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Bronchodilation induced by PGE_2 is impaired in Group-III pulmonary hypertension

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Running title: Loss of PGE_2-induced bronchodilation in pulmonary hypertensive lung

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bph.14854

Abstract

Background and purpose: In patients with pulmonary hypertension (PH) associated with lung disease and/or hypoxia (Group III), a reduction of pulmonary vascular tone and tissue hypoxia are considered therapeutically beneficial. Prostaglandin (PG) E_2 and PGI_2 induce potent relaxation of human bronchi from non-PH (control) patients *via* EP4 and IP receptors, respectively. However, the effects of PGE_2/PGI_2 and their mimetics on human bronchi from PH-patients are unknown. Our aim was to compare the relaxant effects of several PGI_2-mimetics approved for treating PH-Group I with several PGE_2-mimetics in bronchial preparations derived from PH-Group III and control patients.

Experimental approach: Using an organ bath system, the tone of bronchial muscle was investigated in tissue from either control or PH-Group III patients. Expression of prostanoid receptors were analyzed by Western blot and real-time PCR and endogenous PGE_2, PGI_2 and cAMP levels were determined by ELISA.

Key Results: Maximal relaxations induced by different EP4 agonists (PGE_2, L-902688, ONO-AE1-329) were significantly decreased in human bronchi from PH-patients versus controls. In contrast, the maximal relaxations produced by PGI_2-mimetics (iloprost, treprostinil, beraprost) were similar for both groups of patients. Both EP4 and IP receptor protein and mRNA expressions were significantly lower in human bronchi from PH-patients. cAMP levels significantly correlated with PGI_2 but not with PGE_2 levels.

Conclusion and implications: This study shows that PGI_2-mimetics have preserved maximal bronchodilation in PH-Group III patients. The decreased bronchodilation induced by EP4 agonists suggests that restoration of EP4 expression in airways of PH-patients with respiratory diseases could bring additional therapeutic benefit.

Abbreviations: Pulmonary hypertension (PH); chronic obstructive pulmonary disease (COPD); mean pulmonary arterial pressure (mPAP); 6-minute walk distance (6MWD); forced expiratory volume (FEV); forced vital capacity (FVC).

Keywords: human pulmonary hypertension; prostacyclin; prostaglandin E_2; human bronchi relaxation; respiratory diseases; prostanoid receptors.

Bullet point summary:

- 'What is already known': Inhaled prostaglandin (PG)-I_2 mimetics are major vasodilators used in the treatment of pulmonary hypertension (PH).
- 'What this study adds': Relaxation to PGE_2 but not PGI_2 mimetics, is impaired in isolated bronchi derived from PH-Group III patients.
- 'Clinical significance': The impairment of PGE_2-induced bronchodilation via EP4-receptors could be involved in PH-Group III pathogenesis.

Targets

IP receptor

http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=345

EP4 receptor

http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=343

EP2 receptor

https://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=341

DP1 receptor

https://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=338

COX

https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=269

TP receptor

http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=346

DP2 receptor

https://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=339

EP2 receptor

https://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=341

EP1 receptor

https://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=340

EP3 receptor

http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=342

Ligands

PGI_2

http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1915

iloprost

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1895

treprostinil

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=5820

MRE-269

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=5852

beraprost

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?tab=summary&ligandId=1967

PGE_2

http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1883

PGD_2

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1881

histamine

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1204

indomethacin

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1909

BAY u3405

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1911

ONO-AE1-329

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1933

L-902688

https://www.guidetopharmacology.org/GRAC/DatabaseSearchForward?searchString=L-902688+&searchCategories=all&species=none&type=all&comments=includeComments&order=rank&submit=Search+Database

ONO-AE1-259

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1932

CAY10441

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1969

GW 627368

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?tab=summary&ligandId=1953

ONO-8713

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1921

SC-51322

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1924

L-816266

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?tab=summary&ligandId=5844

DG-041

https://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=5822

Declaration of transparency and scientific rigour

Design & Analysis

https://bpspubs.onlinelibrary.wiley.com/doi/full/10.1111/bph.14207

Immunoblotting and Immunochemistry

https://bpspubs.onlinelibrary.wiley.com/doi/full/10.1111/bph.14208

Introduction

Pulmonary hypertension (PH) is , as recently evidenced, defined as mean pulmonary arterial pressure (mPAP) higher than 20 mmHg and is associated with a high rate of mortality (Simonneau *et al.*, 2019). According to classification established by the World Health Organization (Galie *et al.*, 2016), PH Group-III has a high prevalence rate and is associated with chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis, emphysema or bronchial dilatation dysfunction (Hoeper *et al.*, 2016). These patients are mostly hypoxic, and the administration of supplemental oxygen is an important standardized step in their treatment (Fein *et al.*, 2016).

Prostaglandin (PG) I_2 (prostacyclin) and mimetics (iloprost, treprostinil, MRE-269, beraprost) are known to be effective treatment options for Group I pulmonary arterial hypertension (PAH) patients, through their vasodilatory and anti-proliferative properties in pulmonary vessels (Clapp & Gurung, 2015; Hill *et al.*, 2015; Pluchart *et al.*, 2017). In addition, some studies with PH Group-III patients demonstrate that PGI_2-mimetics improve 6-minute walk distance (6MWD), dyspnea, and mPAP (Bourge *et al.*, 2013; Hoeper *et al.*, 2016; Olschewski *et al.*, 1999; Shimizu *et al.*, 2011). These studies and others support that vasorelaxant therapy in Group-III patients with severe PH could be beneficial (Harari *et al.*, 2017; Olschewski *et al.*, 1999; Reichenberger *et al.*, 2007).

Respiratory function is one of the key parameters in PH-patients; therefore, agents that induce bronchorelaxation and reduce hypoxia may provide greater benefit for PH Group-III patients (Harari *et al.*, 2017). In addition, if agents are delivered through the inhaled route, like iloprost or treprostinil, then bronchodilation could enhance both drug and oxygen delivery to the pulmonary vessels and blood circulation, while avoiding untoward ventilation/perfusion mismatch potential (Bourge *et al.*, 2013; Pluchart *et al.*, 2017).

The dilatory effects of PGI_2 and mimetics are mostly mediated via stimulation of the IP-receptor in human pulmonary vessels and bronchi (Haye-Legrand *et al.*, 1987; Norel *et al.*, 1999). However, if we consider the other prostanoid receptors involved in relaxation, iloprost can also bind somewhat to EP4 receptors and treprostinil potently can bind to EP2, DP1, and somewhat to EP4 receptors, which are preferential receptors for PGE_2 (EP1-4) and PGD_2 (DP1) (Abramovitz *et al.*, 2000; Whittle *et al.*, 2012). These affinities exhibited by some PGI_2-mimetics for other PG receptors could have beneficial effects since PGE_2, and in particular EP4-agonists, are known to induce bronchorelaxation in humans (Benyahia *et al.*, 2012; Buckley *et al.*, 2011; Safholm *et al.*, 2015). Furthermore, PGE_2, via the EP4-receptor, has been shown to inhibit proliferation and migration of human airway smooth muscle cells and play an

anti-inflammatory role in lungs (Aso *et al.*, 2013; Birrell *et al.*, 2015; Mori *et al.*, 2011). However, these protective effects of EP4-agonists were shown only in healthy subjects, and it is not known whether these functions will be similar in PH Group-III patients.

Clinically, the efficacy of PH treatments is evaluated by decreased dyspnea and improved capacity to perform physical effort (6MWD). In this perspective, bronchial reactivity could also enhance cardio-respiratory performance, most likely in PH Group-III patients. Given the complexity of adaptation to physical effort and exertion, when the above-mentioned prostanoids are investigated in the context of PH, it is important to understand how these agonists differentially affect airway reactivity and which prostanoid receptors are involved. Therefore, the aim of our study was to assess these complex interactions using bronchial preparations derived from control or PH Group-III patients.

Methods

Human pulmonary bronchial preparations

Human bronchial preparations were collected in Bichat Hospital (Paris) after obtaining patients' informed consent with Ethics Committee approval from INSERM and AP-HP (CEERB du GHU Nord) Institutional Review Board (n° IRB00006477). These investigations conform to the principles outlined in the Declaration of Helsinki. Control bronchi preparations were obtained from patients (25 male, 14 female) who underwent surgery mostly for lung carcinoma while PH bronchial preparations were obtained from patients (13 male, 9 female) who had undergone surgery for lung transplantation. Categories of patients are PH due to lung diseases and/or hypoxia [(Group-III of PH classification (Galie *et al.*, 2016)] with detailed characteristics presented in Table S1 and control patient characteristics were presented in Table S2. PH lungs used in our study were from patients having catheter-measured mPAP $\geq$ 20 mmHg. Bronchi were carefully removed from the macroscopically normal regions of the lungs. All preparations were used within 1–12 h post-surgery.

Organ bath and isometric measurements

Human bronchial specimens derived from control and PH-patients (3- to 6-mm internal diameter) were cut as rings and set up in 10 ml organ baths containing Tyrode's solution (concentration mM): NaCl 139.2, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5, gassed with 5% CO_2 and 95% O_2 at 37°C and pH 7.4. Each ring was initially stretched to an optimal load (~1-2 grams). Following equilibration (90 min), the preparations were pre-contracted with histamine (50 µM) in the presence of the cyclooxygenase inhibitor (indomethacin; 1.7 µM) or TP/DP2 (CRTH2) receptor antagonist (BAY-u3405; 1 µM, when PGE_2 was used as a relaxant agonist). These agents were used to avoid any physiological effects induced by the release of endogenous prostanoids and/or activation of the thromboxane receptor (TP) by PGE_2. When the response reached a plateau, a cumulative concentration of EP receptor agonists [PGE_2 (EP1-4), ONO-AE1-329 (EP4), L-902688 (EP4), or ONO-AE1-259 (EP2)] or PGI_2-mimetics (iloprost, treprostinil, beraprost, or MRE-269) were added to the baths (1 nM to 10 µM).

For the pharmacological studies, control preparations were incubated in the presence or absence of one of the following prostanoid receptor antagonists: RO3244019 (AGN-230933) or CAY10441 (IP), GW627368 (EP4/TP), L-877499 (DP1), BAY-u3405 (TP), ONO-8713 or SC-51322 (EP1), L-826266 or DG-041 (EP3). Following the incubation period, a precontraction was induced with histamine (50 µM) and when the contraction reached a plateau, cumulative concentrations of iloprost and treprostinil were added to the baths.

Measurement of the expression of prostanoid receptors by Western Blot Analysis

The experimental detail provided conforms with British Journal of Pharmacology Guidelines (Alexander *et al.*, 2018). Human bronchial preparations were homogenized under liquid nitrogen using a porcelain mortar. The homogenates were resuspended in RIPA solution containing Tris–HCl buffer (in mM: Tris: 50; NaCl: 150; EDTA: 5; Triton X-100: 1%; sodium desoxycholate 1%; SDS 0.1%) at 4°C (1 ml per 100 mg of tissue) with a protease inhibitor cocktail. The homogenates were centrifuged at 4000 *g* for 20 minutes, at 4°C. The supernatants were assayed for protein content using a bicinchoninic acid (BCA) protein assay kit. 50 µg of protein were loaded on a 13% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes which were subsequently blocked for 1 h in tris-buffered saline (TBS) 0.1% Tween 20, 5% non-fat dry milk. Membranes were then incubated overnight at 4°C with an anti-EP2 receptor antibody (polyclonal, 1/200), an anti-DP1 receptor antibody (polyclonal 1/200), an anti-EP4 receptor antibody (polyclonal, 1/200) or an anti-IP receptor antibody (polyclonal, 1/500). After overnight incubation, the membranes were washed and then incubated with appropriate peroxidase-conjugated secondary antibody (*1/10000*). Bands were visualized using the ECL plus luminescence system. For quantification, the *film* was scanned (GS-800 Calibrated densitometer), and the integrated *optical density* of the *bands* was estimated with *Scion Image* software® [RRID:SCR_008673] and normalized to α-actin. The homogenates of rat brain and pulmonary artery smooth muscle cells samples were used as standards for the IP receptor in our Western blot experiments.

Real-Time PCR analysis of prostanoid receptors mRNA expression

Tissue samples (50-100 mg) were placed into a safe lock tubes containing two beads (tungsten carbide beads, 3mm). The preparations were lysed in 1 ml of Qiazol® Lysis Reagent by using the TissueLyser Adapter Set 2 x 24 and ground for 4 min at 30 Hertz. Addition of 300 µL of chloroform followed by a centrifugation (15 min, 16000 g, 4°C), separated the solution with an aqueous phase containing RNA. Total RNA was extracted using the RNeasy Plus Mini kit according to the manufacturer's instructions. The quantity of RNA was measured using a spectrophotometer (NanoDrop 2000c; Thermo Scientific; Waltham, Massachusetts, USA). The preparations were reverse transcribed using Maxima First Strand cDNA Synthesis Kits according to the manufacturer's standard protocol. Real-time PCR were performed in the CFX96 (Bio-Rad CFX Manage; California USA) device with the iQ SYBR Green Supermix Kit, according to the manufacturer's standard protocol, using specific primers (Table S3). To determine the relative accumulation of the prostanoid receptor transcripts in human bronchi

from control or PH-patients, the threshold cycle (CT) values of each transcript were normalized by subtracting the corresponding CT values obtained from the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control used as the internal standard (ΔCT). The difference in expression of the target genes (EP4-, EP2- and IP- receptors) was analyzed using the formula: $2^{\wedge-\Delta\Delta CT}$, where $\Delta\Delta Ct = (CT_{prostanoid\ receptors} - CT_{GAPDH}) - (\text{mean } CT_{prostanoid\ receptors} - \text{mean } CT_{GAPDH})$.

Measurements of cyclic adenosine monophosphate (cAMP), PGI_2 and PGE_2

The endogenous level of cAMP (after acetylation), 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) and PGE_2 were measured in human bronchial homogenate supernatants using an enzyme immunoassay (EIA) kit according to the manufacturer's instructions. The cAMP and prostaglandins concentrations were expressed as pmol/mg or ng/µg of protein concentrations calculated in these supernatants, respectively. Technical replicates were used to ensure the reliability of single values.

Measurement of functional respiratory tests in patients from the TRIUMPH study

This unpublished data from the pivotal Phase III study of inhaled treprostinil in PH Group-I patients (Benza *et al.*, 2011; McLaughlin *et al.*, 2010) was obtained from United Therapeutics Corporation. The methods used in the TRIUMPH-1 study have been described (Benza *et al.*, 2011; McLaughlin *et al.*, 2010). According to this, eligible patients were between the ages of 18 and 75 years with a confirmed diagnosis of idiopathic or familial PAH or PAH associated with collagen vascular disease, human immunodeficiency virus infection, or anorexigen use. Patients were New York Heart Association (NYHA) functional class III or IV with a baseline 6MWD between 200 and 450 m and were receiving bosentan 125 mg daily or any prescribed dose of sildenafil, 20 mg tid, for at least 3 months before study entry.

Protocol (data on file, courtesy of United Therapeutics, Corp). At Baseline (Visit 1), patients had been assessed to verify that they meet entrance criteria, then underwent a physical exam including a review of PH signs and symptoms and a review of medical history and concomitant medications, chest x-ray, pulmonary function tests (forced expiratory volume, FEV; forced vital capacity, FVC), The Minnesota Living With Heart Failure Questionnaire (MLWHFQ) and vital signs. Patients who had been included in this study provided blood samples and women of childbearing potential (WOCBP) also provided a urine sample for pregnancy testing. Patients had performed a trough (pre-dosing) 6MWD test and had their Borg Dyspnea score noted. Visit 4 had consisted of dosing at the center, measurement of vital signs, and a peak 6MWD with monitoring. Within 24 -72 hours after Visit 4, patients returned (Visit 5) to complete the final study procedures, which included physical examination, including a

review of PH signs and symptoms, vital signs, pulmonary function tests, MLWHF questionnaire, a trough 6MWD, NYHA classification, Borg dyspnea scoring, chest x-ray and provided blood samples.

Data analysis

The data and statistical analysis comply with the recommendations made of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis *et al.*, 2018). For all experiments, the number of observations (group size) is provided in the figure legends, with a minimum of 5 independent observations performed in patient samples. Statistical analysis was undertaken only for studies where each group size was at least n=5. The declared group size is the number of independent values, and that statistical analysis was done using these independent values. ELISA measurements were performed in duplicate and an average taken in each sample to calculate the final mean data. The pharmacological protocol was randomly assigned and pre-determined before mounting the bronchial preparations in each organ bath. Experimental blinding was not used for this study as there was one core experimenter responsible for each of the protocols described, where individuals also performed the subsequent analysis. In order to limit experimental bias, analysis was not routinely performed until experimental data set was complete.

Acquisition and processing of the physiological data (contraction/ relaxation) were performed with the IOX software® (EMKA, Paris, France). The effects induced by the different agonists were expressed in grams (g) or normalized (%) with respect to an initial reference contraction (Histamine, 50 µM) measured just before the addition of the lowest concentration of the vasorelaxant agonist. This allowed for comparison of agonist responses independent of size of bronchial specimens or contraction. The values are positive for contractions and negative for relaxations. Where possible, a four-parameter logistic equation of the form:

$$E = \frac{E_{max}[A]^{nH}}{EC_{50}{}^{nH} + [A]^{nH}}$$

was fitted to data obtained from each organ bath protocols to provide estimates of the maximal relaxation (E_{max}) of the EP or IP receptor ligands [A], the half-maximum effective concentration values (EC_{50}), as well as Hill slope (nH) parameters. All results were analysed using SigmaPlot® [RRID:SCR_003210] for Windows (Systat software, Inc, Richmond, CA, USA 12.0 version). The pEC_{50} values (potency) were calculated as the negative log of EC_{50} values. All data are means ± s.e.mean (SEM) derived from (n) independent patients and

statistical analysis on the curves, on E_{max} and pEC_{50} values, on mRNA expression and on the optic density of the band were performed using two or one-way ANOVA followed by Student Newman-Keuls test or Student's t test with a confidence level of 95 %. Post hoc tests were caried out only if F was significant and there was no variance in homogeneity. Pearson's correlations were performed, correlation coefficient (r) were calculated and P-values less than 0.05 were considered statistically significant. SigmaStat® [RRID:SCR_010285] statistical software (SYSTAT, Richmond, CA, USA) was used.

Compounds and Materials

Iloprost, beraprost, treprostinil, PGE_2, MRE-269, BAY-u3405, CAY10441, SC-51322, GW 627368, anti -EP4 [RRID: AB_327850], -EP2 [RRID: AB_327848], -DP1 [RRID: AB_10078133] antibodies and ELISA kits (PGE_2, 6-keto-$PGF_{1\alpha}$, cAMP) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The IP receptor antibody was made as previously described (Falcetti *et al.*, 2010). Treprostinil was also obtained from United Therapeutics Corporation (Silver Spring, MD, USA). ONO-AE1-329, ONO-AE1-259 and ONO-8713 were gifts from Ono Pharmaceutical Co., Ltd. (Chūō-ku, Osaka, Japan); L-902688, L-877499 and L-826266 were gifts from Merck (Kirkland, Quebec, Canada). RO3244019 (AGN-230933) was a gift from Allergan (Irvine, CA, USA); ECL plus luminescence system and nitrocellulose membranes were purchased from Amersham Biosciences (Glattbrugg, Switzerland). Protease inhibitor cocktail, chloroform, indomethacin and primers were purchased from Sigma–Aldrich (St. Louis, MO, USA). RNeasy Plus Mini kit and Qiazol® Lysis Reagent were obtained from Qiagen (Valencia, CA, USA). BCA protein assay kit and Maxima First Strand cDNA Synthesis Kits were purchased from Thermo (Rockford, Illinois, USA). iQ SYBR Green Supermix Kit was from Bio-Rad (Hercules, CA, USA). The peroxidase-conjugated secondary antibody was from Jackson (West Chester, PA, USA). DG-041 was a gift from deCODE Genetics (Reykjavik, Iceland). All compounds were dissolved in ethanol, dimethyl sulfoxide (DMSO) or Tyrode's solution to give a stock solution of 10 mM. Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS

Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

Results

The mean age of the patients was 62±02 (range: 37-81, n=39) for control patients, and 54±03 (range: 19-66, n=22) for PH-patients. The hemodynamic (clinical) data for control and PH-patients are detailed in Supplementary Methods (Table S1, S2). There is no significant difference in histamine-induced pre-contractions in human bronchial preparations derived from control versus PH-patients [1.54±0.10 g (n=32) in control and 1.62±0.14 g (n=16) in PH-patients].

Bronchodilation induced by EP2/4-agonists (control vs PH preparations)

PGE_2 and the two EP4 selective agonists (L-902688, ONO-AE1-329) induced potent and concentration-dependent relaxations in pre-contracted human bronchial preparations from control patients. However, these relaxations were significantly decreased in the human bronchial preparations derived from PH-patients (Figure 1A-C). In addition, the potency (pEC_{50}) to PGE_2 was significantly lower in human bronchial preparation from PH-patients versus control patients (Table 1). On the other hand, the selective EP2 agonist ONO-AE1-259 induced no or little relaxation with the highest concentrations (≥1 μM) from control and PH patients (Figure 1D) and no difference was observed between control and PH curves for this agonist (Table 1).

Bronchodilation induced by IP agonists (control vs PH preparations)

IP agonists induced concentration-dependent relaxations in pre-contracted human bronchial preparations. The maximum relaxations induced by iloprost and/or treprostinil were significantly greater versus other IP agonists (beraprost and MRE-269) in either control or PH-patients (Figure 2A-D, Table 2). The pEC_{50} of iloprost and relaxation induced by iloprost at 1 μM were significantly lower in human bronchial preparations from PH-patients versus control patients, while there was no difference for the other IP agonists (Figure 2A-D, Table 2). These results comparing relaxation of control bronchial preparations by various PG agonists show that EP4-agonists are 10 to 50-fold more active than IP-agonists (Tables 1-2).

Protein and mRNA levels of prostanoid receptors in human bronchial preparations derived from control and PH-patients.

A significant decrease in both protein and mRNA levels were observed for EP4-receptor and IP-receptor in the human bronchial preparations derived from PH-patients compared to control patients. However, mRNA (preliminary results) and/or protein levels of EP2- or DP1-receptors were not different (Figure 3A-C).

Effects of the prostanoid receptor antagonists on the relaxation induced by IP-receptor agonists

In the presence of IP antagonist (RO3244019, 1 µM), the relaxations induced by treprostinil and iloprost were completely blocked until 0.1 µM and partially blocked at 10 µM in human bronchial preparations from control patients (Figure 4A, B; Table 3). On the other hand, EP4-antagonist (GW627368, 1 µM and 10 µM), DP-antagonist (L-877499, 10 µM), EP1-antagonist (ONO-8713 or SC-51322 10 µM), EP3-antagonist (L-826266, 3 µM or DG-041, 1 µM) or TP-antagonist (BAY-u3405, 1 µM) did not modify the maximal relaxations induced by iloprost- or treprostinil (Figures 4A-D; Table 3). In contrast, only the pEC_{50} values calculated for treprostinil were significantly reduced in presence of the EP4- (GW627368, 10 µM) or DP- (L-877499, 10 µM) antagonists (Table 3). While the EP1 antagonist did not modify iloprost-induced broncodilations, iloprost induced very small dose-dependent contractions in the presence of IP receptor antagonist (CAY10441, 1 µM: E_{max}=0.15±0.04 g, n=5) probably via EP1 activation in our control bronchial preparations at basal tone.

Basal production of cAMP, PGI_2, PGE_2 and correlations

Endogenous levels of PGE_2, PGI_2 (measuring its stable metabolite 6-keto-$PGF_{1\alpha}$) and cAMP in bronchial preparations derived from control and PH patients were not significantly different (Table S4). There was a significant positive correlation between PGI_2 and cAMP or PGE_2 levels, while no correlation was found between PGE_2 and cAMP levels (Figure 5).

Results of Pulmonary Function Tests of the patients from the TRIUMPH trial

Lung function testing with 216 µg/day (nine inhalations four times daily) inhaled treprostinil (Tyvaso®) was performed at baseline and after 12 weeks of treatment in a population of PH Group-I patients (Table 4). There was no evidence of adverse effects of inhaled treprostinil on lung function, as assessed by FVC (median change from baseline 0.0 % in both group) and forced expiratory volume in one second (FEV1, median change from baseline 0.0% in the active treatment group and -0.5 % in the placebo group).

Discussion

In this current work, we have demonstrated that the maximal relaxations induced by PGE_2 and the two potent, selective EP4-agonists (L-902688, ONO-AE1-329) were strongly and significantly decreased by 35-75% in human bronchial preparations derived from PH Group-III patients compared to controls (Figure 1A-C, Table 1). This decreased reactivity could be explained by the reduced EP4-receptor expression (≥50%, mRNA and protein) in PH bronchial preparations (Figure 3A-C). In contrast, the maximal relaxations produced by the PGI_2-mimetics were not modified, even though IP-receptor expression was also reduced in PH bronchial preparations.

Crosstalk between human airways and pulmonary vessels in terms of vascular tone and remodeling has been described for many years (Farah *et al.*, 2009). PH is not only associated with increased pulmonary vascular tone but also with increased respiratory system resistance (Fernandez-Bonetti *et al.*, 1983; Meyer *et al.*, 2002; Schindler *et al.*, 1995). In this context, the efficacy of inhaled PH treatments may be partially related to their direct effect on bronchial tone. Human clinical studies have demonstrated that inhaled PGE_2 exhibited consistent bronchodilation which could be reduced in some pathological conditions (such as asthma) (Kawakami *et al.*, 1973; Melillo *et al.*, 1994; Pavord *et al.*, 1993; Seth *et al.*, 1981; Walters *et al.*, 1982), yet supportive *ex vivo* and *in vitro* studies to further explain these observations are limited.

Although several beneficial effects of PGE_2 or EP4-agonists, such as anti-inflammatory, antiproliferative, and bronchodilatory effects (Aso *et al.*, 2013; Birrell *et al.*, 2015; Mori *et al.*, 2011) have been observed in human airway cells/tissues, their effects in the presence of underlying PH have not been investigated. In the present report we show that EP4-mediated bronchodilation is strongly reduced in PH-patients. This is consistent with observations from a pulmonary model of inflammation, where a downregulation of the EP4-receptor expression has been detected. (Clayton *et al.*, 2005). Inflammation has an important role in the development of PH(Pugliese *et al.*, 2015) , and increased inflammatory mediators may be responsible for the decreased EP4-receptor expression that was observed in PH bronchi (Figure 3). Taken together, our results and those of the literature point to the important and complex roles of the EP4-receptor in different lung diseases.

In PH preparations with end stage pathology, among the agents tested in our study, iloprost and treprostinil induced the greater bronchodilations (Figure 2). The reduction in IP- and EP4-receptor expression slightly affects iloprost (IP-/weak EP4- agonist) induced bronchorelaxation and does not affect treprostinil (DP-/EP2-/IP- and weak EP4- agonist)

responses(Abramovitz *et al.*, 2000; Whittle *et al.*, 2012). The relaxation induced by 1 µM iloprost was significantly decreased (by 33%), as was the pEC_{50} value, in PH patients (Figure 2A, Table 2). On the other hand, relaxations produced by treprostinil in preparations from PH patients were not different from control and may be explained by a compensatory DP-component (Norel *et al.*, 1999; Whittle *et al.*, 2012).

In control preparations, the bronchodilations induced by iloprost and treprostinil were significantly inhibited by RO3244019, a very selective IP-antagonist (Figure 4A, B; Table 2). These antagonistic effects were surmountable with agonist doses >1µM, possibly in a competitive manner or due to some activity of iloprost and treprostinil at EP4- and/or DP-receptors (Abramovitz *et al.*, 2000; Whittle *et al.*, 2012). In particular, using DP- antagonist which significantly decreased the selectivity of treprostinil-induced bronchial (Table 3) or pulmonary vein relaxations (Benyahia *et al.*, 2013). On the other hand, the greater potency of iloprost at the IP-receptor when compared with treprostinil could account for the EP4-antagonist (GW27368) lacking an impact on the iloprost-induced bronchorelaxations in control patients (Figure 4A).

In addition, a significant decrease in IP receptor expression was observed in bronchi derived from PH Group-III patients (Figure 3A-C). Other studies demonstrated similar results either in PASMC derived from PH Group-I patients or in an experimental rat model of PH (Falcetti *et al.*, 2010; Lai *et al.*, 2008). Despite a reduction in IP receptor expression in PH Group-III patients, the ability of PGI_2-mimetics to induce maximal relaxation of *ex vivo* bronchi was not impaired (Figure 2). A similar discrepancy has been demonstrated in PASMC derived from PH Group-I patients, where even in the presence of a strong decrease in IP receptor density, treprostinil was still able to increase cAMP levels (Falcetti *et al.*, 2010). In the current study, the sensitivity (pEC_{50}) and relaxation induced by iloprost at high concentrations were attenuated in bronchi derived from PH Group-III. These results may be explained by the fact that iloprost likely has affinity for both IP- and EP4- receptors at these concentrations, and both receptors are down-regulated in PH patient preparations. Globally, other prostanoid receptors (DP, EP2) and/or peroxisome proliferator-activated receptors, depending of the PGI_2 analogue tested, could account for the maintained bronchorelaxation in PH preparations (Ali *et al.*, 2006; Falcetti *et al.*, 2010; Patel *et al.*, 2018; Turcato & Clapp, 1999).

The different effects of IP and EP4 receptor downregulation on vascular tone may be explained by differences in their signaling pathways and mechanisms of action. For example, cAMP accumulation via G_s activation in vascular smooth muscle cells is thought to be the main

mechanism of IP receptor-induced vasorelaxation, whereas EP4 signaling is not only associated with G_s, but also with G_i (Leduc *et al.*, 2009), phosphatidylinositol 3-kinase (PI3K) (Regan, 2003), β-arrestin (Buchanan *et al.*, 2006; Kim *et al.*, 2010), and β-catenin (Banu *et al.*, 2009; Jang *et al.*, 2012). As shown in Figure 5, cAMP levels were positively correlated with PGI_2 levels but not with PGE_2 levels in homogenates of human bronchial preparations. This suggests that the mechanism of action for IP receptor agonists involving cAMP activation was still functional in PH lungs as shown in hPASMC (Falcetti *et al.*, 2010; Patel *et al.*, 2018), whereas cAMP signaling through EP4 receptor was impaired. The downregulations described here related to PH and/or to the underlying respiratory pathologies could have global implications.

Our *in vitro* results are complemented by *in vivo* data (courtesy of United Therapeutics Corporation) from the TRIUMPH study (Benza *et al.*, 2011), which demonstrated that inhalation of treprostinil does not change respiratory function parameters in patients with PH Group-I. However, in one small prospective study in PH Group-III patients with COPD (Bajwa *et al.*, 2017), the effects of nebulized treprostinil on pulmonary function tests showed a small but statistically significant decrease in FEV1 (median change -0.18 L; P=0.004). Since inhaled treprostinil may cause airway irritation, it is unknown if this change is clinically relevant given the numerical increases in functional improvement. This is in contrast with another study in PH patients (n=7) with pulmonary fibrosis, where no significant changes in pulmonary function tests (FEV, FVC and FEV/FVC ratio) were detected following 12 weeks of parenteral treprostinil monotherapy (Saggar *et al.*, 2014). On the other hand, in these two studies (Benza *et al.*, 2011; Saggar *et al.*, 2014) treprostinil administration improved 6MWD, hemodynamics function or oxygenation in these PH-patients.

The respiratory function data presented from the TRIUMPH study are comparable to data from other studies of nebulized iloprost in PH Group-III patients (Dernaika *et al.*, 2010; Hegewald & Elliott, 2009; Lasota *et al.*, 2013; Olschewski *et al.*, 1999; Reichenberger *et al.*, 2007; Richter *et al.*, 2015). Iloprost treatment demonstrated an absence of effect (or a trend to improvement) on respiratory function and/or oxygenation (Dernaika *et al.*, 2010; Hegewald & Elliott, 2009; Lasota *et al.*, 2013; Reichenberger *et al.*, 2007) and was associated with functional improvement (mPAP, 6MWD in most of the PH Group-III patients with severe PH (mPAP > 35mmHg). However, high doses of PGI_2-analogues could induce airway irritation in some patients (Reichenberger *et al.*, 2007). In terms of dose, one study (Voswinckel *et al.*, 2006) suggests that similar doses of inhaled iloprost (7.5 µg) or treprostinil (7.5-15 µg) result in a similar efficiency to reduce pulmonary vascular resistance (PVR) and mPAP in patients

with severe precapillary PH. These data are surprising as iloprost has been always regarded as more potent than treprostinil (Abramovitz *et al.*, 2000; Benyahia *et al.*, 2013; Hiremath *et al.*, 2010; Hoeper *et al.*, 2009; Kumar *et al.*, 2016; Olschewski *et al.*, 2004; Whittle *et al.*, 2012). Yet, our human bronchial preparation data (see Table 2) are supported by the Voswinckel *et al* results, where *in vivo* the same potency was calculated for these PGI_2-analogues, and airways appear to behave differently from vasculature, with potency differences abolished between iloprost and treprostinil.

These clinical studies and our *in vitro* results support that agonists such as iloprost and treprostinil are the most suitable PGI_2-analogues for patients with (severe) PH Group-III. Inhalation of these PGI_2-analogues may be a preferential administration route, where patients could concurrently benefit from airway dilatation, blood oxygenation, and pulmonary vasodilation to reduce hypoxic pulmonary vasoconstriction observed in PH Group-III patients. Our study also reveals the down regulation of IP- EP4-receptor expression levels in the human airways and loss of EP4-agonist-induced relaxation in PH Group-III bronchial preparation, which could be contributing factors for PH Group-III. For this reason, the most potent therapies to activate IP-receptor and those to target the prevention/reversal of EP4 down regulation may be the most effective for treating respiratory dysfunction in these patients.

Acknowledgements: We would like to thank Elisabeth Brunet and Amina El Hilali from the Anapathology laboratory, CHU X. Bichat for their help. The authors would like to thank Erik Borg, (United Therapeutics Corp) for his helpful corrections on the manuscript. We thank Dr. Takayuki Maruyama for providing the ONO compounds. We would like also to thank, Merck, deCODE Genetics and Allergan companies for some compound gifts. Gulsev Ozen is a recipient of a postgraduate fellowship (BIDEB-2214) from the Scientificfic and Technological Research Council of Turkey (TUBITAK).

Author contributions: Conception and design: XN, GO, CB. Obtaining pulmonary samples and recruitment of patients: YC, HM, CD, DL, XN. Data collection: GO, CB, XN, KB, RB AMS, ACN, SM. Analysis and/or interpretation: XN, GO, CB. Writing of the manuscript for intellectual content: GO, XN, DL, CB. Review of manuscript: DL, AMS, ACN, LHC.

Conflict of interest: This work was funded by an educational research grant from United Therapeutics to XN. LC has received educational research grants from United Therapeutics and honoraria UTC. AMS and ACN are employees of United Therapeutics.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, and Immunoblotting and Immunochemistry, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Figure 1

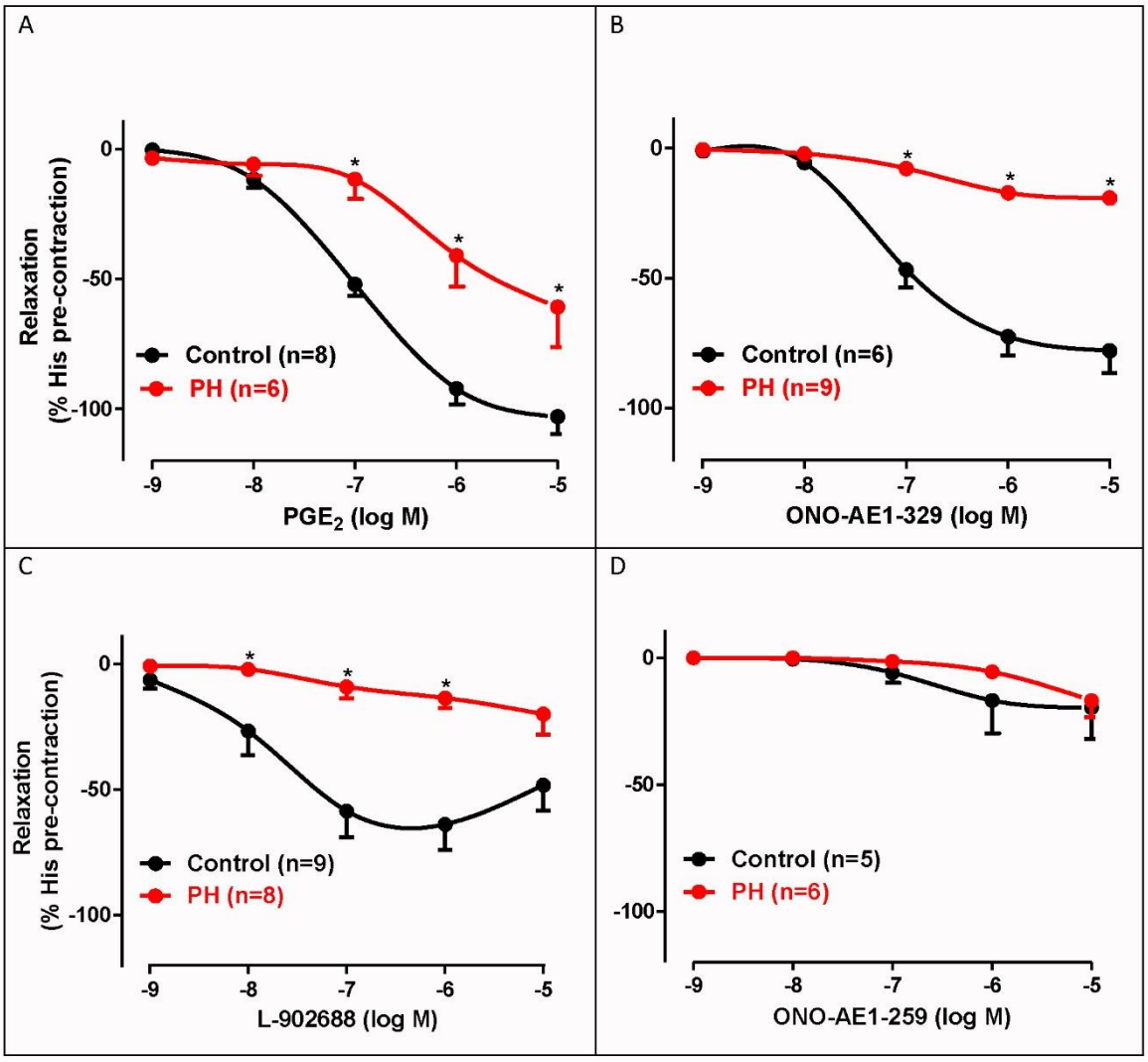

Figure 1. Relaxation induced by EP agonists in human bronchial preparations derived from control and pulmonary hypertensive (PH) Group-III patients. Cumulative concentration-response curves induced by EP receptor agonists [PGE_2 (EP2/4), ONO-AE1-329 (EP4), L-902688 (EP4), ONO-AE1-259 (EP2)]. All rings were treated (30 min) with indomethacin (COX inhibitor, 1.7 µM) and BAY-u3405 (TP antagonist, 1 µM, when PGE_2 concentration-response curve was performed). Responses are expressed as a percentage of pre-contraction induced by histamine (His, 50 µM). Values are means±SEM, (n) indicates the number of patients. *Data significantly different from control patients ($P<0.05$, Two-Way ANOVA) (See Table 1 for pEC_{50}, E_{max} values and statistics).

Figure 2

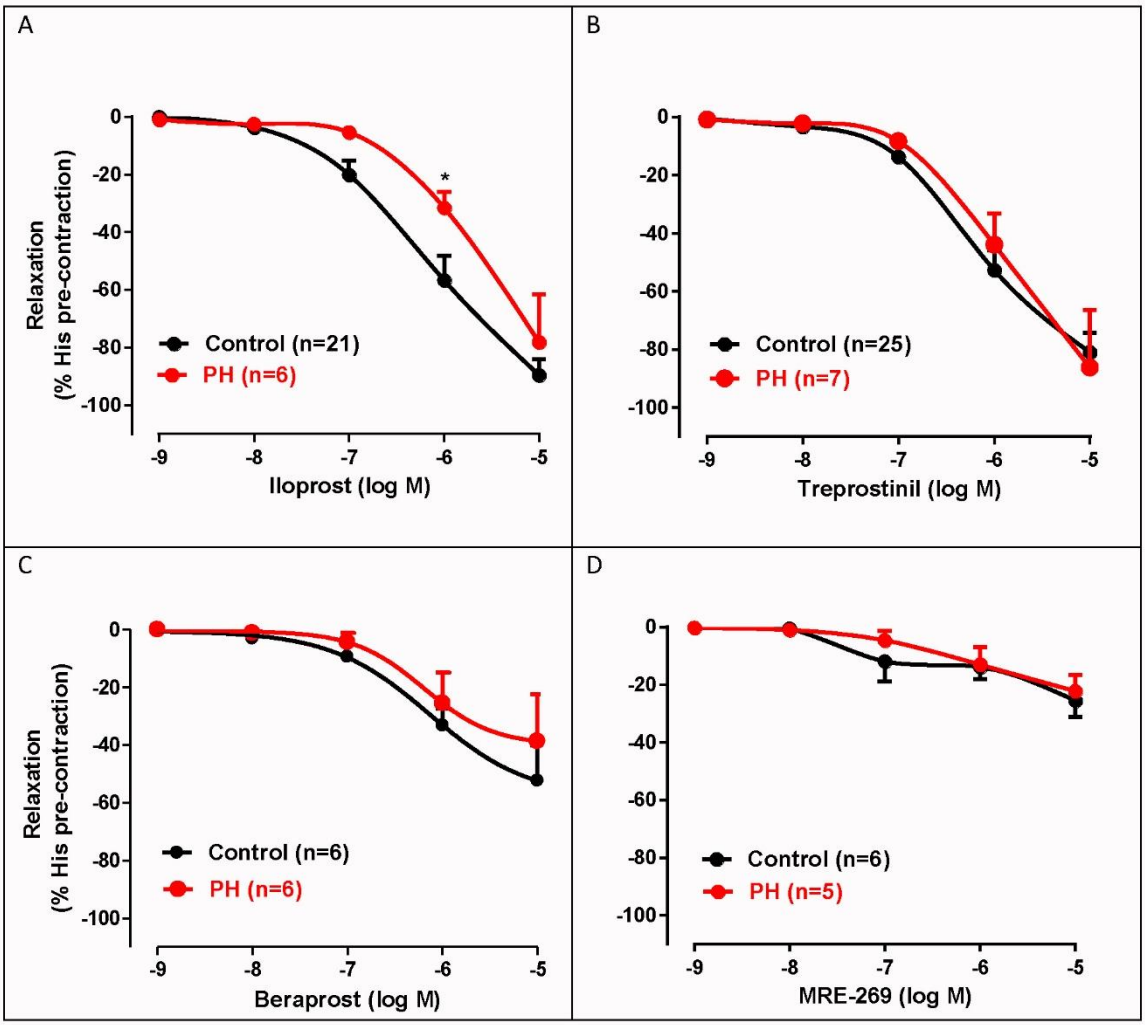

Figure 2. Relaxation induced by IP agonists in human bronchial preparations derived from control and pulmonary hypertensive (PH) Group-III patients. Cumulative concentration-response curves induced by IP receptor agonists (iloprost, treprostinil, beraprost and MRE-269). All rings were treated (30 min) with indomethacin (COX inhibitor, 1.7 µM.) Responses are expressed as a percentage of pre-contraction induced by histamine (His, 50 µM). Values are means±SEM, (n) indicates the number of patients. *Data significantly different from control patients ($P<0.05$, Two-Way ANOVA). See Table 2 for pEC_{50}, E_{max} values and statistics.

Figure 3

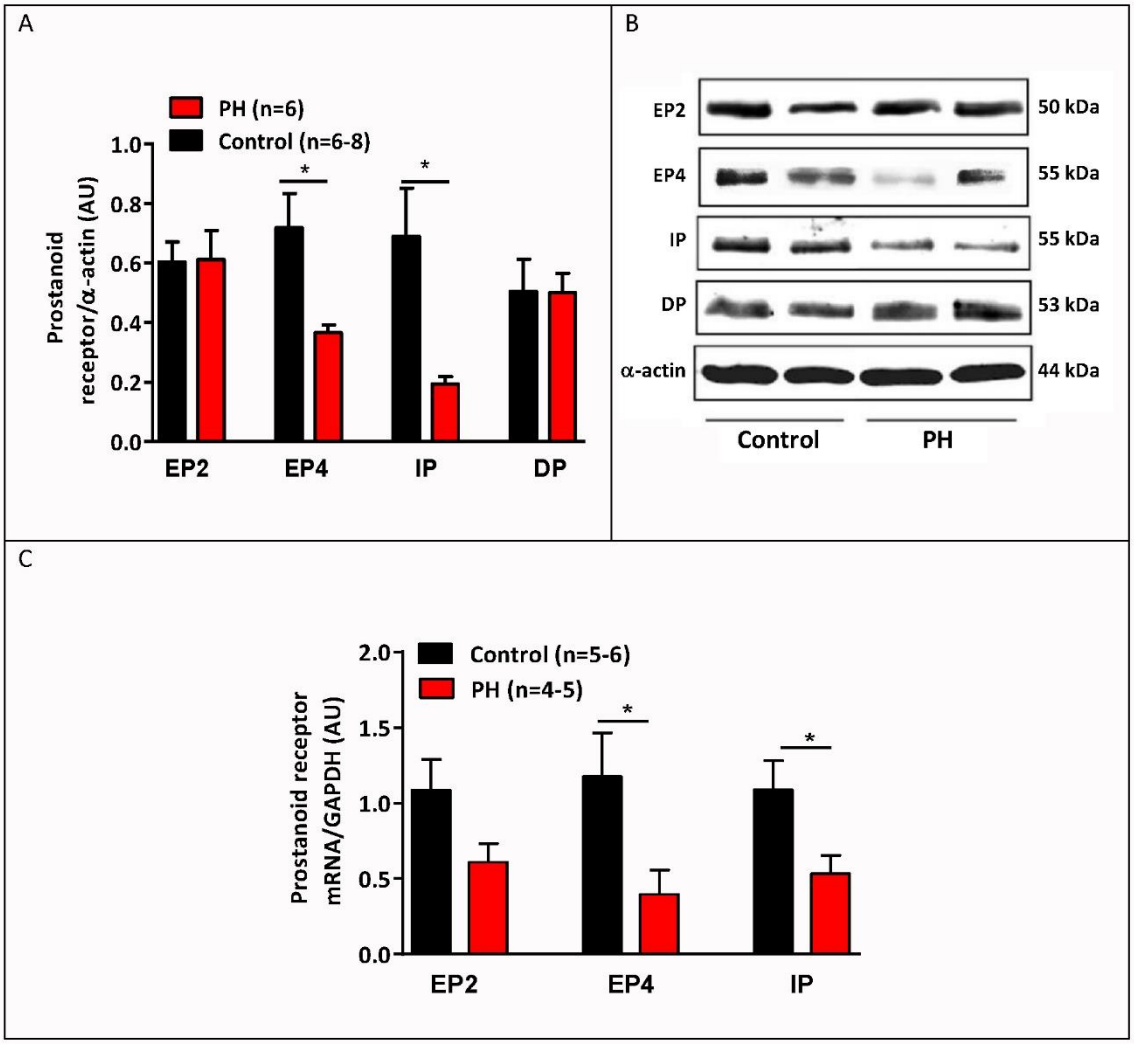

Figure 3. Expression of the prostanoid receptors in human bronchial preparations derived from control and pulmonary hypertensive (PH) Group-III patients. (A) Western blot analysis for prostanoid receptors (EP2, EP4, IP and DP) normalized by α–actin in human bronchial preparations. (B) A representative photograph of Western blot of EP2, EP4, IP, DP receptors and actin. (C) Relative expression of EP2, EP4 and IP mRNA normalized by GAPDH (housekeeping gene) in human bronchial preparations. Values are means±SEM, (n) indicates the number of patients. *Data significantly different from control patients (P≤0.05, Student's t test).

Figure 4

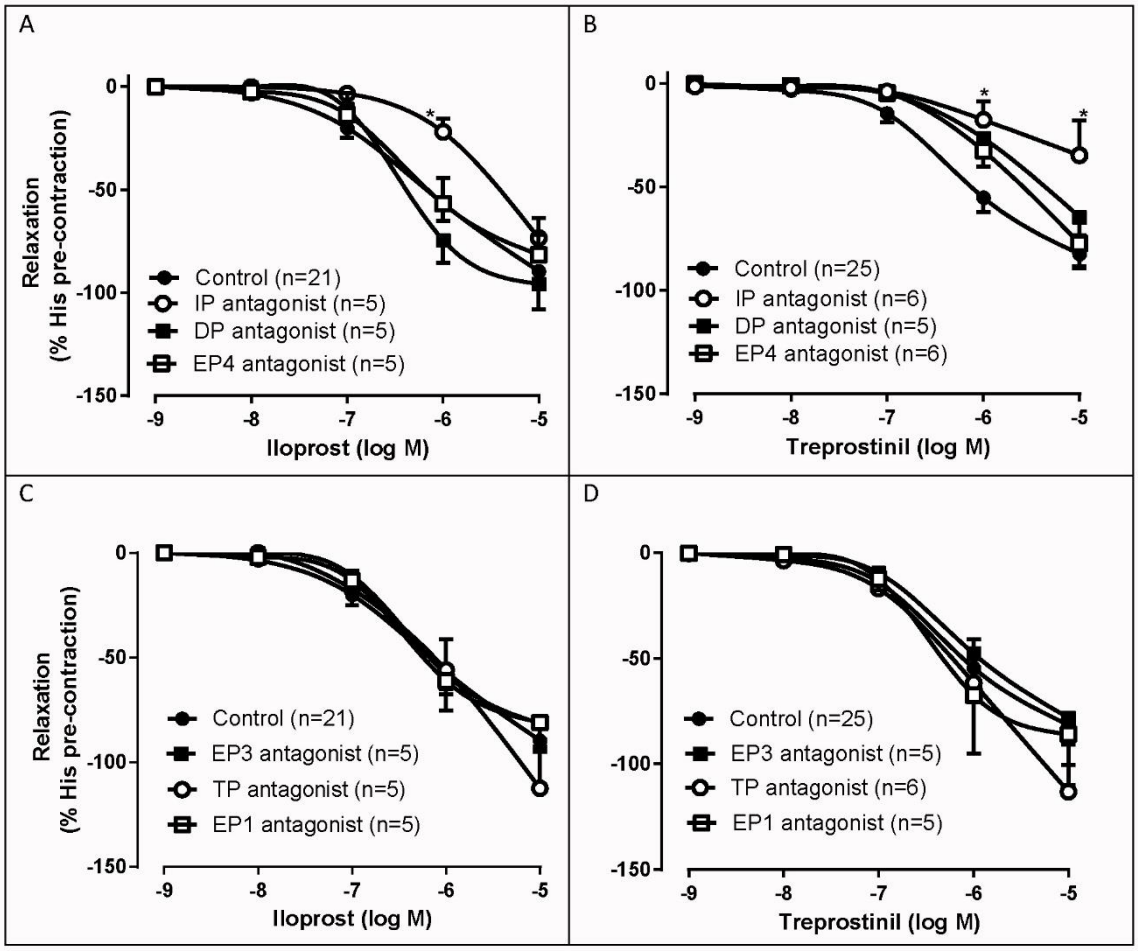

Figure 4. Effect of the prostanoid receptor antagonists on the relaxations induced by IP agonists in human bronchial preparations derived from control patients. Cumulative concentration-response curves induced by IP receptor agonists (iloprost, treprostinil) were performed after an incubation period (30 min) with or without one of the antagonists. The treatments used are DP antagonist (L-877499, 10 µM), EP4 antagonist (GW627368, 10 µM), IP antagonist [(RO3244019 (AGN230933)], 1 µM), EP1 antagonist (ONO-8713 or SC-51322, 10 µM), EP3 antagonist (L-826266, 3 µM or DG-041, 1 µM), TP antagonist (BAY-u3405, 1 µM). Responses are expressed as a percentage of pre-contraction induced by histamine (His, 50 µM). Values are means±SEM, (n) indicates the number of patients. *Data significantly different from control ($P<0.05$, Two-Way ANOVA). See Table 3 for pEC_{50}, E_{max} values and statistics.

Figure 5

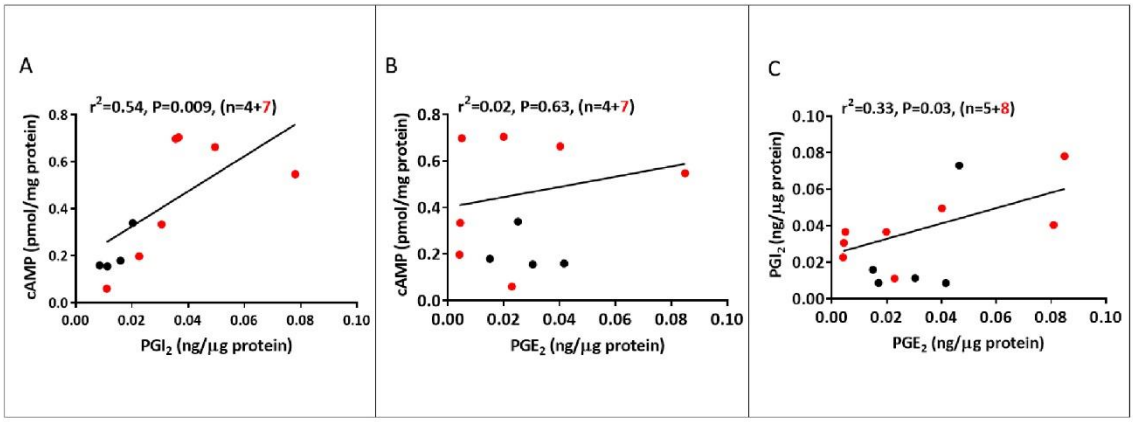

Figure 5. The correlations between the endogenous levels of PGE_2 and PGI_2 (measuring its stable metabolite 6-keto-$PGF_{1\alpha}$) or cAMP in homogenates of human bronchial preparations are presented. Coefficient of determination have been calculated (r^2) and $P<0.05$ indicates significant correlations (Pearson analysis). Data derived from control and pulmonary hypertension Group-III patients, respective n values are presented in parenthesis.

Figure 6

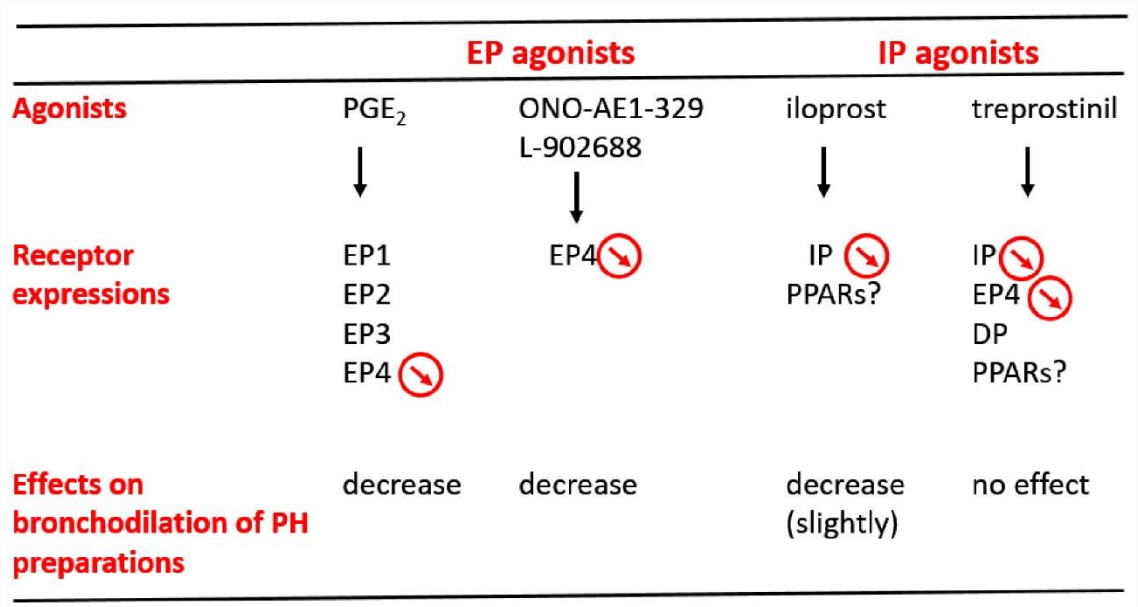

Figure 6. Proposed mechanisms of bronchorelaxation induced by IP and EP receptor agonists in PH Group-III patients. PPAR: peroxisome proliferator activated receptor. Downward arrows indicate decreased expression of prostanoid receptors.

Table 1. Relaxation induced by EP agonists in human bronchial preparations derived from control or pulmonary hypertensive (PH) Group-III patients

	Control patients			PH patients		
	E_{max} (%)	**pEC_{50}**	**n**	**E_{max} (%)**	**pEC_{50}**	**n**
PGE_2	-105±07	7.03±0.12	8	-69±19*	6.21±0.19*	6
ONO-AE1-329 (EP4)	-79±09	7.07 ±0.11	6	-19±03*	NC	9
L-902688 (EP4)	-63±10	8.04±0.22	9	-20±08*	NC	8
ONO-AE1-259 (EP2)	-19±12	NC	5	-16±06	NC	6

Human bronchial preparations were pre-contracted with histamine (50 µM) [Control: 1.56±0.17 g; PH: 1.89±0.16 g]. The rings were incubated for 30 min with indomethacin (1.7 µM) and BAY u3405 (1 µM, when PGE_2 concentration-response curve was performed). The maximal relaxations (E_{max}) and the pEC_{50} values are presented. NC: not calculable. The selectivity of receptor agonists is indicated in parentheses. Values represent means±SEM and are derived from cumulative concentration-response curves induced by EP receptor agonists and from (n) different patients. *Data significantly different ($P<0.05$) from respective control values (One-Way ANOVA or Student's t test).

Table 2. Relaxation induced by IP agonists in human bronchial preparations derived from control or pulmonary hypertensive (PH) Group-III patients

	Control patients			PH patients		
	E_{max} (%)	**pEC_{50}**	**n**	**E_{max} (%)**	**pEC_{50}**	**n**
Iloprost	-94±06[a,b]	6.24±0.13	21	-94±19[b]	5.68±0.15*****	6
Treprostinil	-87±08[b]	6.15±0.10	25	-93±21[b]	6.02±0.05	7
Beraprost	-61±08[b]	6.31±0.18	6	-40±17	5.91±0.21	6
MRE-269	-26±07[a]	NC	6	-24±06	NC	5

Human bronchial preparations were pre-contracted with histamine (50 µM) [Control: 1.41±0.12˚g; PH: 1.44±0.17 g]. The rings were incubated for 30 min with indomethacin (1.7 µM). The maximal relaxations (E_{max}) and the pEC_{50} values are presented. NC: not calculable. Values represent means±SEM and are derived from cumulative concentration-response curves induced by IP receptor agonists and from (n) different patients. * indicates pEC_{50} values significantly different versus respective control values. In each group of patients [a] or [b] indicates E_{max} values significantly different versus beraprost or MRE-269, respectively ($P<0.05$, One-Way ANOVA or Student's t test).

Table 3. Effect of prostanoid receptor antagonists on the relaxation induced by iloprost and treprostinil in human bronchial preparations derived from control patients

IP agonists	Antagonists	E_{max} (%)	pEC_{50}	n
Iloprost	Control	-94±06	6.24±0.13	21
	RO3244019 (IP, 1 µM)	-87±10	5.65±0.14*	5
	GW627368 (EP4)			
	1 µM	-103±08	5.97±0.28	5
	10 µM	-83±10	6.33±0.22	5
	L-877499 (DP, 10 µM)	-97±13	6.38±0.12	5
	ONO-8713 (EP1, 10 µM)	-84±10	6.25±0.20	5
	L-826266 (EP3, 3 µM)	-93±13	6.37±0.12	5
	BAY-u3405 (TP, 1 µM)	-91±10	6.11±0.24	5
Treprostinil	Control	-87±08	6.15±0.10	25
	RO3244019 (IP, 1 µM)	-44±20*	NC	6
	GW627368 (EP4, 10 µM)	-97±13	5.65±0.14*	6
	L-877499 (DP, 10 µM)	-112±18	5.49±0.23*	5
	SC-51322 (EP1, 10 µM)	-92±23	6.18±0.21	5
	DG-041 (EP3, 1 µM)	-89±12	5.82±0.23	5
	BAY-u3405 (TP, 1 µM)	-122±13	6.07±0.21	6

Human bronchial preparations were pre-contracted with histamine (50 µM). The rings were incubated for 30 min with indomethacin (1.7 µM). The maximal relaxations (E_{max}) and the pEC_{50} values are presented. NC: not calculable. The selectivity of receptor agonists or antagonists are indicated in parentheses. Values represent means±SEM and are derived from cumulative concentration-response curves induced by IP receptor agonists (iloprost, treprostinil) and from (n) different patients. *Data significantly different ($P<0.05$) from respective control values (One-Way ANOVA or Student's t test).

Table 4. TRIUMPH study / Respiratory baseline characteristic (Active versus Placebo) for PH Group-I patients receiving treprostinil by inhalation

Statistic	Placebo	Active	P-Value(1)
	Total number of patients		
n	120	115	
	Force vital capacity (FVC) at baseline force		
n	120	115	0.461 NP
Median	82	83	
Min, Max)	(0.0, 123)	(20.0, 139)	
	FVC at week12		
n	109	103	0.741 NP
Median	82	82	
(Min, Max)	(0.0, 127)	(0.0, 149)	
	FVC change from baseline to week12		
n	109	103	0.925 NP
Median	0.0	0.0	
(Min, Max)	(-87, 98)	(-99, 23)	
	Force expiratory volume (FEV) at baseline		
n	120	115	0.201 NP
Median	74.5	76.0	
(Min, Max)	(0.0, 122)	(19.0, 130)	
	FEV at week12		
n	109	103	0.334 NP
Median	73	74	
(Min, Max)	(0.0, 113)	(0.0, 129)	
	FEV change from baseline to week12		
n	109	103	0.917 NP
Median	-1.0	0.0	
(Min, Max)	(-78, 77)	(-86, 23)	

(1) Active versus Placebo; NP=non-parametric test; FEV: Forced expiratory volume; FVC: Force vital capacity.