the expression of EP receptors was assessed by both western blot and quantitative real-time PCR. Similarly to the whole tissue, the results showed no presence of EP_1 on fibroblasts cultured from NM-C and NP-AERD while EP_2, EP_3, and EP_4 protein and mRNA were detected in both NM-C and NP-AERD fibroblast cultures. EP_2 protein expression was significantly lower in fibroblasts from NP-AERD ($p<0.01$) when compared with NM-C (Figure 2A). Likewise, mRNA levels were also lower in fibroblasts from NP-AERD (Figure 2C) when compared with NM-C ($p<0.001$). Protein and mRNA EP_3 levels were similar between fibroblasts cultured from NM-C and NP-AERD (Additional file 2A and 2B). Finally, we found up-regulated protein ($p<0.001$) and mRNA ($p<0.01$) levels of EP_4 in fibroblasts from NP-AERD compared with NM-C (Figure 2B and 2D).

Intracellular cAMP production in nasal fibroblasts upon EP receptor activation

To test whether EP expression correlated with the amount of cAMP released upon receptor activation, cultured fibroblasts from NM-C and NP-AERD were treated with PGE_2 and specific EP receptor agonists.

After 24h in SFM, the concentration of cAMP was almost undetectable. Incubation of fibroblasts with 1mM IBMX significantly increased the levels of cAMP in NM-C and NP-AERD compared with the respective non-treated cells. In these conditions, no differences were found among fibroblasts cultured from NM-C and NP-AERD (Additional file 3).

Incubation with PGE_2 induced a dose-dependent increase in cAMP levels in NM-C and NP-AERD fibroblasts when compared with controls (IBMX, 1mM). Nevertheless, significant differences were found between fibroblasts cultured from NM-C and NP-AERD. Intracellular cAMP was significantly lower in NP-AERD when compared with NM-C at PGE_2 concentrations of 10^{-7}M, 10^{-6}M, and 10^{-5}M. No differences were found between NM-C and NP-AERD at 10^{-8}M PGE_2 (Figure 3).

Stimulation with butaprost induced a dose-dependent increase in cAMP levels in fibroblasts cultured from NM-C and NP-AERD. However, butaprost-stimulated cAMP levels were significantly lower in NP-AERD when compared with NM-C. Significant differences were found at 10^{-7}M, 10^{-6}M, and 10^{-5}M of butaprost (Figure 4A).

CAY10598 also caused a dose-dependent increase in cAMP in fibroblasts cultured from NM-C and NP-AERD. CAY10598-stimulated cAMP levels were significantly higher in fibroblasts from NP-AERD than in NM-C (Figure 4B).

We assessed the additive effects of butaprost and CAY10598 through the results obtained from the individual agonist stimulation produced on cultured fibroblasts. The sum of EP_2 and EP_4

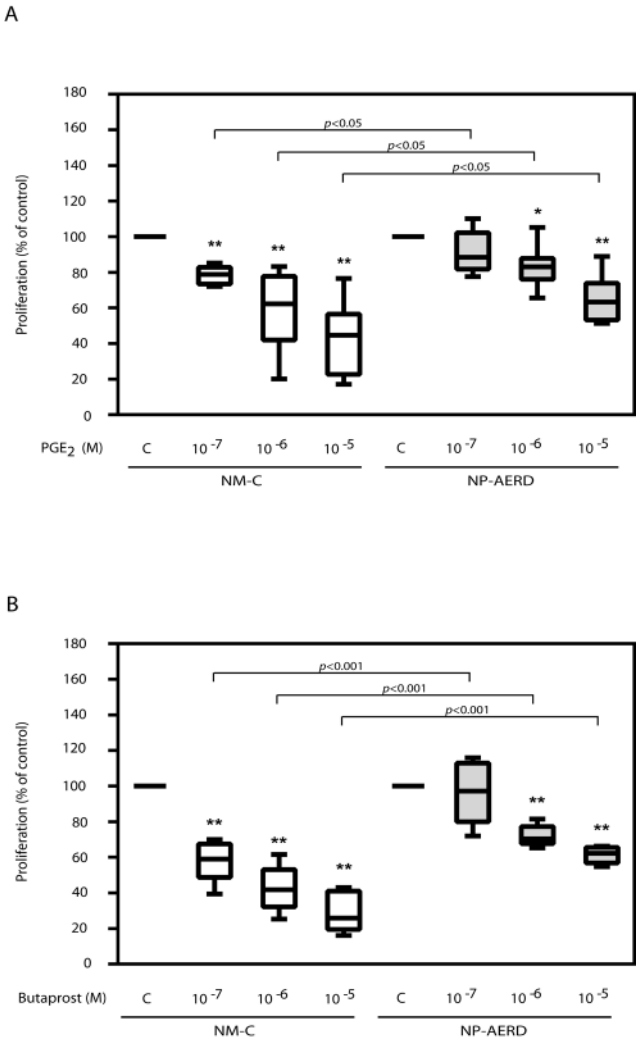

Figure 6. PGE_2 and butaprost effect on nasal fibroblast proliferation. Fibroblasts from NM-C and NP-AERD (n=7 each) were incubated with (A) PGE_2 or (B) butaprost from 10^{-7}M to 10^{-5}M for 18h. Proliferation of fibroblasts was assessed by analysing the proportion of cells that incorporated EdU using flow cytometry. Samples were normalized to the control group (cells incubated with DMEM 2.5% FBS) which is set as 100. * p<0.05, ** p<0.01 compared to control group.

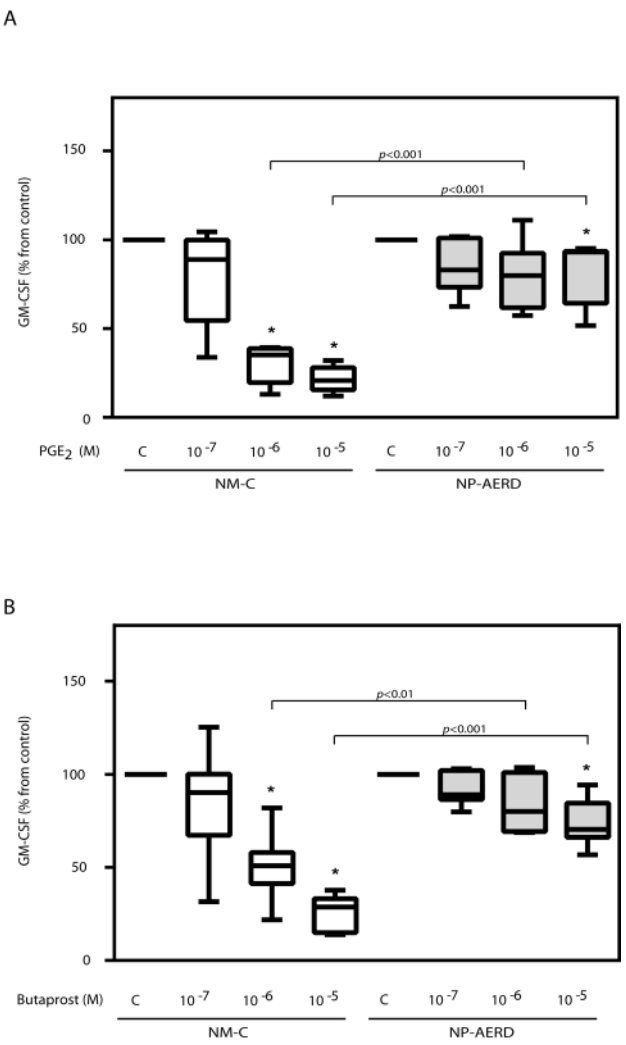

Figure 7. PGE_2 and butaprost effect on GM-CSF release. Fibroblasts from NM-C and NP-AERD (n=7 each) were incubated with or without (A) PGE_2 or (B) butaprost from 10^{-7}M to 10^{-5}M for 24h in media containing 5% FBS. GM-CSF release in cell culture supernatants was measured by ELISA and cytokine production was corrected by cell number using XTT assay. Samples were normalized to the control group (cells incubated with DMEM 5% FBS) which is set as 100. * p<0.05 compared to control group.

agonists induced significantly lower levels of cAMP in NP-AERD at 10^{-6}M and 10^{-5}M when compared with NM-C in the same conditions (Additional file 4).

Differences observed in the amount of cAMP released upon EP_2 and EP_4 activation between NM-C and NP-AERD could be related to the different expression of the aforementioned receptors.

Cultured fibroblasts were stimulated with or without different concentrations of forskolin (5μM to 40μM) and cAMP levels were measured. The results showed that forskolin significantly and similarly stimulated cAMP in NM-C and NP-AERD when compared with their respective controls (Figure 5).

To determine the effect of specific EP_3 activation on cAMP release, cultured fibroblasts were treated with or without forskolin (20μM) and with or without sulprostone at different concentrations for 15min. Forskolin-stimulated cAMP levels were significantly and similarly reduced by sulprostone in fibroblasts cultured from both NM-C and NP-AERD (Additional file 5).

Effect of PGE_2 and butaprost on nasal fibroblast proliferation

Incubation with PGE_2 provoked a dose-dependent inhibition of cell proliferation in cultured fibroblasts from both NM-C and NP-

AERD (Figure 6A). However, the inhibition of cell proliferation by PGE_2 was significantly lower in NP-AERD when compared with NM-C. As seen for PGE_2, butaprost also produced a significant inhibition on NM-C and NP-AERD fibroblast proliferation (Figure 6B). The capacity of butaprost to inhibit cell proliferation was significantly lower in NP-AERD compared with NM-C. Without treatment, we found no significant differences between NM-C and NP-AERD cultured fibroblasts.

Effect of PGE_2 and butaprost on GM-CSF release

As shown in Figure 7, stimulation of fibroblasts with PGE_2 (Figure 7A) or butaprost (Figure 7B) at 10^{-6}M and 10^{-5}M for 24h inhibited FBS-induced GM-CSF release in NM-C. In NP-AERD the inhibition of GM-CSF release was observed at 10^{-5}M of PGE_2 or butaprost. Inhibition of FBS-induced GM-CSF release was significantly lower in NP-AERD when compared with NM-C. Without treatment, we found no significant differences in GM-CSF release between NM-C and NP-AERD cultured fibroblasts.

Discussion

The main findings of our study were the following: (i) at baseline, there is decreased EP_2 and increased EP_4 expression in NP tissue samples and in cultured fibroblasts obtained from NP-AERD; (ii) stimulation with PGE_2, specific EP_2 and EP_4 agonists differentially increased cAMP levels in fibroblasts from NM-C and NP-AERD; (iii) cAMP levels increased similarly between fibroblasts from NM-C and NP-AERD when stimulated with a strong cAMP activator; (iv) stimulation with PGE_2 and EP_2 agonist differentially inhibited cell proliferation in fibroblasts from NM-C and NP-AERD; and (v) stimulation with PGE_2 and EP_2 agonist differentially inhibited GM-CSF release in NM-C and NP-AERD.

We first confirmed the baseline expression of each EP receptor on whole tissue and fibroblasts isolated from NM-C and NP-AERD. Using two different techniques (western blot and quantitative real-time PCR) we found reduced expression of EP_2 and enhanced expression of EP_4 in either nasal tissue or cultured fibroblasts obtained from NP-AERD. Moreover, we observed no expression of EP_1 and no differences in EP_3 in both NM-C and NP-AERD.

EP_1 expression has been reported by some previous studies in NP of AERD and of NSAID-tolerant asthmatics [13] but was not found in other studies [20, 21]. Differences in the methodology (immunohistochemistry vs PCR and western blot) and in the quality and affinity of EP_1 antibodies used probably account for these differences.

Our results are in accordance with previous studies that show low expression of EP_2 receptor in a variety of samples from lower [17] and upper [18,22,23] airways of patients with AERD. Moreover, various polymorphisms in the EP_2 gene have been described [24, 25] in these patients, resulting in a low transcription level of the respective receptor.

The molecular basis of reduced EP_2 expression is still under investigation. Cahill and co-workers [26], in a study performed in fibroblasts isolated from NP-AERD, showed that epigenetic factors contribute to down-regulate the expression of the EP_2 receptor in AERD.

The reduced expression of EP_2 has downstream consequences upon receptor activation

The repercussion of alterations in EP_2 receptor expression found in AERD on the downstream signalling pathway is unclear. Effectively, in a study performed with peripheral blood cells, Corrigan and co-workers [17] hypothesized that the reduced expression of EP_2 on AERD patients has functional effects resulting in a reduced anti-inflammatory activity of PGE_2. Nevertheless, the authors reported no differences in the global mRNA expression of EP_2 in peripheral blood cells in AERD when compared with non-AERD asthma patients. In consequence, the use a specific EP_2 agonist did not result in any significant difference in their inhibitory effects between subjects. This study suggests that the abnormal regulation of EP_2 takes place in the inflamed airway milieu of AERD but is not present in peripheral blood cells that cannot be used as surrogates of cells or tissues collected from either the upper or the lower airways. Based on the concept that EP_2 drives signalling messages by increasing cAMP release [1] we investigated the effects of an EP_2 agonist on cAMP release. Our study shows that the low expression of EP_2 in AERD is associated with a significant reduction in the release of cAMP, a finding that supports that the altered expression regulation has downstream signalling consequences.

Airway remodelling and chronic inflammation are processes characteristic of AERD. PGE_2 signals through EP_2 to dampen the proliferation of fibroblasts and their production of collagen[1]. PGE_2 also modulates the production of several cytokines involved in the inflammatory and reparative processes. Previous studies have reported that PGE_2 down-regulates the production of important pro-inflammatory cytokines, such as GM-CSF [27]. In our study we demonstrated that PGE_2 via EP_2 is able to inhibit cell proliferation in both NM-C and NP-AERD. However, inhibition in NP-AERD is significantly lower when compared with NM-C, probably due to the low levels of EP_2 on these cells. Effectively, Huang and co-workers [28] described that in control lung fibroblasts, PGE_2 suppression of proliferation occurs through ligation of the EP_2 receptor, resulting in activation of AC, increased cAMP production, and activation of cAMP-dependent signalling pathways. Thus, alterations in EP_2 receptor expression could contribute to a reduced suppression of proliferation mediated by PGE_2.

Our study also demonstrated that PGE_2 through EP_2 inhibits GM-CSF release induced by FBS on NM-C fibroblasts. In cultured fibroblasts from NP-AERD the level of GM-CSF inhibition was significantly lower when compared with NM-C. AERD pa-

tients frequently exhibit a massive eosinophilic inflammation, resulting from an increase migration, activation, and survival of eosinophils into tissue [29]. Effectively, several cytokines such GM-CSF sustained these processes. Our results are consistent with those obtained by Clarke et al. [30] which showed that the EP_2 receptor subtype mediates the inhibitory effect of PGE_2 on GM-CSF release from human airway smooth muscle cells. In fibroblasts from NP-AERD, we described that the inhibition of GM-CSF release by PGE_2 is lower than that verified in NM-C. These data indicated that the low expression of EP_2 receptor in these cells could contribute to perpetuating the chronic eosinophilic inflammation characteristic of AERD patients.

EP_4 up-regulation in AERD does not completely compensate the deficient EP_2

In our experiments we found augmented baseline expression of EP_4 in cultured fibroblasts from NP-AERD when compared with NM-C. As expected, we observed an increased capacity of an EP_4 agonist to induce cAMP in AERD. In agreement with our findings, high immunoreactivity has been reported for EP_4 receptor on induced sputum macrophages of patients with asthma [31], despite that they also reported increased levels of EP_2 on these cells.

Considering the convergent signalling cascade of EP_2 and EP_4, it is possible that the high levels of EP_4 found in NP-AERD in this study resulted from a compensatory up-regulation mechanism to counteract the deficient EP_2 receptor. To investigate whether the up-regulated EP_4 can compensate the deficient regulation of EP_2 we compared the effects of PGE2 and the additive effects of EP_2 and EP_4 agonists on cAMP biosynthesis. We observed that the capacity of fibroblasts from NP-AERD to increase cAMP in the presence of PGE_2 is significantly lower when compared with NM-C. Moreover, the enhanced capacity of EP_4 agonist to increase the production of cAMP in AERD was not sufficient to compensate for the inefficient EP_2.

Despite the elevated expression of EP_4 receptor, when fibroblasts from NP-AERD were stimulated with PGE_2, the levels of cAMP were lower in these cells compared to those obtained from NM-C. Thus, high levels of EP_4 receptor may not be sufficient to compensate for the low expression of EP_2. This is in accordance with previous studies that compared signalling through EP_2 and EP_4 receptors and showed that EP_4 has a less efficient functional coupling to cAMP [32].

cAMP biosynthesis in AERD through an EP receptor-independent pathway

Since the observed alteration in downstream signalling was attributed to alterations in EP expression, we studied the effect of a cAMP-elevating agent on cultured fibroblasts from both NM-C and NP-AERD. Forskolin acts via a pathway which does not involve EP_2 or EP_4. We demonstrated no differences in intracellular cAMP between fibroblast cultures when stimulated with forskolin. Stumm et al. [33] studied responsiveness to PGE_2 in lung fibroblasts isolated from asthmatic mice and reported that responses to forskolin mirrored those to PGE_2 and they attributed these findings to the unaltered expression of EP_2 in these cells, which preserves responsiveness to PGE_2. According to these data and since the amount of cAMP released was recovered in NP-AERD after forskolin stimulation, we corroborated that the differences observed between NM-C and NP-AERD are most probably due to alterations in EP expression and their coupling with the cAMP biosynthesis machinery.

Similar expression of EP_3 in AERD and control subjects

EP_3 activation normally reduces cAMP levels. We have not detected any difference in the expression of EP_3 between NM-C and NP-AERD. In keeping with this observation we found that the activation of EP_3 through sulprostone inhibits forskolin-stimulated cAMP in fibroblasts and this effect was similar in NM-C and NP-AERD. Thus, we confirmed previous studies that described the capacity of EP_3 activation to decrease cAMP production in vitro [34,35] and we excluded the potential involvement of EP_3 in the differences observed in cAMP levels between NM-C and NP-AERD. The use of NM as control group could be taken as a potential limitation of this study. However, we sought to highlight the functional repercussion of the low expression of EP_2 receptor in AERD, more than to establish a comparison between the phenotypes of NPs that has already been reported [18].

Conclusion

The altered expression of PGE_2 receptors on AERD has downstream functional repercussions. These events associated with the reduced production of PGE_2 reported in AERD could contribute to perpetuating the chronic inflammatory and remodelling processes usually present in upper and lower airways of these patients. EP agonists represent potential new therapies for inflammatory airways diseases. Reduced EP_2 and the limited capacity of an enhanced EP_4 to counteract the deficient EP_2, suggests that a combination of EP_2 and EP_4 agonists, might prove more helpful that isolated agonists for the treatment of asthma variants such as AERD, in which airway PGE_2-mediated homeostasis is deficient.

Acknowledgements

We are indebted to the Cytometry and Cell Sorting core facility (IDIBAPS) for their technical assistance.

LMC was supported by a grant from the Centro de Investigaciones Biomédicas en Red de Enfermedades Respiratorias (CIBERES) from Instituto de Salud Carlos III, Madrid, Spain. The study was supported by grants from the Spanish Ministry of Health (FIS PI12/254), FEDER, and Fundación Respira (Spanish Respiratory Society).

Authorship contribution

Conceived and designed the experiments: LMC, RT, LP, JRF, and CP. Performed the experiments: LMC and MPG. Analysed the data: LMC, RT, JM, LP, JRF, and CP. Provision of study material or patients: IA. Wrote the paper: LMC, RT, JRF, and CP. Final approval of manuscript: LMC, RT, MPG, IA, JM, LP, JRF, and CP.

Conflict of interest

The authors declared that there are no competing interests in relation to this manuscript.

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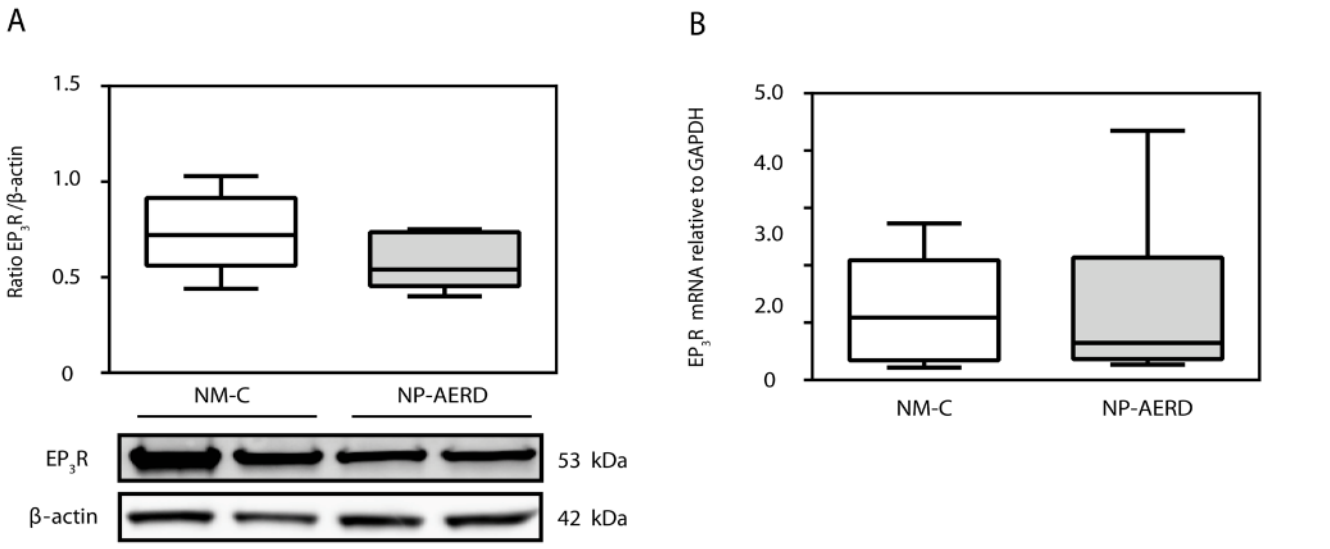
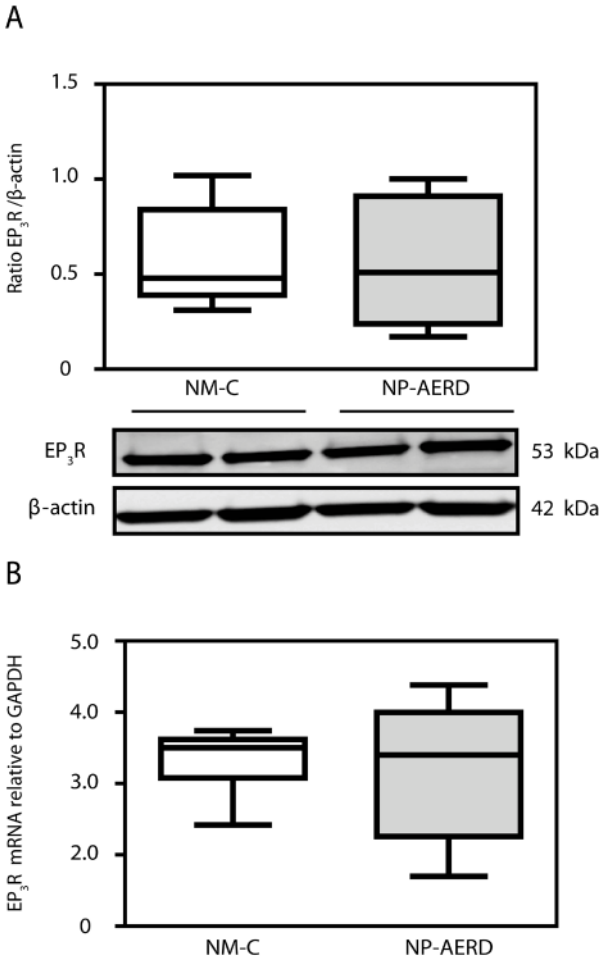
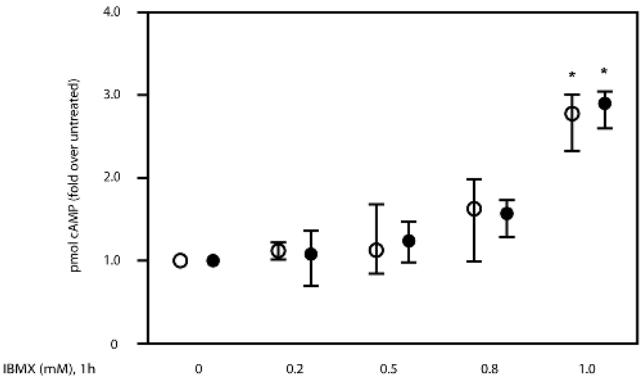
APPENDIX ADDITIONAL FILES



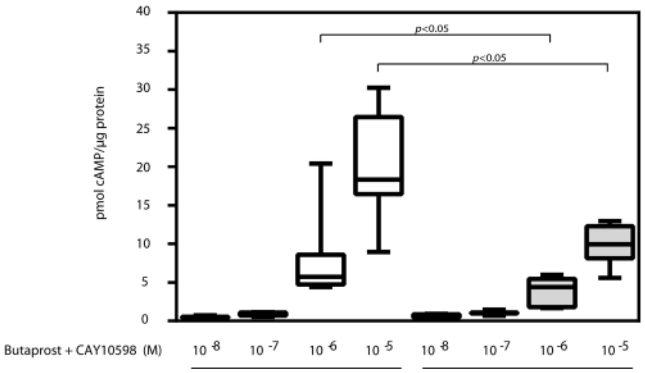
Additional file 1. Basal expression of EP_3 receptors in nasal samples. (A) Densitometric analysis and representative western blot of EP_3 expression normalized by β-actin in nasal samples from NM-C and NP-AERD (n=7 each). (B) EP_3 mRNA expression in nasal samples from NM-C and NP-AERD (n=7 each) was analysed by quantitative real-time PCR and normalized to the GAPDH constitutive gene.



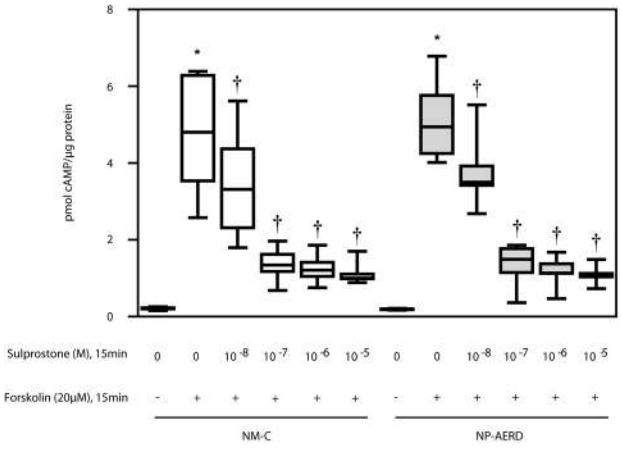
Additional file 2. Basal expression of EP_3 receptors on cultured fibroblasts. Quiescent fibroblasts from NM-C and NP-AERD (n=7 each) were incubated for 24h with cell media without FBS. (A) Densitometric analysis and representative western blot of EP_3 expression normalized by β-actin. (B) Quantitative real-time PCR analysis of EP_3 mRNA expression normalized to the GAPDH constitutive gene.



Additional file 3. IBMX effect on intracellular cAMP levels on cultured fibroblasts. Fibroblasts from NM-C and NP-AERD (n=7 each) were incubated with or without IBMX at different concentrations (0.2, 0.5, 0.8, and 1mM) for 1h. Intracellular cAMP levels were measured by ELISA. Results were normalized to the total protein concentration present in each sample. White circle: NM-C; Black circle: NP-AERD. * p<0.05 compared with respective untreated cells.



Additional file 4. Additive effects of butaprost and CAY10598 on intracellular cAMP levels on cultured fibroblasts. The values of intracellular cAMP concentration obtained through the single stimulation of fibroblasts from NM-C and NP-AERD with different concentrations of butaprost and CAY10598 were summed.



Additional file 5. Sulprostone effect on forskolin-stimulated intracellular cAMP levels on cultured fibroblasts. Fibroblasts from NM-C and NP-AERD (n=7 each) were preincubated with 1mM IBMX for 1h and treated for 15min with forskolin at 20μM and sulprostone (EP_3 agonist) at 10^{-8}M to 10^{-5}M. Intracellular cAMP levels were measured by ELISA. Results were normalized to the total protein concentration present in each sample. * p<0.05 compared with respective control (IBMX, 1mM); † p<0.05 compared with respective cells treated with forskolin at 20μM.