Role of the Prostanoid EP4 Receptor in Iloprost-mediated Vasodilatation in Pulmonary Hypertension

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***Rationale*: Iloprost is effective for the treatment of pulmonary hypertension. It acts through elevation of cAMP by binding to the prostacyclin receptor (IP receptor). However, there is evidence that patients with severe pulmonary hypertension have decreased expression of the IP receptor in the remodeled pulmonary arterial smooth muscle.**

***Objectives*: We hypothesized that prostanoid receptors other than the IP receptor are involved in signal transduction by iloprost.**

***Methods*: Immunoblotting was used to detect the IP and prostanoid EP4 receptor in lung tissue from patients with idiopathic pulmonary arterial hypertension, and immunohistochemistry was used to detect these receptors in lung sections from rats treated with monocrotaline (MCT28d). Protein and mRNA were isolated from pulmonary arterial smooth muscle cells (PASMCs) from control and MCT28d rats treated with AH6809 (an EP2 receptor antagonist) and AH23848 (an EP4 receptor antagonist) in combination with iloprost. Intracellular cAMP was also assessed in these tissues.**

***Measurements and Main Results*: IP receptor expression was reduced in idiopathic pulmonary arterial hypertension patient lung samples and MCT28d rat lungs compared with the controls. Reverse transcriptase–polymerase chain reaction and immunoblotting of MCT28d rat PASMC extracts revealed scant expression of the IP receptor but stable expression of EP4 receptor, compared with controls. Iloprost-induced elevation in intracellular cAMP in PASMCs was dose-dependently reduced by AH23848, but not by AH6809.**

***Conclusions*: Iloprost mediates vasodilatory functions via the EP4 receptor in the case of low IP receptor expression associated with pulmonary arterial hypertension. This is a previously unrecognized mechanism for iloprost, and illustrates that the EP4 receptor may be a novel therapeutic approach for the treatment of pulmonary arterial hypertension.**

Keywords: prostanoid EP4 receptor; iloprost; pulmonary artery hypertension

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Iloprost can be effective for the treatment of pulmonary hypertension (PH), but many patients are only partially responsive to therapy. Iloprost acts through elevations of cAMP after binding to the prostacyclin receptor, but the lungs of patients with PH have decreased expression of the IP receptor.

What This Study Adds to the Field

Iloprost mediates vasodilatory functions via the EP4 receptor in the case of low IP receptor expression associated with pulmonary arterial hypertension. This finding indicates the EP4 receptor may be a potentially novel therapeutic target for the treatment of PH.

Pulmonary vascular remodeling is a hallmark of pulmonary arterial hypertension (PAH) and is characterized by hypertrophy and hyperplasia of various cell types within the vessel, including medial smooth muscle cells, fibroblasts, and endothelial cells. Several signaling pathways have been shown to be dysregulated in this disease including the following: (*1*) an imbalance between prostacyclin and thromboxane as evidenced by a reduced production of prostacyclin, mainly by down-regulation of prostacyclin synthase and increased excretion of thromboxane (1, 2); (*2*) an increased expression of growth factors such as endothelin (3), serotonin (4, 5), and platelet-derived growth factor (PDGF) (6, 7); and (*3*) an up-regulation of cyclic nucleotide phosphodiesterases (PDEs) such as PDE5 (8, 9) and PDE1 (10). Some of these pathways have been addressed therapeutically by the application of prostanoids (or analogs), endothelin antagonists, or PDE5 inhibitors. In particular, prostacyclin and its analogs (iloprost, beraprost, and treprostinil) have been shown to exert beneficial effects in PAH. Inhalation of aerosolized iloprost has been shown to cause selective pulmonary vasodilatation in pulmonary hypertension (11–13). Long-term use of nebulized iloprost is reported to improve exercise capacity, event-free survival, and hemodynamics in severe pulmonary hypertension. This finding was supported by a randomized, controlled, phase III study in patients with NYHA (New York Heart Association) class III and IV disease (14), which resulted in the regulatory approval of inhaled iloprost for PAH.

The major signaling mechanism of iloprost in smooth muscle cells involves binding to a G-protein–coupled receptor (GPCR), the IP receptor, which directly stimulates the adenylyl cyclase (AC) via Gsα, which converts ATP to cyclic adenosine monophosphate (cAMP). The prostanoid receptor family consists of eight distinct rhodopsin-like receptor proteins termed the IP, EP1, EP2, EP3, EP4, DP, FP, and TP receptors. In addition, the prostanoid receptors may be grouped according to the G-protein to which they preferentially couple. Receptors normally associated with smooth muscle relaxation (the IP, EP2, EP4, and DP receptors) couple via Gs to elevate intracellular cAMP. The receptors EP1, EP3, FP, and TP couple via both Gi and Gq to either reduce intracellular cAMP or elevate Ca^{2+} (15). However, there is evidence that the lungs of patients with PAH have decreased expression of the IP receptor (16). It was therefore hypothesized that prostanoid receptors other than

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the IP receptor may be involved in the signal transduction initiated by iloprost.

The aim of the present study was to investigate the expression of the IP receptor in lung sections from patients with idiopathic PAH (IPAH) and from an experimental pulmonary hypertension study conducted by the injection of monocrotaline (MCT) in rats. In addition, functional experiments were performed in pulmonary arterial smooth muscle cells (PASMCs) to investigate whether prostanoid receptors other than the IP receptor are involved in the vasorelaxant effects of iloprost.

METHODS

Patient Characteristics and Measurements

Human lung tissue was obtained from three donors and three patients with IPAH undergoing lung transplantation. Lung tissue was snap-frozen directly after explantation for mRNA and protein extraction (7). The study protocol for tissue donation was approved by the Ethik-Kommission am Fachbereich Humanmedizin der Justus-Liebig-Universitaet Giessen of the University Hospital Giessen (Giessen, Germany) in accordance with national law and with the Good Clinical Practice/International Conference on Harmonisation guidelines. Written, informed consent was obtained from each individual patient or the patient's next of kin.

MCT-induced Pulmonary Hypertension

The experimental design for adult male Sprague-Dawley rats (300–350 g in body weight; Charles River, Sulzfeld, Germany) was randomized for treatment 28 days after a subcutaneous injection of saline or 60 mg/kg MCT (Sigma, Deisenhofen, Germany) to induce pulmonary hypertension (10). All protocols were approved by the Animal Care Committee of the University of Giessen.

Immunohistochemistry

Fixation was performed by immersion of the lungs in 3% paraformaldehyde solution. After dehydration (automatic vacuum tissue processor, Leica TP 1050; Leica, Bensheim, Germany) and paraffin embedding, the 3-μm sections were immersed in blocking solution containing 1% bovine serum albumin (BSA) (Sigma, Deisenhofen, Germany) and 1% goat serum in phosphate-buffered saline (PBS) for 30 minutes after washing three times in PBS. Sections were incubated, respectively, with polyclonal antibodies against the prostanoid receptors, including anti-IP receptor (Acris, Hiddenhausen, Germany), or anti-EP4 receptor antibody (Cayman, Ann Arbor, MI) for 1 hour. The Dako labeled streptavidian-biotin system (Dako, Hamburg, Germany) was used to detect the signal, and color development was performed by incubation with diaminobenzidine substrate-chromogen for 2 minutes. Blocking solution was used instead of the primary antibody for negative controls.

Isolation and Culture of PASMCs

The PASMCs were isolated from Sprague-Dawley rats 28 days after MCT injection, as described previously (7). To obtain proximal and distal PASMCs, the main pulmonary artery was dissected free from lung and cardiac tissue, and a single full-length incision was made. Hank's balanced salt solution (HBSS) (Gibco, Karlsruhe, Germany) was used. The diameter of the distal part of pulmonary arteries was smaller than 100 μm. The intima and adventitia layers were carefully removed. The central pulmonary artery was separated, and the distal artery tissue was then cut into small pieces and washed with HBSS. Cells were resuspended in culture medium Dulbecco's modified Eagle medium–F12 (Gibco), supplemented with 100 U/ml penicillin and 100 g/ml streptomycin (PAN-Biotech, Aidenbach, Germany), 0.5 mM L-glutamine (Gibco), and 20% fetal calf serum for subsequent culture in 6-well plates and incubated at 37°C in 5% CO_2–95% air. After 24 hours, the medium was changed and thereafter every 2–3 days. The PASMCs were studied at the primary passage stage. Characterization of PASMCs was done at the primary passage using immunocytochemical staining for α-smooth muscle actin (Sigma) and desmin (NeoMakers, Fremont, CA).

Analysis of Prostanoid Receptor Expression by Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was isolated from PASMCs at the primary passage with Trizol reagent (Life Technologies, Rockville, MD), after a determination of the concentration by spectrophotometry and quality by electrophoresis on agarose gel as well as spectrophotometry. The first-strand cDNA was synthesized with the ImProm-II reverse transcription system (Promega, Madison, WI), using oligo(dT) primers according to the manufacturer's instructions. Subsequently, 1 μg of cDNA product was used as a template in polymerase chain reaction (PCR) amplifications together with the primers following the manufacturer's manual. Primers for PCR were designed with the Primer3 program (https://sourceforge.net/projects/primer3). Gene-specific primers were used according to Table 1. After an initial PCR activation step for 10 minutes at 95°C, the following thermal profile was used: 1 minute at 94°C, 1 minute at 55°C annealing, 1 minute elongation at 72°C (30 cycles), The amplicons were resolved in a 1.5% agarose gel and detected by ethidium bromide staining. The expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were monitored as a loading control and quantified by densitometry.

Western Blot Assay

After removing the medium, the PASMCs were washed with HBSS and lysed in 20 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.1% vol/vol Nonidet P-40, 0.05% wt/vol sodium deoxycholate, 0.025% wt/vol sodium dodecyl sulfate, and 0.1% vol/vol Triton X-100 supplemented with phenylmethanesulfonyl fluoride (PMSF) (0.1 mg/ml), leupeptin (10 μg/ml), and aprotinin (25 μg/ml) (Sigma) (17). Insoluble proteins were removed by centrifuging at 10,000 rpm for 3 minutes. The supernatants were assayed for protein content using Dye Reagent Concentrate (Bio-Rad, Munich, Germany). Extracts containing equal amounts of protein were denatured by boiling for 5 minutes in Laemmli's buffer containing β-mercaptoethanol and separated on 12% sodium dodecyl sulfate–polyacrylamide gels at 130 V, and the resolved proteins were transferred to nitrocellulose membranes. The membranes were then immunoblotted with rabbit polyclonal antibody to the IP receptor (Cayman) at 1:500 dilution, or the EP4 receptor (Sigma). The secondary antibodies were specific to peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Sigma).

TABLE 1. PRIMER SEQUENCES USED IN REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION

Primer Name		Sequence	Amplicon Size (*bp*)
α-SM-actin	Sense	5′-CGATAGAACACGGCATCATC-3′	525
	Antisense	5′-CATCAGGCAGTTCGTAGCTC-3′	
Desmin	Sense	5′-ACCTGCGAGATTGATGCTCT-3′	368
	Antisense	5′-CGGGTCTCAATGGTCTTGAT-3′	
COX-2	Sense	5′-ACTGTACCGGACTGGATTCTA-3′	580
	Antisense	5′-CCATCCTGGAAAAGTCGAAG-3′	
IP	Sense	5′-TCACGATCAGAGGATTCACG-3′	358
	Antisense	5′-ATTCCCACAGAACAGCCATC-3′	
EP1	Sense	5′-ACTGCCACCTTCCTGTTGTT-3′	373
	Antisense	5′-GCCCAAGGCTAATGAAACAC-3′	
EP2	Sense	5′-CTTGTTCCACGTGGTAA-3′	306
	Antisense	5′-AAGAGCAAGGCGACCCCATA-3′	
EP3	Sense	5′-TATGCCAGCCACATGAAGAC-3′	374
	Antisense	5′-CACATGATCCCCATAAGCTG-3′	
EP4	Sense	5′-AGTGACCATCGCCAGATACA-3′	339
	Antisense	5′-ATGTAAGAGAAGGCGGCGTA-3′	
TP	Sense	5′-TGTGAGGTGGAGATGATGGT -3′	369
	Antisense	5′-AGGTCGTTAGCAGTCACCAA-3′	
FP	Sense	5′-TCACGGGAGTCACATTTTG-3′	342
	Antisense	5′-TGAGTTCCCAGATGTGCAAG-3′	
GAPDH	Sense	5′-TTCATTGACCTCAACTACAT-3′	469
	Antisense	5′-GAGGGGCCATCCACAGTCTT-3′	

Definition of abbreviations: α-SM-actin = α-smooth muscle actin; COX-2 = cyclooxygenase 2; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Blots were visualized using the enhanced chemiluminescence detection system (Amersham, Dreieich, Germany). Samples were normalized to GAPDH and quantified by densitometry.

Determination of cAMP Accumulation

The EP4 receptor antagonist (AH23848; Sigma) effect on cAMP accumulation mediated by iloprost was measured by a commercial radioimmunoassay (RIA) cyclic AMP (^{125}I) kit (Immunotech, Marseille, France) following the manufacturer's protocol. The PASMCs were grown to 90% confluence in 48-well plates, as described (18). After preincubation in 500 μM 3-isobutyl-1-methylxhantine (IBMX) (Sigma) for 30 minutes at 37°C, PASMCs were incubated with AH23848 or the EP2 antagonist AH6809 (1, 10, 100 μM) (Sigma) for 15 minutes at 37°C. Next, cells were stimulated by iloprost (100 nM) for 15 minutes. After removing the medium, cAMP measurements were performed as described below. Reactions were stopped by aspiration and the addition of ice-cold 96% ethanol. Dried samples were added with 200 μl RIA buffer (150 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.4) and frozen at −80°C. The cAMP in the supernatant was determined by RIA. Protein determination was performed according to the method of Bradford. RIA for cAMP was performed according to the manufacturer's instructions and the mean of cAMP concentration was calculated. Results were expressed as pmol/mg protein for each treatment dose point.

Statistical Analysis

Data from multiple experiments expressed as the mean and standard error (SE) were calculated. All statistical analysis was performed with Student's *t* test. Difference among groups was considered significant when *P* was less than 0.05.

RESULTS

Expression of IP and EP4 Receptor Protein in Human Donor and IPAH Lungs

As shown in the Western blots of Figure 1A, the IP receptor band was detected at 52 kD. The ratio of the IP receptor to GAPDH exhibited a decreased expression of the IP receptor in IPAH lungs compared with human donors (***$P < 0.01$), whereas the EP4 receptor was detected at 78 kD and displayed a similar level of expression between the human donors and IPAH lung samples (Figure 1B). The results reveal the expression of IP receptor protein to be decreased but the expression of EP4 receptor was stable in the IPAH patient's lung tissue as compared with donor lung tissue.

Immunohistochemical Localization of IP and EP4 Receptor in Control Rat and MCT28d Rat Lungs

In MCT-challenged rats, prominent medial wall hypertrophy is evident in the muscular pulmonary arteries. The thick medial layer displays smooth muscle proliferation. The pulmonary artery from the control rat lung section demonstrated IP and EP4 receptor–positive staining (Figures 2A and 2D) in the medial smooth muscle wall. The MCT28d rat lung section exhibited only scant IP receptor–positive staining (Figure 2B), but stable EP4 receptor–positive staining (Figure 2E). No labeling was seen in negative controls in immunohistochemical experiments (Figures 2C and 2F).

Prostanoid Receptors and the Relative Gene Expression Changes at Passage 2 in PASMCs

Semiquantitative reverse transcriptase–PCR was used to survey prostanoid receptors and the relative gene expression from the primary passage to passage 5 of control rat PASMCs (Figure 3). The PASMCs were isolated from the distal pulmonary artery regions and cultured in the presence of 10% fetal bovine serum. To characterize PASMCs, we used the smooth muscle cell–specific gene markers α-smooth muscle actin and desmin. Desmin was down-regulated at passage 3. The primers and product sizes of the prostanoid receptors and relative genes are listed in Table 1. IP, EP2, EP3, and FP receptors were down-regulated at passage

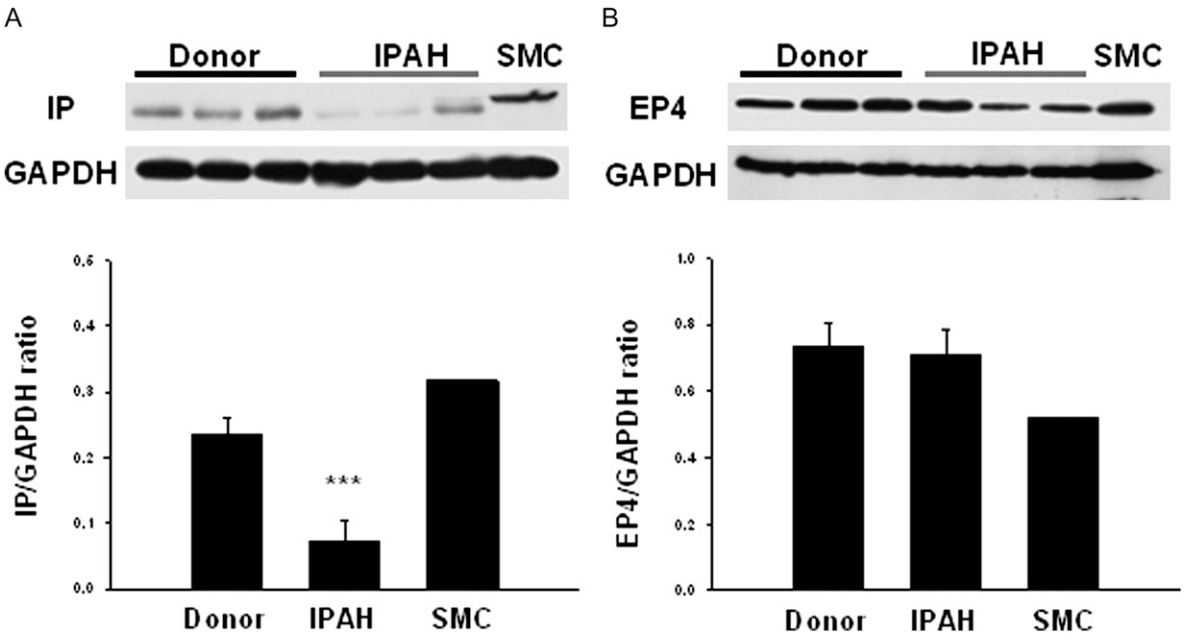

Figure 1. IP and EP4 receptor protein level in human donor and idiopathic pulmonary arterial hypertension (IPAH) lung. (*A*) The IP receptor protein was detected in lung tissues as a 52-kD band, and was decreased in IPAH lung tissues as compared with donor lung tissue. (*B*) The EP4 receptor protein was detected as a 78-kD band and exhibited stable expression in IPAH as compared with donor lung tissue. The *bars* represent mean ± SEM of three samples in each group, with human pulmonary arterial smooth muscle cells (SMC) as a positive control. ***$P < 0.01$ as compared with donor. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

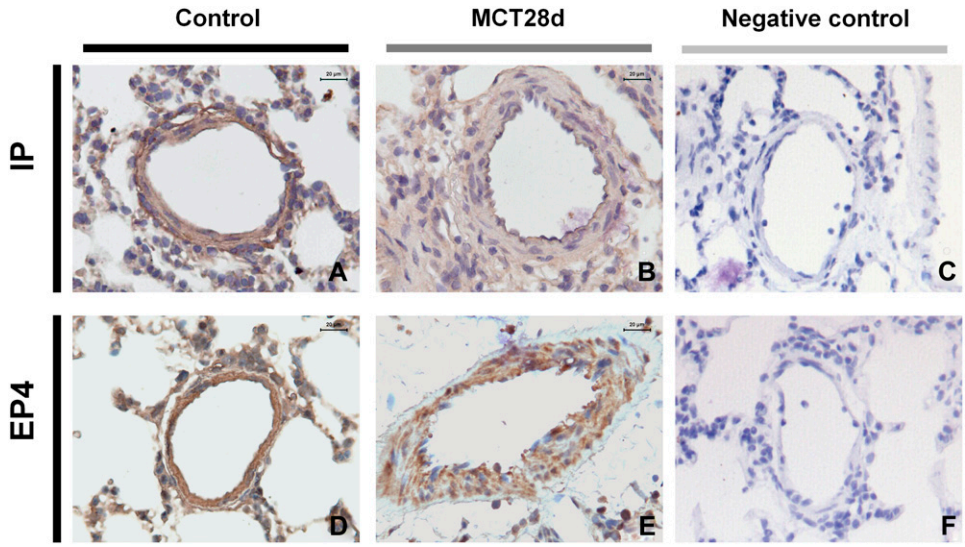

Figure 2. Immunohistochemical localization of IP and EP4 receptor in control and MCT28d rat lungs An analysis by immunohistochemistry of the IP and EP4 receptor was performed on lung sections of control and MCT28d rats. The IP receptor was expressed in the pulmonary arteries of control lungs (*A*). The IP receptor expression was decreased in the pulmonary arteries of MCT28d rats (*B*). EP4 receptor was detected in pulmonary arteries and it was stably expressed in both the control (*D*) and MCT28d rat lung sections (*E*). The cells stained in *brown* were considered positive for the expression of the IP and EP4 receptors and stained with blocking solution instead of the primary antibody as negative controls (*C*) and (*F*). *Bar* = 20 mm, original magnification: ×400.

2. Therefore, PASMCs were used before passage 2 for all of the *in vitro* experiments.

Gene Profiling of the Prostanoid Receptors and the Relative Gene Expression in Distal and Proximal PASMCs from Control and MCT28d Rats

The PASMCs were isolated from MCT28d and control rats. To obtain proximal and distal PASMCs, a single full-length artery incision was made and the main pulmonary artery was dissected free from lung and cardiac tissue. Proximal PASMCs were obtained from trunk and lobar arteries (>2 mm external diameter), and distal PASMCs were isolated from peripheral arteries (<1 mm external diameter). Prostanoid receptors and the relative gene expression profiles were compared in four groups of PASMCs (Figure 4A): control rat proximal and distal PASMCs and MCT28d rat proximal and distal PASMCs. The mRNA expression was separately analyzed in three individual rats in each group of PASMCs, and this revealed variability in the pattern of gene expression and the pattern associated with the pulmonary artery hypertrophy. Densitometry quantification of prostanoid receptors in the gene expression of these four groups was performed (Figure 4B). The data are shown as the mean ± SEM for the same group of three individual PASMCs. In primary or secondary pulmonary hypertension, because of the characteristic changes in vascular structure, the muscular arteries and arterioles exhibit smooth muscle proliferation leading to further medial hypertrophy in the distal musculature (19). Within these four PASMC groups (the MCT28d rat proximal or distal PASMCs and control rat proximal or distal PASMCs), COX-2 was unchanged. The IP was down-regulated in both the proximal and distal PASMCs groups of MCT28d compared with control groups. The EP1 and TP receptors were down-regulated in the MCT28d distal group. The EP2 and EP4 receptors were not significantly changed. The EP3 and FP receptors were down-regulated in the proximal and distal groups of MCT28d, and in the distal group of the control. To the best of our knowledge, these findings are the first to identify that the prostanoid receptor genes presenting in the pulmonary hypertension animal model exhibit different behaviors in the distal and proximal PASMCs.

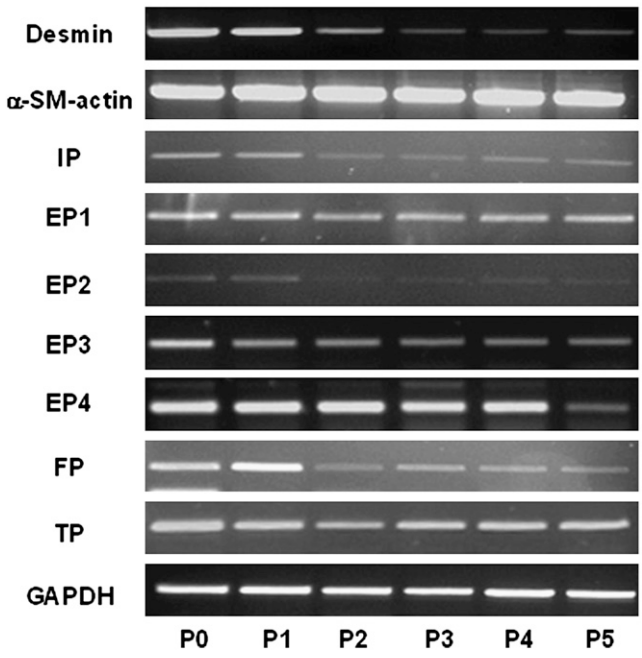

Figure 3. Prostanoid receptor gene profile of control rat pulmonary arterial smooth muscle cells (PASMCs) in different passages. Representative reverse transcriptase–polymerase chain reaction analysis. After passage 2, the mRNA expression levels of the IP, EP2, EP3, and FP receptors were reduced in rat PASMCs. α-SM-actin = α-smooth muscle actin.

Immunoblotting of IP and EP4 Receptor Expression in Distal PASMCs of Control and MCT28d Rats

To evaluate the protein expression of the IP and EP4 receptors, protein was prepared from the distal PASMCs of control and MCT28d rats. As is evident in the Western blots (Figure 5A), the IP receptor protein band was detected at 52 kD. The ratio of IP receptor to GAPDH was shown to have decreased IP receptor expression in MCT28d compared with control PASMCs ($P < 0.05$). However, the EP4 receptor was detected at 78 kD, indicating stable expression in the control and MCT28d rats (Figure 5B). There is evidently reduced IP receptor protein expression in the remodeled vessels in patients with pulmonary hypertension (16). Taken together, the results indicate the expression IP receptor protein was decreased but EP4 receptor protein expression was stable in both the pulmonary hypertension animal model (MCT28d) and IPAH lung samples.

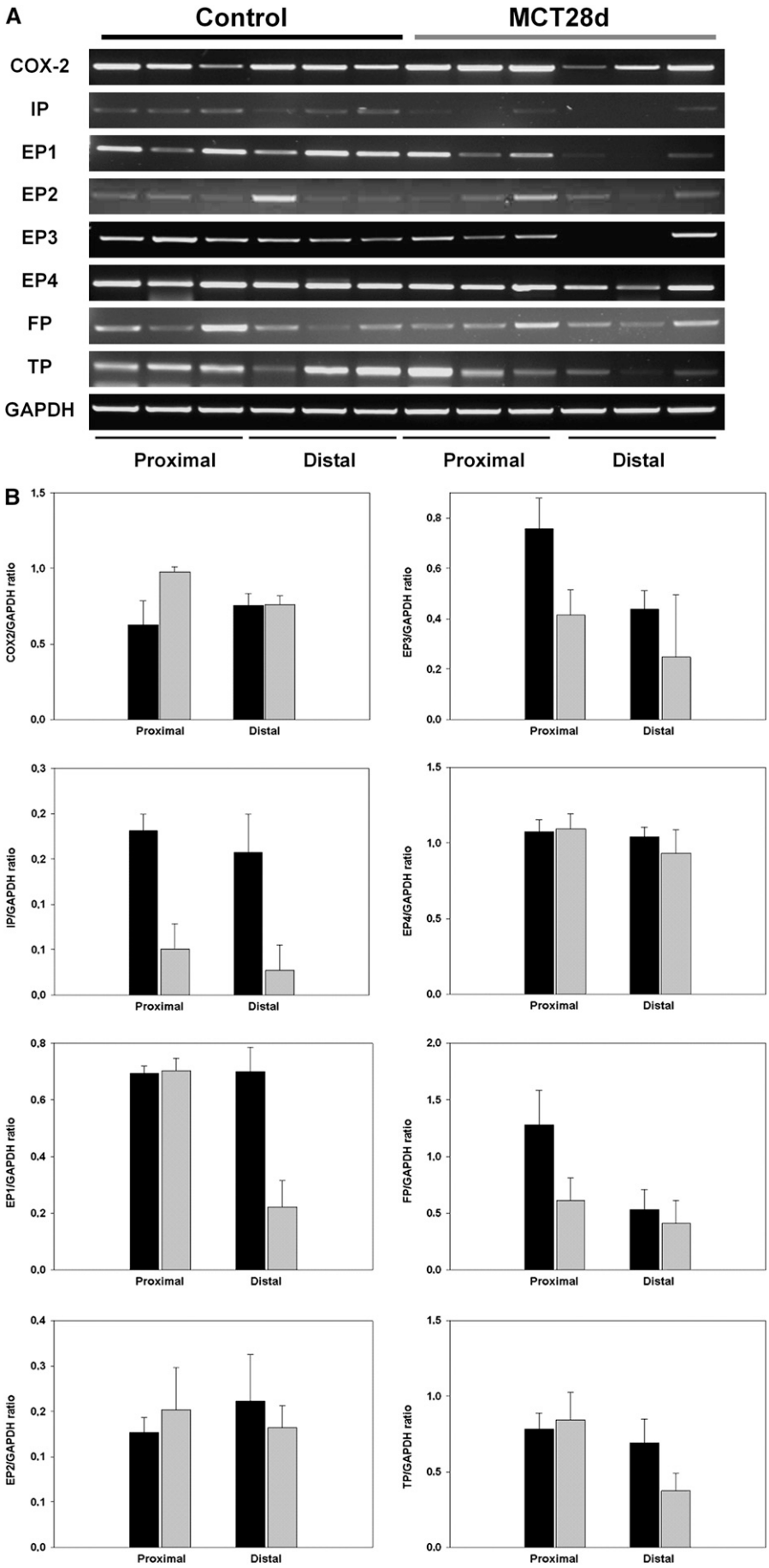

Figure 4. The prostanoid receptor gene profile of distal and proximal pulmonary arterial smooth muscle cells (PASMCs). (*A*) Representative reverse transcriptase–polymerase chain reaction analysis. The mRNA expression of prostanoid receptors in the proximal and distal portion of PASMCs that were isolated from either control or MCT28d pulmonary arterial hypertension rat pulmonary arteries. The expression differences were compared with GAPDH as a loading control, n = 3. The PASMCs were harvested for RNA in the primary passage. Densitometry quantification of prostanoid receptors in terms of the gene expression of these four groups (*B*). Data are shown as the mean ± SEM in the same group of three individual PASMCs. The *black bars* represent the proximal or distal PASMCs of the control groups. The *gray bars* represent the proximal or distal PASMCs of the MCT28d groups.

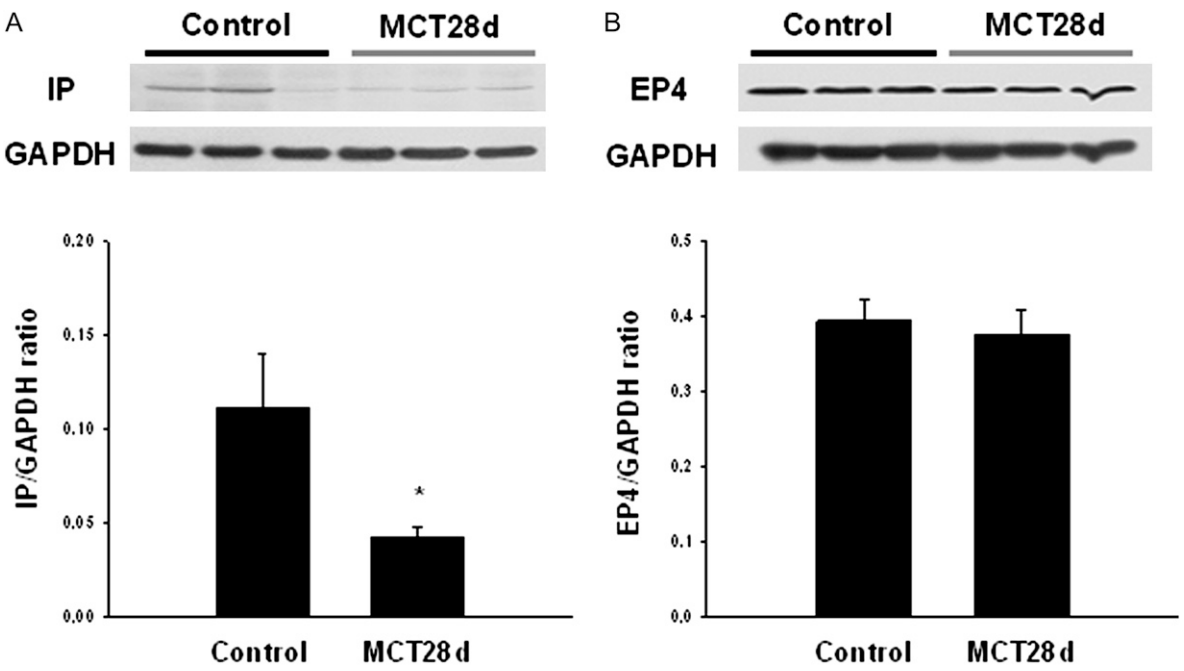

Figure 5. Immunoblotting for IP and EP4 receptors in primary pulmonary arterial smooth muscle cells (PASMCs) from control and MCT28d rats. (*A*) Densitometric analysis from three different experiments in each group. The IP receptor was identified as a 52-kD immunoreactive band that was decreased in MCT28d rat PASMCs compared with control PASMCs. Data are mean ± SEM, n = 3 in each group. *$P < 0.05$ as compared with control. (*B*) The EP4 receptor was identified as a 78-kD immunoreactive band and was stably expressed in MCT28d PASMCs compared with control PASMCs.

Effect of EP4 Receptor Antagonist (AH23848) and EP2 Receptor Antagonist (A6809) on cAMP Accumulation in MCT28d Rat PASMCs

The PASMCs from MCT28d rats exhibited scant IP receptor, but stable EP4 and EP2 receptor expression. Prostanoids (mainly PGE2 and PGI2) activate the IP and EP4 receptors, which are coupled via G-stimulatory proteins to adenylyl cyclase to generate cAMP (20–22), leading to mediation of vasodilatory functions. The EP2 and EP4 receptors are both coupled via Gαs to induce elevations in intracellular cAMP, leading to smooth muscle relaxation (15). To delineate the contribution of the EP2 and EP4 receptor in view of scant IP expression to iloprost-induced intracellular cAMP accumulation, we performed additional functional experiments in MCT28d rat PASMCs using AH6809 (a selective EP2 receptor antagonist) and AH23848 (a selective EP4 receptor antagonist) in combination with iloprost. Preincubation with AH23848 was used to block the EP4 receptor, whereas AH6809 was used to block the EP2 receptor. Preincubation with IBMX (23) excluded a role for PDEs in these experiments. The MCT28d rat PASMCs were stimulated for 30 minutes at various AH23848 or AH6809 concentrations (0, 1, 10, 100 μM), whereas IBMX (500 μM) was applied, then incubated with or without iloprost (100 nM) for 15 minutes. Iloprost-induced intracellular cAMP accumulation was inhibited in a dose-dependent manner by AH23848 (the EP4 receptor antagonist) (Figure 6A), but not by AH6809 (the EP2 receptor antagonist) (Figure 6B). These results indicated that iloprost may mediate vasodilatory functions via the EP4 receptor in substitution on the IP receptor in MCT28d rat PASMCs.

DISCUSSION

One of the key pathways that is altered in PAH is the prostacyclin signaling pathway. It is known that disturbances to prostacyclin synthesis (1, 2), as well as polymorphisms in the genes encoding PGI_2 synthase (PGIS) (24) contribute to severe pulmonary hypertension. Substitution of prostacyclin, either by overexpression of PGIS (25) in experimental pulmonary hypertension or application of the stable prostacyclin analogs iloprost (26, 27) or beraprost (28), decreased pulmonary arterial pressure and vascular remodeling. Prostacyclin is a product of cyclooxygenases (COX) and mediates potent antiplatelet, vasodilator, and antiinflammatory actions by activating the IP receptor (29). However, there is evidence that the lungs of patients with PAH have decreased expression of the IP receptor (16). In this study, the question of how iloprost may work under conditions of low IP receptor expression was addressed.

These prostanoid receptors are members of the GPCR superfamily and are coupled to AC and phospholipase C (30–32). To delineate the contribution of prostanoid receptors in iloprost signal transduction, prostanoid receptor gene expression was profiled, and EP1 and the EP3 receptors were demonstrated to be down-regulated in MCT28d rat PASMCs. The EP1 and EP3 receptors couple via both Gi and Gq to either reduce intracellular cAMP or elevate Ca^{2+}, and are involved primarily in vascular contraction via the Ca^{2+}/phospholipase C pathway (15). Thus, the role of EP1 and EP3 receptor in the iloprost-induced increase of intracellular cAMP in MCT28d rat PASMCs was excluded. The EP2 and EP4 receptors both couple via Gαs to induce elevations in intracellular cAMP, leading to smooth muscle relaxation (15). Interestingly, prostanoid receptor gene profiling revealed that the EP2 and EP4 receptors were stably expressed, suggesting the possibility that EP2/EP4 receptors may be involved in the iloprost-induced increase in intracellular cAMP levels, when IP receptor expression is reduced in MCT28d rat PASMCs. The functional pharmacology of EP2 and EP4 receptors, studied using various prostanoid receptor agonists, suggested that iloprost is an agonist of the human EP4 receptor (33, 34).

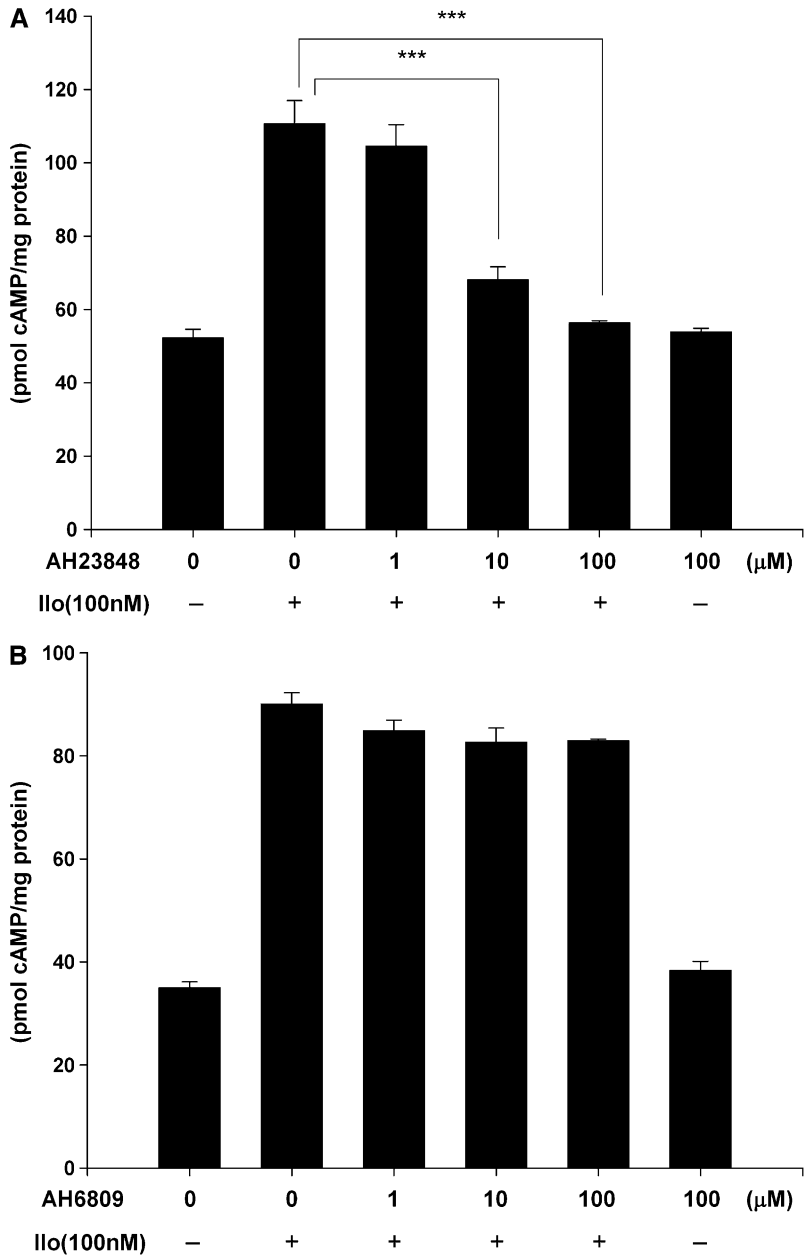

Figure 6. The EP4 antagonist AH23848 blocks the cAMP accumulation mediated by iloprost in MCT28d rat pulmonary arterial smooth muscle cells (PASMCs). The intracellular cAMP accumulation induced by iloprost was inhibited by AH23848 but not AH6809. The MCT28d rat PASMCs, which exhibit scant IP receptor but stable EP4 receptor expression, were stimulated for 30 minutes at various concentrations (0, 1, 10, 100 μM) of AH23848 (*A*) or the EP2 antagonist AH6809 (*B*), with or without iloprost (100 nM) for 15 minutes. Data are the mean ± SEM of three different experiments. ***$P < 0.01$ as compared with iloprost treatment alone.

In addition, to delineate the contribution of the EP2 and EP4 receptor to iloprost-induced intracellular cAMP accumulation when IP expression is low, additional functional experiments were performed in MCT28d rat PASMCs using AH6809 (a selective EP2 receptor antagonist) and AH23848 (a selective EP4 receptor antagonist) in combination with iloprost. As a result, the iloprost-induced intracellular cAMP accumulation was inhibited in a dose-dependent manner by AH23848 but not by AH6809, clearly demonstrating the contribution of EP4 receptors in mediating the effects of iloprost.

The EP4 receptor is stably expressed in both human PAH and MCT-induced pulmonary hypertension in rat lungs, suggesting that it may be an interesting therapeutic target. The signaling mechanism is similar to the IP receptor and involves the well-known cAMP–protein kinase A axis, which results in vasodilatation and antiproliferation. Interestingly, iloprost has been documented as an EP4 receptor agonist (35, 36). Apart from the IP, iloprost activates the EP4 receptor, which may overcome the effects of down-regulation of the IP receptor under disease conditions. The IP receptor is down-regulated in human PAH, as is evident from data presented in the current study, which are in accordance with a previous report that describes the decreased expression of the prostacyclin receptor in PAH (16). In addition to perturbations to receptor expression, other components of the prostacyclin system are also affected in PAH, including decreased levels of the prostacyclin metabolite 6-keto-PGF1α in urine (2), decreased expression of prostacyclin synthase (1), and polymorphisms of PGIS (24). Therapeutic application of prostanoids does result in the improvement of survival and hemodynamics in patients with PAH, as has been shown in several clinical trials (12, 37–39). These effects of prostanoids on clinical improvement of patients with severe pulmonary hypertension may be related to non–receptor-mediated effects in the pulmonary vessels (e.g., antithrombotic effects) or the vasodilatation of the less heavily remodeled pulmonary arteries, which may have preserved prostacyclin receptor signaling (40).

Receptors other than the prostacyclin receptor could be involved in the mediation of these vasodilatory and vasculoprotective effects (20, 35). The regulation of pulmonary vascular tone under physiologic conditions is mainly controlled by prostacyclin and nitric oxide, and mediators such as natriuretic peptides (ANP, BNP), vasoactive intestinal polypeptide (VIP), endothelin, or thromboxane. Important information regarding the role of any of the vasodilating pathways can be earned from the pathophysiologic situation of pulmonary hypertension. In this line, disturbances of prostacyclin synthesis, as well as polymorphisms of PGIS (24), have been related to pulmonary hypertension. In addition, there is evidence that the nitric oxide system is dysfunctional as well, either by decreased expression of NO synthase (41) or low NO bioavailability due to increased oxidative stress (42). This pathway is currently targeted by PDE5 inhibition, which amplifies the remaining NO signal by stabilization of the downstream second messenger cyclic guanosine monophosphate (cGMP) (43). New pharmacologic activators of the soluble guanylate cyclase may thus further amplify the NO signaling cascade (44). Alternatively, peptides including the natriuretic peptides (ANP, BNP) or VIP counteract vasoconstriction, and substitution of these vasodilative and antiproliferative peptides is under clinical development. Petkov and colleagues have recently shown that both receptors of VIP, namely VPAC-1 and VPAC-2, are up-regulated in patients with IPAH (45). Both receptors were localized in PASMCs and believed to be compensatory up-regulated in response to a pathologic decrease of circulating VIP. In addition, VIP knockout mice develop more severe pulmonary hypertension (46) and exogenous VIP either delivered as aerosol or intravenous infusion reduces pulmonary hypertension (45, 46). However, because PAH is a complex disease, targeting a single pathway cannot be expected to be uniformly successful.

Prostacyclin and its analogs (iloprost, beraprost, treprostinil) have offered beneficial effects in PAH. Iloprost is the first-line drug of PAH therapy; therefore, it is the more important vasodilator–antiproliferative pathway alternative PGI2 receptor, compared with others. However, it is not yet clear if prostacyclin analogs operate only via a single prostanoid receptor or via multiple prostanoid receptor or nonprostanoid pathways. To investigate the expression profile of prostanoid receptors and to perform functional experiments, proximal (vessels >2 mm external diameter) and distal (vessels <1 mm external diameter) pulmonary smooth muscle cells were isolated from MCT-treated rats. This animal model of pulmonary hypertension is characterized by remodeling of the precapillary vessels (medial thickening, *de novo* muscularization of small pulmonary arterioles). Due to this mimicry of clinical pulmonary arterial hypertension, the rat MCT model has repeatedly been used for investigating the acute hemodynamic effects of vasodilators and the chronic antiremodeling effects of pharmacologically active agents (7, 10, 47). As expected, the expression of the differentiation marker desmin decreased during the passage of the cells, whereas α-smooth muscle actin remained constant. Along these lines, certain receptors (including IP, EP_2, EP_3, FP) have been shown to be regulated, whereas others stay constant in their expression profile. Previous *in vitro* studies have already suggested the substantial antiproliferative potency of prostacyclin analogs in human PASMCs (48). Interestingly, distal human PASMCs, isolated from pulmonary arteries (<1 mm external diameter), seem to be more susceptible to prostacyclin analog–induced inhibition of proliferation than PASMCs from proximal pulmonary arteries (>8 mm of external diameter) (19). In distal and proximal PASMCs, the expression of IP, EP_3, FP, and TP was decreased in MCT-treated rats as compared with control rats. In contrast, the EP2 and EP4 receptors were stably expressed.

In conclusion, the EP4 receptor may take over the function of the IP receptor in the remodeled vessels of pulmonary hypertensive subjects. Furthermore, the prostacyclin analog iloprost increases cAMP in smooth muscle cells by binding to the EP4 receptor. This finding provides an unrecognized mechanism for iloprost and the prospect that the EP4 receptor may be a novel therapeutic approach for the treatment of PAH.

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