# The Prostanoid EP4 Receptor in Prostacyclin Sensing by Pulmonary Arterial Smooth Muscle Cells in Monocrotaline-Induced Pulmonary Hypertension in Rats

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## IV. Abbreviations:

| | |
|---|---|
| 5-HT | 5-hydroxytryptamine |
| 5-HTT | 5-hydroxytryptamine transporter |
| AA | Amino acid |
| AC | Adenylate cyclase |
| α-SM-actin | Alpha smooth muscle actin |
| APS | Ammonium persulfate |
| ATP | Adenosine 5'-triphosphate |
| BMPs | Bone morphogenetic proteins |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| cDNA | Complementary deoxyribonucleic acid |
| cGMP | Cyclic guanosine monophosphate |
| COX-2 | Cyclooxygenase-2 |
| CRE | cAMP response elements |
| CREB | CRE binding protein |
| DEPC | Diethypyrocarbonate |
| DMSO | Dimethyl sulfoxide |
| DP | Prostaglandin D receptor |
| DTT | Dithiothreitol |
| EDTA | Ethylendinitriloprost-N,N,*N´*,*N´*,-tetra-acetate |
| EC | Endothelial cell |
| EP receptor | The prostaglandin E receptor |
| ERK | Extracellular signal-regulated kinase |
| ET | Endothelin |
| $ET_A$ | Endothelin receptor A |
| $ET_B$ | Endothelin receptor B |
| FCS | Fetal calf serum |
| FDA | Food and Drug Administration |

| | |
|---|---|
| FP | Prostaglandin F receptor |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| Gi | Inhibitory adenylate cyclase g protein |
| Gq | Guanine nucleotide binding protein, q polypeptide |
| GPCR | G-protein coupled receptor |
| Gs | Stimulating adenylate cyclase g protein |
| HEPES | 2-(-4-2-hydroxyethyl)-piperazinyl-1-ethansulfonate |
| HIV | Human immunodeficiency virus |
| HRP | Horseradish peroxidase |
| IBMX | 3-isobutyl-1-methylxanthine |
| ICC | Immunocytochemistry |
| IHC | Immunohistochemistry |
| Ilo | Iloprost |
| INO | Inhaled NO |
| IPAH | Idiopathic pulmonary arterial hypertension |
| IP receptor | Prostacyclin receptor or prostaglandin I receptor |
| JNK | c-Jun N-terminal kinase |
| Kv | Voltage-gated potassium channels |
| MAP kinases | Mitogen-activated protein kinases |
| MCT | Monocrotaline |
| MCT28d rat | Monocrotaline–induced rat 28 day |
| MMP | Matrix metalloproteinase |
| mRNA | Messenger ribonucleic acid |
| NEST | Nuclear envelope signal transduction |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NYHA | New York Heart Association |
| OD | Optical density |
| ODC | Ornithine decarboxylase |

| | |
|---|---|
| PAH | Pulmonary arterial hypertension |
| PAP | Pulmonary arterial pressure |
| PASMC | Pulmonary arterial smooth muscle cell |
| PBS | Phosphate-buffered saline |
| PBST | Phosphate-buffered saline + 0.1 % Tween 20 |
| PCR | Polymerase chain reaction |
| PDE | Phosphodiesterases |
| PDE5 | Phosphodiesterase type 5 |
| PDGF | Platelet-derived growth factor |
| $PGI_2$ | Prostacyclin, prostaglandin $I_2$ |
| PGD | Prostaglandin D |
| PGE | Prostaglandin E |
| PGI | Prostaglandin I |
| PGIS | $PGI_2$ synthase |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PKG | Protein kinase G |
| PMSF | Phenylmethylsulfonylfluoride |
| PPAR | Peroxisome proliferator-activated receptor |
| PPRE | PPAR-responsive element |
| PVR | Pulmonary vascular resistance |
| RIA | Radioimmunoassay |
| RT-PCR | Reverse transcription PCR |
| SD | Sprague-Dawley |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | SDS polyacrylamide gel electrophoresis |
| SLE | Systemic lupus erythematosus |
| SMA | Smooth muscle actin |
| TAE | Tris-acetate-EDTA |

| | |
|---|---|
| TCA | Trichloroacetic acid |
| TGF-β | Transforming growth factor-beta |
| TIE2 | The receptor for angiopoietin-1 |
| TE | Tris EDTA |
| TEMED | *N*,*N*,*N'*,*N'*-tetramethyl-ethane-1,2-diamine |
| Trep | Treprostinil |
| TP | Thromboxane A receptor |
| VIP | Vasoactive intestinal peptide |
| VEGF | Vascular endothelial growth factor |
| VSMCs | Vascular smooth muscle cells |
| V/V | Volume per total volume |
| W/V | Weight per total volume |

# 1. Introduction

In pulmonary hypertension associated with chronic pulmonary arterial disease, a key pathological characteristic is narrowing of the lumen of the pulmonary arteries. Prostacyclin and its analogs, such as iloprost, have been shown to extend the survival of patients with pulmonary arterial hypertension (PAH) inhaled iloprost is the treatment of choice for pulmonary hypertension. It is not only convenient, but also reduces the infection risk associated with intravenous infusion. Iloprost acts through elevation of cAMP levels which occur after binding to the prostacyclin receptor (IP receptor). However, recent evidence has suggested that the lungs of some patients with pulmonary hypertension exhibit decreased expression of the IP receptor. The mechanism of action of prostacyclin analogs in pulmonary hypertension have not been elucidated, therefore, it is not known whether the effects of prostacyclin are mediated by a single prostanoid receptor pathway, or operate by various prostanoid receptors or non-prostanoid receptor pathways. Therefore, the major hypothesis in my thesis is "prostanoid receptors other than the IP receptor are involved in the signal transduction induced by prostacyclin".

**The literature section of this thesis will summarize** the following: 1) The pathophysiology of pulmonary arterial hypertension 2) The cellular changes associated with pulmonary arterial hypertension 3) Prostacyclin therapy for pulmonary hypertension 4) Prostacyclin signal transduction, focusing on the prostanoid EP4 receptor. 5) Signaling mechanisms of prostacyclin: the prostanoids receptor and peroxisome proliferator-activated receptor (PPAR) 6) Animal models of PAH: monocrotaline-treated rats.

**In the methods section of this thesis,** the methods are described which were applied to investigate the mechanism of action of iloprost and the prostanoid receptors. Lung samples from pulmonary hypertension patients were examined for expression of the IP and EP4 receptors. Tissues from rats with monocrotaline-induced pulmonary hypertension were examined for the expression of prostanoid receptors by immunohistochemistry. Proximal and distal pulmonary arterial smooth muscle cells (PASMCs) were isolated and cultured in vitro study to prostanoid receptors and prostacyclin effects in PAH. To identify smooth muscle cells, specific smooth muscle markers were identified by immunocytochemistry. Protein and mRNA

were isolated from PASMC from control and monocrotaline-treated rats, and analyzed by immunoblotting and RT-PCR. A Cell proliferation assay was used to determine the appropriate dose of iloprost for the *in vitro* studies and intracellular cyclic AMP (cAMP) levels were analyzed after prostacyclin stimulation.

**In the results section,** an attempt is made to describe the prostacylin signaling pathway from the cell surface to the nucleus in PASMC from rats with monocrotaline-induced pulmonary hypertension. (1) the prostacyclin analog iloprost mediates vasodilator functions through the EP4 receptor, in the case of the low prostacyclin receptor expression associated with pulmonary hypertension. The first part of the results suggests a previously-unrecognized mechanism of action for iloprost, and the prospect that the EP4 receptor might be a novel therapeutic target for the treatment of PAH. (2) Patients with idiopathic PAH (IPAH) lack PPARs, and a similar expression pattern was observed in MCT-induced PAH. Treprostinil might be a ligand for the nuclear receptor PPARs and mediates antiremodeling effects through PPAR-α and PPAR-γ associated with PAH.

**In the discussion section of my thesis,** I discuss my work according to the two directions suggested by the results. The major focus of thesis is on the specific contribution of the EP4 receptor in iloprost-mediated signal transduction associated with PAH. In addition, it is shown that treprostinil might be a ligand for the nuclear receptor PPARs. There is also discussion of the prostacylin signaling pathway from the cell surface to the nucleus in PASMC from rats with monocrotaline-induced pulmonary hypertension.

Prostacyclin analogs are powerful vasodilators and antiproliferative agents in smooth muscle cells. The major contribution of this thesis is the identification of a previously unrecognized mechanism of action of prostacyclin analogs, and the prospect that the EP4 receptor might be a novel therapeutic target for the treatment of PAH. The major results of my thesis were published in the *Am J Respir Crit Care Med.* in July 2008.

# 2. The Review of the literature

## 2. 1. The Pathophysiology of pulmonary arterial hypertension

Pulmonary hypertension is a disease of the vasculature where the to pulmonary artery pressure rises above normal values.. Clinically defined PH requires an increase in the mean pulmonary artery pressure of more than 25 mm Hg at rest, or 30 mm Hg during exercise. The arteries in the lung create increased resistance to blood flow and blood pressure that increases the right ventricle pressure and thus, the workload of heart. The major five symptoms of pulmonary hypertension are 1.) shortness of breath with minimal exertion, 2) fatigue, 3) chest pain, 4) dizzy spells and 5) fainting. [Simonneau et al., 2004].

Pulmonary arterial hypertension has a multifaceted pathobiology. The important issue of pulmonary artery pressure rising above the normal levels can be attributed to vasoconstriction, remodeling of the pulmonary artery vessel wall, and thrombosis leading into increased pulmonary vascular resistance in PAH [Humbert et al., 2004]. The endothelial cells, smooth muscle cells and fibroblasts, as well as inflammatory cells and platelets, may play important roles in PAH. Meanwhile, several signaling pathways have been shown to be dysregulated in PAH including the following: (1) an imbalance between prostacyclin and thromboxane, as evident by reduced production of prostacyclin, mainly by down-regulation of prostacyclin synthase and increased excretion of thromboxane [Tuder et al., 1999;Christman et al., 1992]; (2) an increased expression of growth factors such as endothelin [Giaid et al., 1993] and platelet-derived growth factor (PDGF) [Humbert et al., 1998;Schermuly et al., 2005a] and (3) up-regulation of cyclic nucleotide phosphodiesterases (PDEs) such as PDE1 [Schermuly et al., 2007b], PDE3/4 [Dony et al., 2008b], and PDE5 [Schermuly et al., 2005b;Wharton et al., 2005].

### 2.1.1 The clinical classification of pulmonary arterial hypertension

**Table 1. Pulmonary Hypertension Classification System from the 2003 World Symposium on Pulmonary Hypertension [Simonneau et al., 2004]**

**1. Pulmonary arterial hypertension**
1.1. Idiopathic pulmonary arterial hypertension
1.2. Familial pulmonary arterial hypertension
1.3. Associated with pulmonary arterial hypertension
  1.3.1. Collagen vascular disease
  1.3.2. Congenital systemic to pulmonary shunts
  1.3.3. Portal hypertension
  1.3.4. Human immunodeficiency virus
  1.3.5. Drugs and toxins
  1.3.6. Other (thyroid disorders, glycogen storage disease, Gaucher's disease, hemoglobinopathies, hereditary hemorrhagic telangiectasia, myeloproliferative disease,splenectomy)
1.4. Associated with venous or capillary involvement
  1.4.1. Pulmonary veno-occlusive disease
  1.4.2. Pulmonary capillary hemangiomatosis
1.5. Persistent pulmonary hypertension of the newborn
**2. Pulmonary hypertension with left heart disease**
2.1. Left-sided atrial or ventricular heart disease
2.2. Left-sided valvular heart disease
**3. Pulmonary hypertension associated with lung disease and/or hypoxemia**
3.1. Chronic obstructive pulmonary disease
3.2. Interstitial lung disease
3.3. Sleep-disordered breathing
3.4. Alveolar hypoventilation disorders
3.5. Long-term exposure to high altitude
3.6. Developmental abnormalities
**4. Pulmonary hypertension due to chronic thrombotic/embolic disease**
4.1. Thromboembolic obstruction of proximal pulmonary arteries
4.2. Thromboembolic obstruction of distal pulmonary arteries
4.3. Nonthrombotic pulmonary embolism
**5. Miscellaneous; sarcoidosis, histiocytosis X, lymphangiomatosis, compression of pulmonary vessels (adenopathy, tumor, fibrosing mediastinitis)**

## 2.2 The cellular changes associated with pulmonary arterial hypertension

Pulmonary arterial hypertension has a complex cellular and molecular pathobiology. Vasoconstriction, remodeling of the pulmonary vessel wall, and thrombosis, contribute to increased pulmonary vascular resistance in PAH [Humbert et al., 2004]. Endothelial cells, smooth muscle cells and fibroblasts, as well as inflammatory cells and platelets, may play a significant role in PAH.

One of the major elements of PAH remodeling is **smooth muscle cell proliferation** in distal parts of pulmonary arteries. The cellular processes of this hyperproliferation are incompletely understood. In addition, a hallmark of severe pulmonary hypertension is the formation of a layer of myofibroblasts and extracellular matrix between the endothelium and the internal elastic lamina, termed the neointima. In some model systems, particularly in hypoxia models, the adventitial fibroblasts appear to be the first cells activated to proliferate and to synthesize matrix proteins in response to the pulmonary hypertensive stimulus [Stenmark et al., 2002]. **Disorganized endothelial cell** proliferation, leading to the formation of plexiform lesions is described in many cases of PAH [Cool et al., 1999;Voelkel and Cool, 2004]. In response to hypoxia, shear stress, inflammation, or drugs or toxins, endothelial cells may react in various ways, affecting the process of vascular remodeling. Injury can alter not only cell proliferation and apoptosis but also homeostatic functions of the endothelium (including coagulation pathways, and the production of growth factors and vasoactive agents). Endothelial cells also express markers of angiogenesis, such as vascular endothelial growth factor (VEGF) and its receptors in PAH [Cool et al., 1999]. In addition, cells comprising plexiform lesions of idiopathic PAH are monoclonal in origin. Therefore, although the lesions themselves are probably hemodynamically irrelevant, they may represent more than simply the result of severe elevation of intravascular pressures [Lee et al., 1998]. Moreover, several factors, including transforming growth factor-beta (TGF-β) receptor-2 and the apoptosis-related gene, Bax [Yeager et al., 2001] are downregulated in 90% of plexiform lesions while abundant expression was observed in endothelial cells outside these lesions. Human herpesvirus 8 infection may also contribute to the growth of monoclonal endothelial cells in plexiform lesions from patients with idiopathic PAH [Yeager et al., 2001;Cool et al., 2003]. These

findings suggest that triggers, including vasculotropic viruses, can stimulate the growth of endothelial cells by dysregulating cell growth or growth factor signaling.

The mechanisms that enable the **adventitial fibroblasts** to migrate into the media (and ultimately into the intima) are currently unclear, but there is good evidence to suggest that upregulation of matrix metalloproteinases (MMP2 and MMP9) occurs, and that these molecules are involved in migration. This neovascularization occurs primarily in the adventitia, and then it extends into the outer parts of the media. This adventitial vessel formation could provide a factor for circulating progenitor cells to access the vessel wall from the adventitial side. It is unknown whether circulating progenitor cells derived from the bone marrow contribute directly to the adventitial thickening (and perhaps medial thickening), or whether bone marrow-derived progenitor cells simply enhance the proliferative and migratory activity of the local adventitial fibroblasts. Significant attention in the future will have to be focused on the role of circulating precursor cells to vascular remodeling [Davie et al., 2004].

**Thrombotic lesions and platelet dysfunction** are potentially important processes in PAH [Herve et al., 2001]. Biological evidence shows that intravascular coagulation is a continuous process in PAH patients, characterized by elevated plasma levels of fibrinopeptide A- and D-dimers. In addition, procoagulant activity and fibrinolytic function of the pulmonary endothelium are altered in PAH. Evidence also exists to suggest that enhanced interactions between platelets and the pulmonary artery wall may contribute to the functional and structural alterations of pulmonary vessels. Vascular abnormalities in PAH may lead to release by platelets of various procoagulant, vasoactive, and mitogenic mediators. Indeed, in addition to its role in coagulation, the platelet stores and releases important contributors to pulmonary vasoconstriction and remodeling such as thromboxane A2, platelet-activating factor, serotonin (5-hydroxytryptamine [5-HT]), platelet-derived growth factor (PDGF), TGF-β, and VEGF. However, it remains unclear whether thrombosis and platelet dysfunction are causes or consequences of the disease [Herve et al., 2001].

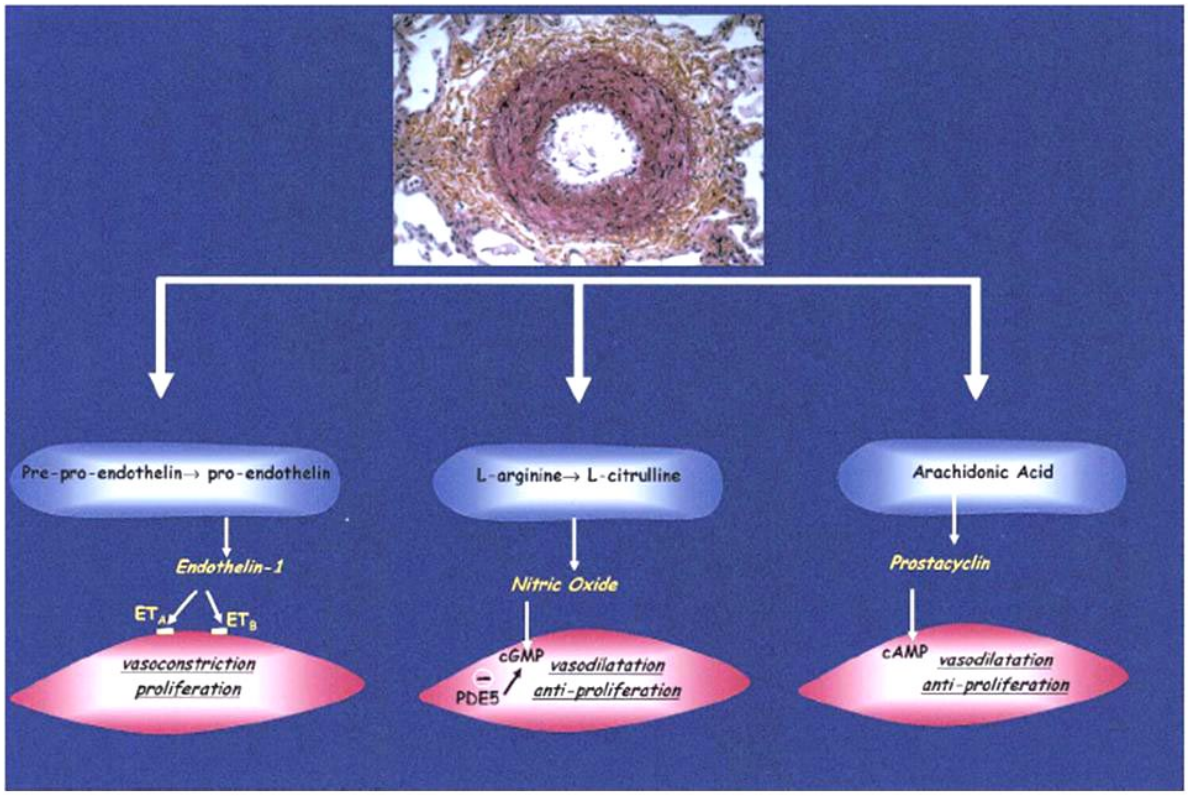


**Figure 1. Consequences of pulmonary arterial endothelial cell dysfunction on pulmonary artery smooth muscle reaction [Humbert et al., 2004].**

Dysfunctional pulmonary artery endothelial cells (blue) have decreased production of prostacyclin and nitric oxide, with an increased production of endothelin-1 promoting vasoconstriction and proliferation of pulmonary artery smooth muscle cells (red). cAMP = cyclic adenosine monophosphate; cGMP = cyclic guanosine monophosphate; ET = endothelin; $ET_A$ = endothelin receptor A; $ET_B$ = endothelin receptor B; PDE5 = phosphodiesterase type 5.

## 2.3 Treatment strategies using prostacyclins in pulmonary hypertension

Pulmonary arterial hypertension (PAH) has a multifaceted pathobiology. The important issue of pulmonary artery pressure rising above normal level is attributed to vasoconstriction, remodeling of the pulmonary vessel wall and thrombosis, leading to increased pulmonary vascular resistance in PAH [Humbert et al., 2004]. Different signal pathways have been shown to be dysregulated in PAH, including the following: (1) an imbalance between prostacyclin and thromboxane, as evident by a reduced production of prostacyclin, mainly by down-regulation of prostacyclin synthase and increased excretion of thromboxane [Tuder et al., 1999;Christman et al., 1992]; (2) an increased expression of growth factors such as endothelin [Giaid et al., 1993] and PDGF [Humbert et al., 1998;Schermuly et al., 2005a] and (3) up-regulation of cyclic nucleotide PDEs such as PDE1 [Schermuly et al., 2007b], PDE3/4 [Dony et al., 2008a], and PDE5 [Schermuly et al., 2005b;Wharton et al., 2005]. In this thesis, I focus on prostacyclin sensing in pulmonary arterial smooth muscle cells from rats with monocrotaline-induced pulmonary arterial hypertension [Lai et al., 2008].

Prostacyclin is an important endogenous pulmonary vasodilator, acting through activation of cAMP-dependent pathways. Prostacyclin also inhibits the proliferation of vascular smooth muscle cells and decreases platelet aggregation. Prostacyclin synthesis is decreased in endothelial cells from PAH patients. Analysis of urinary metabolites of prostacyclin showed a decrease in the amount of excreted 6-ketoprostaglandin F1$\alpha$, a stable metabolite of prostacyclin, in patients with idiopathic PAH [Christman et al., 1992]. Moreover, endothelial cells of PAH patients are characterized by reduced expression of prostacyclin synthase [Tuder et al., 1999], and prostacyclin therapy has been shown to improve hemodynamics, clinical status, and survival of patients displaying severe PAH.

Prostaglandins (prostaglandin I 2 ($PGI_2$) and prostaglandin E-1 ($PGE_1$)) are naturally occurring prostanoids that are endogenously produced as metabolites of arachidonic acid in the vascular endothelium [Kerins et al., 1991]. In vascular smooth-muscle cells, prostaglandin stimulates adenylate cyclase which converts adenosine triphosphate to cyclic adenosine monophosphate (cAMP). Thus, protein kinases mediate a cAMP-induced decrease in

intracellular calcium leading to relaxation and vasodilation [Badesch et al., 2004]. Both $PGI_2$ and $PGE_1$ are potent pulmonary vasodilators and inhibitors of platelet aggregation. A deficiency in endogenous prostacyclin may be a contributing factor to the pathogenesis of some forms of PAH [Christman et al., 1992]. In addition, there is evidence that the lungs of PAH patients have decreased expression of the IP receptor [Hoshikawa et al., 2001]. Clinical studies have focused on the potential benefit of long-term supplementation of exogenous $PGI_2$. Several prostacyclin analogs, administrated through different routes, are currently available for the treatment of PAH. Epoprostenol, a short-acting $PGI_2$ analog, improved hemodynamic function, exercise capacity, and survival in patients, but the problems and adverse effects related to this treatment are due primarily to complicated delivery system and characteristics of the drug. Pain and infection associated with the long-term presence of an indwelling intravenous catheter are common. Furthermore, epoprostenol has a short half-life (3–6 min) [Barst et al., 1996]. Therefore, stable long-acting prostacyclin analogs can resolve some of these problems and improve the prospects of long-term pulmonary vasodilator therapy.

Iloprost is the first $PGI_2$ analog that is FDA approved for the treatment of PAH through direct pulmonary delivery by aerosol inhalation. Iloprost is a stable $PGI_2$ analog, with a half-life of 20-30 min and duration of effect up to 120 min using a specified breath-actuated nebulizer system [Olschewski et al., 1996]. In a randomized controlled trial, inhaled doses of 2.5-5.0 g administered six to nine times daily improved functional classification, exercise tolerance, and quality of life [Olschewski et al., 2002]. Inhaled iloprost has been shown to be effective for the treatment of PAH and may provide an alternative to the use of intravenous epoprostenol. When the clinical effects of inhaled iloprost and intravenous epoprostenol are compared, iloprost inhalation has clear advantages but also certain drawbacks. Most importantly, inhalation provides potent pulmonary vasodilatation with minimal systemic side effects, and no risk of catheter-related complications. However, inhaled iloprost last only 30 to 90 min, and thus six to nine inhalations are needed to achieve good clinical results. Treprostinil is another long-acting stable $PGI_2$ analog, with a duration of action up to four hours, and is FDA approved for subcutaneous infusion. The safety and effectiveness of treprostinil were demonstrated in smaller clinical trials and one large randomized, controlled trial with 470

patients [Simonneau et al., 2002]. Improvement in exercise capacity, improved indices of dyspnea, a reduction in signs and symptoms of pulmonary hypertension, and improved hemodynamics were noted in the patients who received subcutaneous treprostinil [Simonneau et al., 2002]. In addition, the patients experienced improved functional classification and exercise tolerance, without reported adverse effects [Voswinckel et al., 2006]. An inhaled liposomal treprostinil formulation that may improve therapeutic response is also currently undergoing pre-clinical trials [Dhand, 2004].

## 2.4 Prostacyclin signal transduction

### 2.4.1 Molecular characteristics of prostanoid receptors

Cyclooxygenases metabolize arachidonate to five primary prostanoids: $PGE_2$, $PGF_{2\alpha}$, $PGI_2$, $TxA_2$, and $PGD_2$ [Breyer et al., 2001;Needleman et al., 1986]. Prostanoids that consist of the prostaglandins (PG) and the thromboxanes (Tx) are cyclooxygenase products derived from C-20 unsaturated fatty acids (Figure 2). These autocrine lipid mediators interact with specific members of a family of distinct G-protein-coupled prostanoid receptors, which divide into five subtypes (EP1-4, FP, IP, TP, and DP) [Breyer et al., 2001;Negishi et al., 1995]. In addition, the eight subtypes of prostanoid receptors are each encoded by an individual gene. Phylogenetic analyses indicate that receptors sharing a common signaling pathway have higher sequence homology than receptors sharing a common prostanoid as their preferential ligand. The effects of prostanoid receptors on smooth muscle reflect this relationship. Thus $EP_2$, $EP_4$, DP, and IP induce smooth muscle relaxation and are more closely related to each other than to the other prostanoid receptors. Similarly, $EP_1$, FP, and TP receptors cause smooth muscle contraction and form another group based on sequence homology. The $EP_3$ receptors usually stimulate smooth muscle contraction and define a third group. On the basis of these phylogenetic analyses, it has been suggested that the COX pathway may have evolved from $PGE_2$ and an ancestral EP receptor [Narumiya et al., 1999]. The evolution of the different EP receptor types from this ancestral prostanoid receptor would have linked $PGE_2$ to different signal transduction pathways. The receptors for the other prostanoids might then have evolved by gene

duplication of these different EP receptor subtypes [Narumiya et al., 1999]. Alternative splicing of the exon encoding the seventh transmembrane domain occurs at a position approximately 9-12 amino acids into the carboxy terminus of the $EP_3$, FP, and TP receptors of various species. The rat $EP_1$ receptor is also subject to alternative splicing but instead diverges midway into the sixth transmembrane domain. The variant form ($rEP_{1\text{-variant}}$) contains none of the amino acids that are highly conserved within the seventh transmembrane domain of the other prostanoid receptors. Generally, prostanoid receptor isoforms exhibit similar ligand binding but differ in their signal pathways, their sensitivity to agonist-induced desensitization, and their tendency toward constitutive activity, as will be discussed in the next section. While there is homology between the $EP_3$ receptor isoforms of different species, the human and mouse TP receptor isoforms demonstrate no homology. This may be indicative of other TP isoforms [Narumiya et al., 1999]. The receptors that are subject to alternative splicing ($EP_1$, $EP_3$, FP, and TP) are phylogenetically related, perhaps suggesting the evolutionary conservation of the sequence(s) involved in this process [Narumiya et al., 1999;Pierce and Regan, 1998].

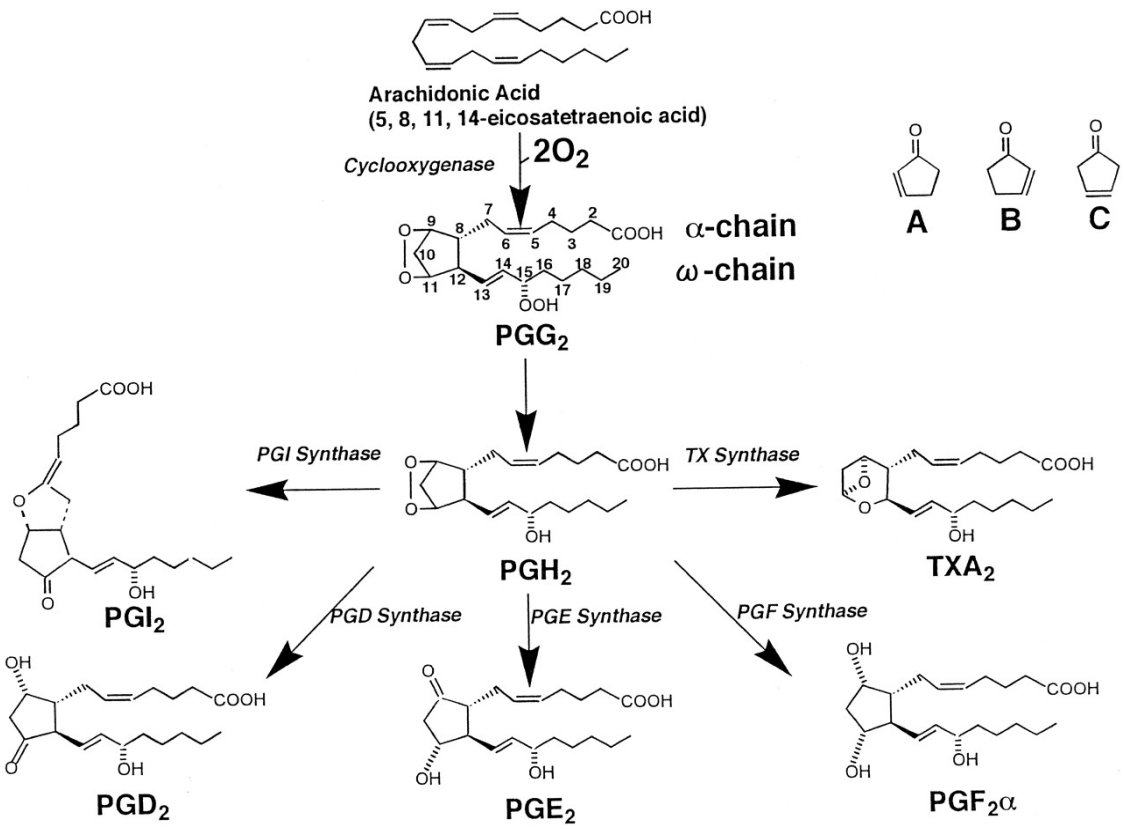


**Figure 2. Biosynthetic pathways of prostanoids [Narumiya et al., 1999]**

Formation of series 2 prostaglandins (PG), $PGD_2$, $PGE_2$, $PGF_{2\alpha}$, $PGG_2$, $PGH_2$, and $PGI_2$, and a thromoboxane (Tx), $TxA_2$, from arachidonic acid is shown.

The first two steps of the pathway, (conversion of arachidonic acid to $PGG_2$ and then to $PGH_2$), are catalyzed by cyclooxygenase, and subsequent conversion of $PGH_2$ to each PG is catalyzed by respective synthase as shown.

Ring structures of A, B, and C types of PG are shown separately.

### 2.4.2 Prostanoid signal transduction in smooth muscle cells

Signal transduction pathways of prostanoid receptors have been studied by examining agonist-induced changes in the levels of second messengers (cAMP, free $Ca^{2+}$, and inositol phosphates), and by identifying G protein coupling by various methods. These results are summarized in Table 2. Prostanoid receptors sharing a common signal pathway have higher sequence homology than do receptors sharing a common prostanoid as their preferential ligand. Thus three groups of related receptors have been defined: 1) DP, IP, $EP_2$, and $EP_4$; 2) $EP_1$, FP, and TP; and 3) $EP_3$[Wright et al., 2001].

Prostanoid receptors in group 1) are linked to heterotrimeric G proteins that are composed of a $G_\alpha$-subunit that stimulates adenylate cyclase to produce cAMP. An increase in intracellular cAMP concentration is observed after stimulation of the recombinant human DP [Boie et al., 1995], IP [Boie et al., 1994;Nakagawa et al., 1994], $EP_2$ [Regan et al., 1994] and $EP_4$ [Wright et al., 2001] receptors, in addition to their species homologs. The results obtained with recombinant receptors corroborated those obtained previously in isolated tissues. For instance, prostaglandin D (PGD)-, prostaglandin E (PGE)-, and prostaglandin I (PGI)-responsive receptors cause the stimulation of cAMP production in platelets and in the vasculature [Hardy et al., 1998]. However, the recombinant human IP receptor can also mediate inositol phosphate production and increases in free $Ca^{2+}$ levels by coupling with $G_{\alpha q}$ [Namba et al., 1994]. Likewise, $EP_2$, $EP_4$, and DP receptors in choroid tissue do not couple to adenylate cyclase, but rather to eNOS; this may be evoked by $G_{\beta\gamma}$ action on phosphatidylinositol 3-kinase, which in turn activates, sequentially, protein kinase B (PKB) and eNOS [Wright et al., 2001].

Prostanoid receptors in group 2) couple to increases in intracellular free $Ca^{2+}$ through the activation by $G_{\alpha q}$ of phospholipase C, with subsequent inositol phosphate liberation. This pathway has been demonstrated for FP using anti-$G_{\alpha q}$ antibodies, which corroborates earlier results demonstrating inositol phosphate turnover in isolated luteal cells on $PGF_{2\alpha}$ administration. In the case of TP, $G_{\alpha q}$ activation is the primary effector pathway as shown during stimulation of native TP receptors in platelets [Wright et al., 2001;Namba et al., 1994;Shenker et al., 1991]. However, the previously described TP receptor splice variants TP and $TP_\beta$ also can signal through $G_{i\alpha}$ and $G_{s\alpha}$ to inhibit and stimulate adenylate cyclase,

respectively [Hirata et al., 1996]. The $EP_1$ preferentially couples to $G_{\alpha q}$. An increase in inositol phosphate after its stimulation in brain and ocular vasculature is clearly observed [Wright et al., 2001].

The $EP_3$ subtypes constitute group 3) of the prostanoid receptor family, and employ as their primary effector pathway the inhibition of adenylate cyclase through the $G_{i\alpha}$ -family [Negishi et al., 1988]. However, the molecular cloning of the bovine $EP_3$ receptor splice variants demonstrates the array of second messengers to which these receptors are coupled. Four subtypes of bovine $EP_3$ have been cloned (designated A, B, C, and D), and all show identical agonist binding properties [Namba et al., 1993]. However, $EP_{3A}$ acts through $G_{i\alpha}$ to inhibit adenylate cyclase, $EP_{3B}$ and $EP_{3X}$ signal through $G_{s\alpha}$ to activate adenylate cyclase, and $EP_{3D}$ is coupled to $G_{\iota\alpha}$, $G_{\sigma\alpha}$, and $G_{\alpha\theta}$, resulting in the inhibition and activation of adenylate cyclase as well as the activation of phospholipase C. Alternatively, nuclear $EP_{3\alpha}$ receptors seem to be G protein dependent but not coupled to adenylate cyclase or phospholipase C activation [Bhattacharya et al., 1999]. A novel type of G protein regulation has also been reported for the $EP_{3B}$ and $EP_{3X}$ receptors**.** In addition to their stimulatory effects on $G_s\alpha$, they are thought to negatively regulate G protein activity by specifically inhibiting the GTPase activity of $G_\alpha$, a member of the $G_{i\alpha}$-family[Negishi et al., 1993]. Along the same lines, $EP_{3D}$-induced ductus arteriosus relaxation is pertussis toxin-, NO-, and endothelin- insensitive but if dependent on ATP-sensitive potassium channel activation [Bouayad et al., 2001]; while the mechanisms remain to be elucidated, direct receptor-channel interaction is a possibility. The $EP_3$ receptor subtypes may also differ in their levels of constitutive activity, the agonist-independent activity of the receptor [Wright et al., 2001].

**Table 2. Signal transduction of prostanoid receptors** [Narumiya et al., 1999]

Data obtained from receptors of various species are summarized, and representative signal transduction of each receptor is shown. PI, phosphatidylinositol; ↑, increase; ↓, decrease

| Type | Subtype | Isoform | G Protein | Second Messenger |
|---|---|---|---|---|
| DP | | | $G_s$ | cAMP↑ |
| EP | $EP_1$ | | Unidentified | $Ca^{2+}$↑ |
| | $EP_2$ | | $G_s$ | cAMP↑ |
| | $EP_4$ | | $G_s$ | cAMP↑ |
| | $EP_3$ | $EP_{3A}$ | $G_i$ | cAMP↓ |
| | | $EP_{3B}$ | $G_s$ | cAMP↑ |
| | | $EP_{3C}$ | $G_s$ | cAMP↑ |
| | | $EP_{3D}$ | $G_i$, $G_s$, $G_q$ | cAMP↓, cAMP↑, PI response |
| FP | | | $G_q$ | PI response |
| IP | | | $G_s$, $G_q$ | cAMP↑, PI response |
| TP | | TPα | $G_q$, $G_i$ | PI response, cAMP↓ |
| | | TPβ | $G_q$, $G_s$ | PI response, cAMP↑ |

**The prostanoid receptors are classified into**

**DP: Prostaglandin D receptor**
**EP (EP1-4): Prostaglandin E receptor**
**TP: Thromboxane receptor**
**IP: Prostacyclin receptor**
**FP: prostaglandin F receptor**

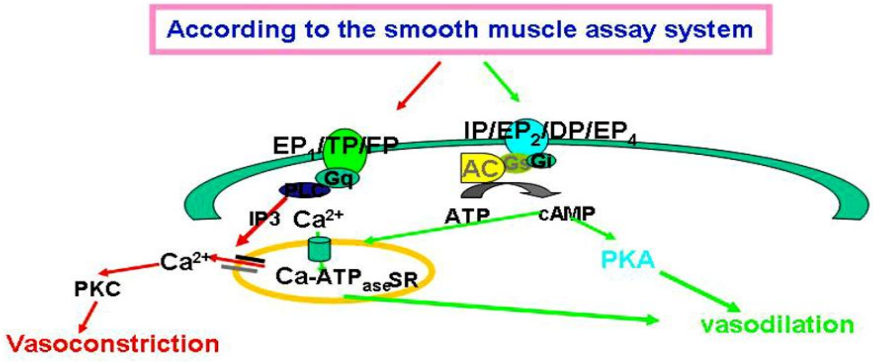


**Figure 3** Major signal transduction pathways in vascular smooth muscle cells.

Receptors for vasodilatory prostaglandins are coupled to different intracellular signaling cascades via different G-proteins. At least three transduction systems are involved: Gs- or Gi-coupled control of adenylate cyclase activity, Gq-coupled activation of phospholipase C (PLC) which inducesphospholipid breakdown and generates the signal molecules $IP_3$ and diacylglycerol and results in $Ca^{2+}$-mobilisation.

### 2.4.3 The Prostanoid EP4 receptor

The prostanoid receptors classification in the early literature somewhat confuses the molecular identities of the prostanoid EP2 receptor (EP2 receptor) and the prostanoid EP4 receptor (EP4 receptor). After 1995, the EP4 receptor was defined more clear [Nishigaki et al., 1995;Breyer et al., 2001;Wilson et al., 2004]. The human $EP_4$ receptor cDNA encodes a 488 amino acid polypeptide with a predicted molecular mass of ~78 kDa. The $EP_4$ receptor mRNA is widely distributed, with a major species of ~3.8 kb detected by Northern analysis in different tissues, such as lung, adrenal, and kidney tissues [Sando et al., 1994;Breyer et al., 1996]. Important vasodilator effects of $EP_4$ receptor activation have been described in venous and arterial beds through increased cAMP production [Coleman et al., 1994b;Coleman et al., 1994a].

A particular role for the $EP_4$ receptor in regulating the pulmonary ductus arteriosus has also been suggested by the recent studies in mice harboring a targeted disruption of the $EP_4$ receptor gene [Segi et al., 1998;Nguyen et al., 1997]. The $EP_4$ receptor has a preference for analogs with a C-1 carboxylate that is >50-fold higher than that observed for the corresponding methyl ester [Abramovitz et al., 2000;Breyer et al., 1996;Breyer et al., 2001], and $EP_4$ receptor may be pharmacologically distinguished from the $EP_1$ and $EP_3$ receptor by the $EP_4$ receptor insensitivity to sulprostone [Abramovitz et al., 2000;Breyer et al., 1996], and from $EP_2$ receptors by $EP_4$ insensitivity to butaprost and relatively selective activation by $PGE_1$-OH [Kiriyama et al., 1997;Boie et al., 1997]. Currently, pharmacological researchs on piglet saphenous veins reveal that they contain multiple relaxatory prostanoid receptors, and suggesting that IP receptor agonists are also prostanoid EP4 receptor agonists [Wilson and Giles, 2005]. Iloprost and cicaprost are effective agonists of the human prostanoid EP4 receptor. The pharmacological agonist binding data reveal hight binding of iloprost ($pK_i$=6.6) and cicaprost ($pK_i$=7.4) to the EP4 receptor and lower affinity binding to the EP2 receptor($pK_i$=5.9, <5.9, respectively). Therefore, $PGI_2$ is an agonist of human EP4 receptors [Wilson et al., 2004].

The structural difference between the two $G_{\alpha s}$-coupled EP receptors is the length of the C-terminal tail: the $EP_4$ receptor has a long (156 amino acid residue) C-terminal sequence

and contains 38 serine and threonine residues that might serve as multiple phosphorylation sites, whereas the $EP_2$ receptor has a shorter tail sequence. The $EP_4$ receptor was found to undergo rapid agonist-induced desensitization, whereas the $EP_2$ receptors did not [Nishigaki et al., 1996]. Similarly, EP4 receptors were rapidly internalized, but $EP_2$ receptors did not [Desai et al., 2000]. The $EP_4$ receptors would be a target for agonist-dependent phosphorylation and desensitization [Bastepe and Ashby, 1999;Bastepe and Ashby, 1997]. The $EP_4$ receptors may play variable physiologic roles based on the persistence of the signal generated by the receptor upon ligand activation.

The signaling properties of $EP_4$ receptors are in the activation of two different pathways. The $EP_4$ receptor may activate the cAMP/PKA pathway and also there is a concomitant activation of the PI3K and ERK signaling pathways [Fujino et al., 2005]. The pathways of activation of cAMP−PKA signaling can inhibit smooth muscle cell proliferation [Indolfi et al., 1997]. In this signaling cascade, the release of $G_{\alpha s}$ after receptor stimulation leads to adenylyl cyclase (AC) activation, which leads to an increase in the intracellular cAMP levels. The subsequent activation of PKA by cAMP can result in the phosphorylation of the CRE binding protein (CREB), which is a transcription factor that interacts with CREs and is central to the regulation cAMP-responsive gene expression [Mayr and Montminy, 2001;Johannessen et al., 2004]. Cyclooxygenase-2 (COX-2) expression is regulated by cAMP. The catalytic product of COX-2 is $PGH_2$, is the immediate precursor for the biosynthesis of the prostaglandins and thromboxanes. In PASMCs, the activation of endogenous $EP_2$ and $EP_4$ prostanoid receptors can occur through an autocrine signaling pathway [Bradbury et al., 2003]. Interestingly, recent evidence suggests that the lungs of some patients with pulmonary hypertension exhibit decreased expression of the IP receptor [Lai et al., 2008]. The mechanisms of action of prostacyclin analogs in pulmonary hypertension are not yet clear. Whether they activate only a single prostanoid receptor pathway, or operate through multiple prostanoid receptors or non-prostanoid receptor pathways is not known. Many data have shown that prostacyclin analogs are also agonists of human EP4 receptors. The signaling mechanisms of EP4 are thus very complex, and require further analysis.

### 2.4.4 Intracellular trafficking of prostanoids receptors

The biological actions of $PGE_2$ are thought to result from its interaction with plasma membrane G protein-coupled receptors termed EP, which include the $EP_1$, $EP_2$, $EP_3$, and $EP_4$ subtypes [Coleman et al., 1994b]. The most well-known signal transduction pathways of prostacyclin agonists are mediate by prostanoids receptors on the cell surface. The receptors for vasodilatory prostaglandins are coupled to different intracelluarlar signaling cascades via different G-proteins to act on the cAMP-dependent pathways [Schrör and Weber, 1997;Breyer et al., 2001]. Recent data have implied that GPCRs transducer signals not only through secondary messengers, but also through agonist-induced receptor endocytosis [Breyer et al., 2001;Zhang et al., 1999;Tsao et al., 2001].

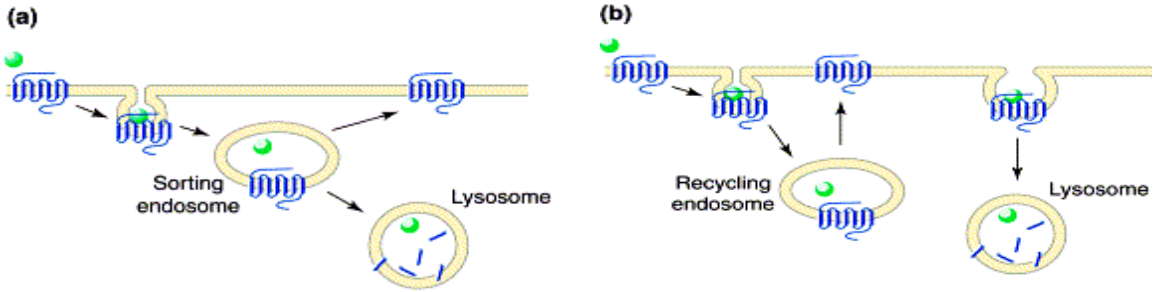


**Figure 4.** The membrane pathway mediating rapid and reversible internalization (sequestration) of G-protein-coupled receptors (GPCRs) might be related to the membrane pathway mediating GPCR trafficking to lysosomes in two principal ways [Tsao et al., 2001].
(a) GPCRs could follow divergent pathways after endocytosis by a common membrane mechanism. This hypothesis suggests that distinct GPCRs are sorted between divergent downstream trafficking pathways after endocytosis.
(b) The membrane pathway mediating rapid and reversible internalization of GPCRs might be completely separate from the pathway mediating receptor trafficking to lysosomes.
This suggests that GPCRs are sorted before endocytosis, such as by physical segregation of receptors in distinct microdomains of the plasma membrane which later endocytose.

It is usually assumed that the signal transduction cascades are initiated at the plasma membrane, and not the nuclear membranes. However, some studies have revealed that, EP3, EP4 receptors are present in nuclear envelope [Bhattacharya et al., 1998;Bhattacharya et al., 1999]. The nuclear membrane contains high levels of cyclooxygenase-1 and -2 and of $PGE_2$ [Spencer et al., 1998]. Cytosolic phospholipase $A_2$ undergoes a calcium-dependent translocation to the nuclear envelope [Schievella et al., 1995], and COX-2 translocates to the nucleus in response to certain growth factors [Coffey et al., 1997]. It is thus possible that prostanoids may induce some of their effects via intracellular EP receptors, to have a direct nuclear action [Goetzl et al., 1995;Morita et al., 1995]. Several studies have revealed that the nuclear envelope plays a major role in signal transduction cascades [Malviya and Rogue, 1998;Nicotera et al., 1989]. In fact, a nuclear lipid metabolism that is a part of unique nuclear signaling cascade termed NEST (nuclear envelope signal transduction) [Baldassare et al., 1997]. Both heterotrimeric and low molecular weight G proteins [Baldassare et al., 1997;Saffitz et al., 1994], phospholipase C [Malviya and Rogue, 1998], phospholipase D [Baldassare et al., 1997], and adenylate cyclase [Lepretre et al., 1994] can be localized at the nucleus. Evidence exists the demonstrate EP3, and $EP_4$ receptors in the nuclear envelope, and reveals that these receptors are functional, and their actions appear to involve pertussis toxin (PTX)-sensitive G proteins [Bhattacharya et al., 1999].

In conclusion, the mechanisms of action of prostacyclin analogs in pulmonary hypertension are not yet clear whether they are activated only by a single prostanoid receptor pathway, or operate via various prostanoid receptor or non-prostanoid receptor pathways. The presence of prostanoid receptors in the nuclear membrane suggests differential signaling pathways of prostacyclin actions involving both cell surface and nuclear receptors. For these reasons, it is important to investigate the regulation of prostanoid receptor intracellular trafficking and the function of nuclear prostanoid receptor in prostacyclin agonist-induced signal transduction

## 2.5 Signaling mechanisms of prostacyclin: prostanoids receptors and peroxisome proliferator-activated receptors (PPARs) in pulmonary arterial hypertension

Prostacyclin and its analogs activate G-protein-coupled cell–surface prostacyclin (IP) receptors, leading to the inhibition of smooth muscle cell proliferation [Breyer et al., 2001]. Additionally, the lungs of some patients with pulmonary hypertension have decreased expression of the IP receptor [Lai et al., 2008] and the absence of IP receptors worsens pulmonary hypertension [Hoshikawa et al., 2001]. The studies have suggested that some of these effects of prostacyclin analogs in pulmonary hypertension are mediated by nuclear receptor pathways. Data have shown that prostacyclin and its analogs can also activate the nuclear receptor family of peroxisome proliferator-activated receptors (PPARs) [Ali et al., 2006;Falcetti et al., 2007].

The PPARs are transcription factors belonging to the nuclear receptor superfamily, the three different PPAR subtypes have been identified, $PPAR_{\alpha}$, $PPAR_{\gamma}$, and $PPAR_{\delta}$. The PPAR ligands range from free fatty acids and their derivatives produced by the cyclooxygenase or lipoxygenase pathway to certain hypolipidemic drugs. The PPARs regulate gene expression by binding to the retinoid receptor RXR, and then, as a heterodimeric complex, to specific DNA sequence elements termed PPAR-responsive elements (PPREs) in the promoter regions of target genes, to regulate their expression. Fatty acid derivatives and eicosanoids have been identified as natural ligands for PPARs [Bishop-Bailey, 2000;Bishop-Bailey et al., 2002;Bishop-Bailey and Wray, 2003].

Prostacyclin ($PGI_2$) is generated from arachidonic acid by the action of the cyclooxygenase (COX) system coupled to $PGI_2$ synthase (PGIS). The presence of the COX-2/PGIS at the nuclear and endoplasmic reticular membrane suggests differential signaling pathways of $PGI_2$ actions involving both cell-surface and nuclear receptors [Liou et al., 2000;Smith et al., 1983]. The $PGI_2$ signaling through $PPAR_{\delta}$ plays an important role in embry implantation [Lim and Dey, 2002], tumourgenesis [Gupta et al., 2000], and apoptosis [Hatae et al., 2001]. Prostacyclin agonist treatment of pulmonary disease is gradually becoming being more

important [Falcetti et al., 2007;Hansmann et al., 2007;Hansmann et al., 2008] To date, studies show that $PGI_2$ agonists can regulate PPARs [Falcetti et al., 2007;Hatae et al., 2001], indicating that a signaling mechanism for this abundant eicosanoid is operative in certain systems. The $PGI_2$ agonists such as iloprost can effectively induce DNA binding and transcriptional activation by $PPAR_\alpha$ and $PPAR_\delta$ [Forman et al., 1997] but other $PGI_2$ agonists, such as cicaprost, are incapable of inducing dimerization between $PPAR_\alpha$ or $PPAR_\delta$ and the retinoid X receptor [Reginato et al., 1998]. The $PGI_2$ itself also failed to induce dimerization under these experimental conditions, possibly because the chemical instability of this PG prevents it from reaching the nuclear target. Alternatively, while cell-permeable cPGI makes its way into the nucleus more efficiently, a specific PG transporter may be required for intracellular delivery of $PGI_2$. Leukotriene $B_4$, a product of arachidonic acid generated by the lipoxygenase pathway, has also been reported as a $PPAR_\alpha$ ligand [Orie et al., 2006]. As for PPARγ, 15-deoxy-$\Delta^{12,14}$-$PGJ_2$, a $PGD_2$ metabolite, was first proposed as a ligand in an adipocyte differentiation model [Ameshima et al., 2003]. Because of the important role of $PPAR_\alpha$ and $PPAR_\gamma$ in metabolic diseases [Howard and Morrell, 2005], many synthetic ligands of PPARs are being continuously developed. However, the question of endogenous ligand utilization by these receptors remains unanswered. In addition, pulmonary hypertension researches has shown reduced expression of the PPARγ gene and protein in the lungs of patients with severe pulmonary hypertension and loss of PPARγ expression in the complex vascular lesions present in these patients. Total PPARγ mRNA was decreased in patients with severe pulmonary hypertension when compared with normal lung tissue or tissue from patients with emphysema. Thus, a lack of endothelial cell PPARγ expression may be a marker of an abnormal endothelial cell phenotype, and lack of PPARγ expression inhibits apoptosis and facilitates endothelial cell growth and angiogenesis [Ameshima et al., 2003;Hansmann et al., 2008]. The mechanisms of prostacyclin analogs in pulmonary hypertension are not yet clear. Whether they activate a single prostanoid receptor pathway, or operate via various prostanoid receptors or nuclear receptor pathways has not been defermined. These studies raise the possibility that regulation of PPARs by $PGI_2$ represent differential signaling pathways of prostacyclin actions involving both cell-surface and nuclear receptors.

## 2.6 The monocrotaline-induced animal model of pulmonary vascular diseases

Pulmonary arterial hypertension (PAH) has a multifaceted pathobiology. The important issue of pulmonary artery pressure rising above the normal levels is accredited to vasoconstriction, remodeling of the pulmonary vessel wall, and thrombosis, leading increased pulmonary vascular resistance in PAH [Humbert et al., 2004].

Monocrotaline (MCT) is a toxic pyrrolizidine alkaloid of plant origin. Injecting small doses of MCT into rats causes delayed and progressive lung injury characterized by pulmonary vascular remodeling, pulmonary hypertension, and compensatory right heart hypertrophy. The lesions induced by MCT administration in rats are similar to those observed in chronic pulmonary vascular diseases of people [Todd et al., 1985;Rabinovitch et al., 1978;Rabinovitch et al., 1979]. In a study of hypoxia-inducible factor (HIF)-1α and pulmonary hypertension. Two models were applied 1) prolonged hypoxia and 2) MCT treatment. These studies demonstrated that both hypoxia and MCT induced temporal increases in the Ppa, the ratio RV/(LV + S) and HIF-1α levels. In addition, the $PaO_2$ level significantly decreased in rats one to three weeks after MCT treatment [Lai and Law, 2004].

Structural characteristics of muscular pulmonary arteries and arterioles in two classic models of pulmonary hypertension, the rat hypoxia and monocrotaline models, have been assessed. Studies demonstrated that MCT and chronic hypoxia both induced right ventricular hypertrophy. Monocrotaline increased the medial cross-sectional area of pulmonary arteries with an external diameter of between 30-100 μm and 101-200 μm, and reduced the lumenal area of pulmonary arteries with an external diameter of 101-200 μm. Chronic hypoxia slightly increased the medial cross-sectional area without a change in the lumenal area. Both MCT and hypoxia increased the percentage of partly muscularized and fully muscularized arterioles. The MCT, in contrast to chronic hypoxia, induced structural changes to muscular pulmonary arteries with an external diameter of 101-200 μm, which may contribute to increased pulmonary arterial pressure (PAP) and right ventricular hypertrophy [Lai and Law, 2004;van Suylen et al., 1998].

In conclusion, the comparison between hypoxia- and MCT-induced remodeling demonstrates

that hypoxic vasoconstriction causes an immediate increase in PAP that is followed by vascular remodeling. In contrast, MCT primarily causes injury, induceing structural changes to the muscular pulmonary arteries which then results in an increase in PAP. In this thesis, the major interest is in the role of the prostanoid EP4 receptor in prostacyclin sensing in pulmonary arterial smooth muscle cells. For that reason, I applied the animal model of MCT-induced pulmonary hypertension in this study.

## 2.7 Aims of the work

The excessive muscularization of pulmonary arteries is the hallmark of severe pulmonary hypertension. Prostacyclin agonists are powerful vasodilators and antiproliferative agents in smooth muscle cells. However, it is not yet clear if prostacyclin analogs exert activity only by a single prostanoid receptor pathway or if they can activate multiple prostanoid receptor or non-prostanoid receptor pathways (such as PPAR pathways). Therefore, this study was divided into to two parts described below, in order to investigate the signaling pathways of prostacyclin analogs. In addition, functional experiments were performed with PASMCs from rats with MCT-induced pulmonary hypertension.

1) The major purpose of this study was to investigate whether prostanoid receptors other than the IP receptor are involved in the vasorelaxant effects of iloprost, and the role of the prostanoid EP4 receptor in prostacyclin sensing by PASMC in MCT-induced pulmonary hypertension in rat. This aspect of the thesis has been published in *Am J Respir Crit Care Med.* 2008 Jul 15;178(2):188-96.

2) There are multiple signaling possibilities for prostacyclin. Stimulation by the prostanoid pathway is cell specific, depending not only on the ability of prostacyclin to activate the cell surface prostacyclin receptor, but also on its ability to act intracellularly via the nuclear peroxisome proliferator-activated receptors (PPARs). The second direction study of this thesis was an investigation of prostacyclin analog activity via PPARs, a non-prostanoid receptor pathway, in PASMCs from MCT-induced pulmonary hypertension.

# 3. Methods

## 3.1 Patient characteristics and measurements.

Human lung tissue was obtained from three donors and three idiopathic pulmonary arterial hypertension (IPAH) patients undergoing lung transplantation. Lung tissue was snap-frozen directly after transplantation for mRNA and protein extraction [Schermuly et al., 2005a]. The study protocol for tissue donation was approved by the Ethics Commission of the Faculty of medicine of the Justus-Liebig- University, Giessen 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.

## 3. 2 Animal models of monocrotaline-induced pulmonary hypertension

Pulmonary hypertension is characterized by hemodynamic abnormalities such as high PAP, vascular remodeling, and right ventricular hypertrophy.

The animal model of MCT-induced pulmonary hypertension has been applied to investigate the pathological mechanisms of pulmonary hypertension [Lai et al., 2008;Schermuly et al., 2005a]. Monocrotaline, a pyrrolizidine alkaloid, is an extract from the crushed seeds of *Crotalaria spectabilis* (Figure 5), a warm-climate garden plant, and induces multi-organ toxicity, harming the kidney, heart, and liver. To induce pulmonary hypertension, adult male Sprague-Dawley (SD) rats (300–350 g) (Charles River, Sulzfeld, Germany) were randomized to two groups, receiving a single subcutaneous injection of either saline or 60 mg/kg MCT (Sigma, Germany) [Schermuly et al., 2007b]. The MCT was dissolved in 1 *N* HCl, neutralized to pH 7.4 with 0.5 *N* NaOH for subcutaneous injection. All protocols were approved by the Animal Care Committee of the University of Giessen.




**Figure 5. Seeds and the plant of *Crotalaria spectabilis.***

The animal model of monocrotaline-induced pulmonary hypertension has been applied to investigate the pathologenic mechanism of pulmonary hypertension. Monocrotaline was extracted from the seeds of *Crotalaria spectabilis*, a warm-climate garden plants which can induces multi-organ toxicity harming the kidney, heart, and liver.

### 3.3 Tissue preparative and immunohistochemistry

The lung tissues were fixed by immersion of the lungs into a 3% paraformaldehyde solution overnight. The samples were then dehydrated (automatic vacuum tissue processor, Leica TP 1050, Bensheim, Germany) and paraffin embeddied. After deparaffinisation and dehydration, trypsin 0.1% (GIBCO, Germany) was used to enhance penetration of the antibody into the sections for were immunohistochemistry. Next, the endogenous peroxidase of tissue sections was blocked with 3% hydrogen peroxide and sections washing three times in PBS. After that the section was immersed in blocking solution containing 1% bovine serum albumin (BSA) (Sigma, Germany) and 1% goat serum in PBS for 30 min. Sections were incubated with polyclonal antibodies against the prostanoid receptors, including anti-IP receptor (Acris, Germany) or anti-EP4 receptor antibody (Cayman, USA) for 1 h. The DAKO labelled streptavidian-biotin system (DAKO, Germany) was used to detect the signal, and colour development was undertaken by incubation with diaminobenzidine (DAB) substrate-chromogen for 2 min. As a negative control, 1% BSA diluted in PBS was used instead of the primary antibody [Chen et al., 2004]. The staining protocol was performed according to the DakoCytomation LSAB2 System-HRP manufacturer's instructions as follows:

1 Peroxidase block: hydrogen peroxide was applied to cover sections. Which were incubated for 5 min, and rinsed gently with distilled water and placed in fresh 1× PBS buffer.

2. Blocking: A solution containing 1% bovine serum albumin (BSA) and 1% goat serum was applied in 1× PBS for 30 min.

3 Primary antibody or negative control reagent: The primary or negative control reagent was applied to cover the specimen. After solution was applied a 1-h incubation, the section was rinsing gently with 1× PBS buffer.

4 Biotinylated link: The yellow solution was applied to cover the specimen. After 30 min, slide was rinsing as in step 3.

5 Streptavidin-HRP: The red streptavidin reagent was applied to cover the specimen, which was incubated for 30 min, and rinsed as before.

6 Substrate-chromogen solution: The DAB substrate-chromogen solutions were removed from 2-8 °C storage. The DAB solution: was prepared by adding one drop (or 20 µl) of the DAB chromogen solution per ml of substrate buffer. After 2 min incubation, the brown colour development was performed, and the section was rinsed gently with distilled water.

7 Hematoxylin counterstain: slides were immersed in the bath of hematoxylin, and incubated for two or five min, depending on the strength of hematoxylin used. Slides were rinsed in a bath of distilled water for 2 min twice.

8 Mounting: Specimens were mounted and coverslipped with an aqueous-based mounting medium.

### 3.4 Isolation and culture of pulmonary arterial smooth muscle cells

The PASMCs were isolated from SD rats twenty-eight days after MCT injection, as described previously [Schermuly et al., 2005a]. Animals were anesthetized with a mixture of ketamine and xylazine (100 mg/kg, i.p) (Pfizer, Germany). 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 (Figure 6A). Hank's balanced salt solution (HBSS) (GIBCO, Germany) was used to flush the vessel. The diameter of the distal part of the pulmonary arteries was smaller than 100 µm (Figure 6B). The intimal and adventitial 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 (Figure 6C, D). After about 72 h, smooth muscle cells started to migrate out from the small pieces of pulmonary artery. Cells were resuspended in culture medium DMEM-F12 (GIBCO, Germany), supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (PAN, Germany), 0.5 mM L-glutamine (GIBCO, Germany), and 20% fetal calf serum for subsequent culture in 6-well plate and incubated at 37 °C in 5% $CO_2$-95% air. After 24 h, 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, Germany) and desmin (Neomarkers,USA).

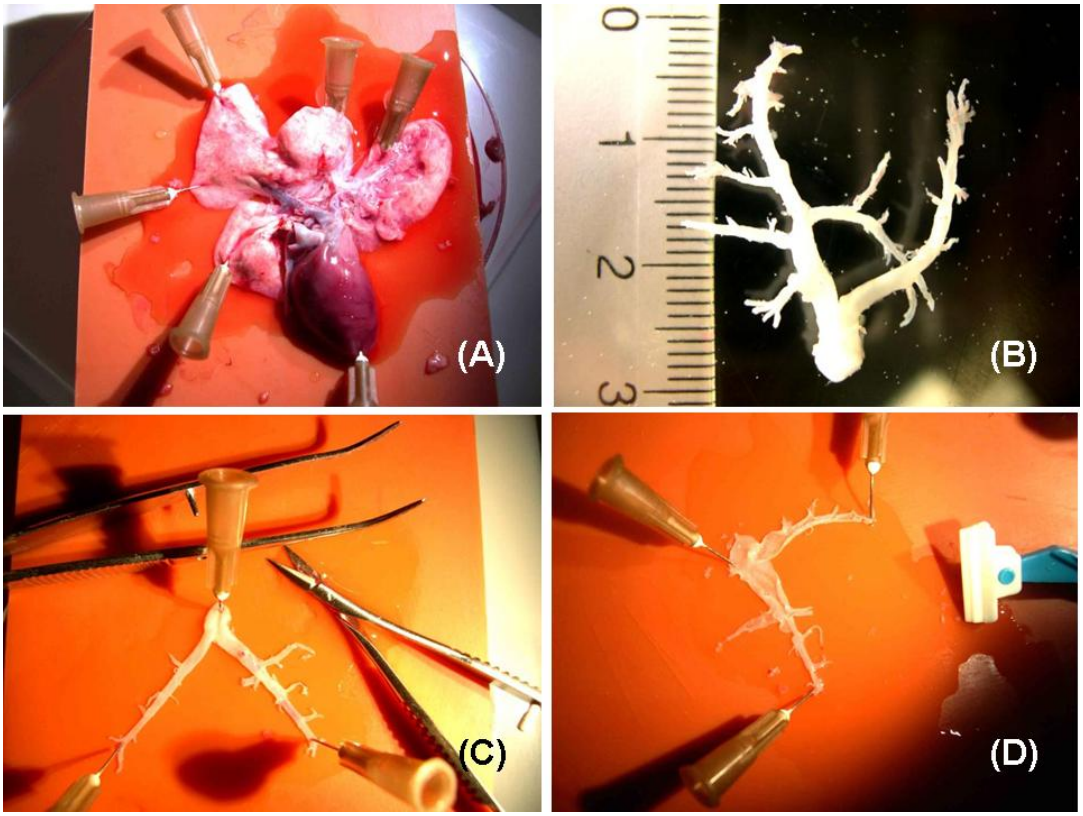


**Figure 6. Isolation of pulmonary artery smooth muscle cells from rat lung**

The main pulmonary artery was dissected free from lung and cardiac tissue, and a single full-length incision was made (A). Hank's balanced salt solution (HBSS) (GIBCO, Germany) was used to flush the arteries. The diameter of the distal part of pulmonary arteries was smaller than 100 μm (B). The intimal and adventitial 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 (C, D).

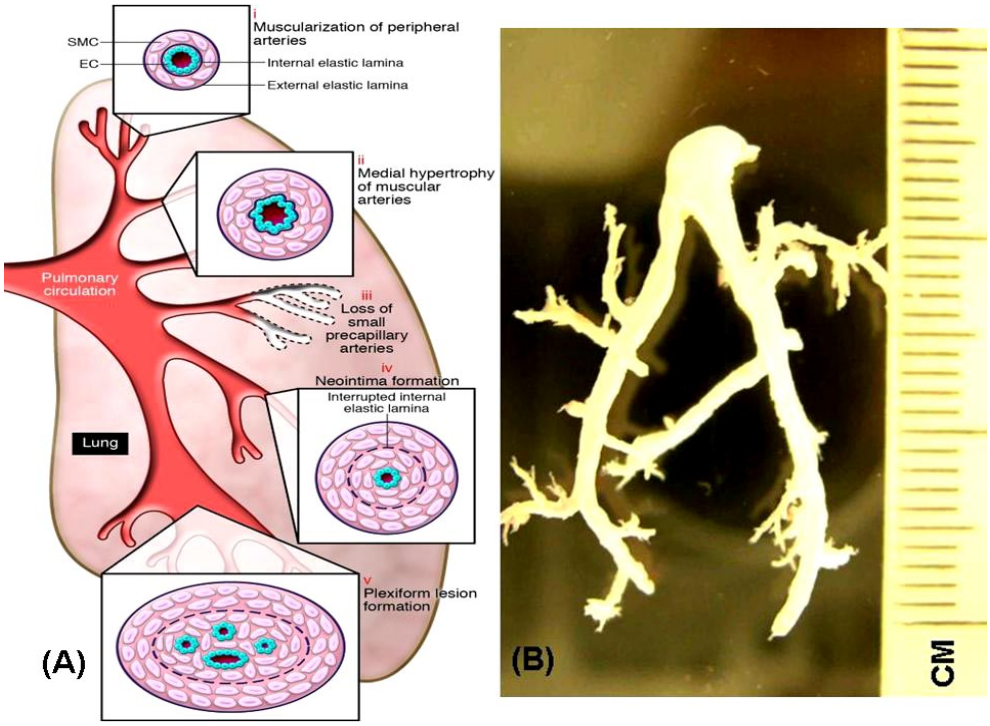


**Figure 7. Diameter of isolated pulmonary arteries from rat lungs**

(A) Pathobiology of PH. Scheme illustrating the different vascular abnormalities associated with PH compared with normal pulmonary circulation. This scheme depicts the abnormalities throughout the pulmonary circulation, including (i) abnormal muscularization of distal precapillary arteries, (ii) medial hypertrophy (thickening) of large pulmonary muscular arteries, (iii) loss of precapillary arteries, (iv) neointimal formation that is particularly occlusive in vessels 100–500 μM, and (v) formation of plexiform lesions in these vessels [Rabinovitch, 2008].

(B) Representative illustration of isolated pulmonary artery after 28 days monocrotaline injection in rats (MCT28d). The diameter of the distal portion of pulmonary arteries was smaller than 200 μm.

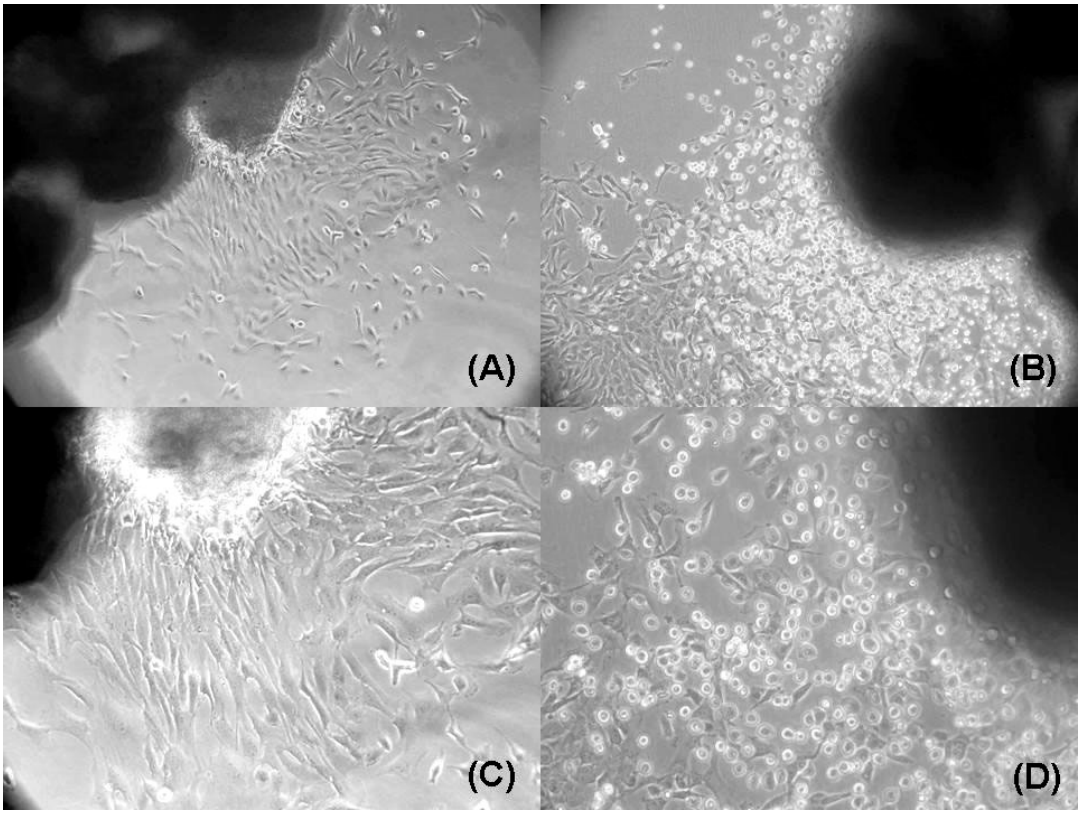


**Figure 8. Migrated cells from rat pulmonary arteries**

The main pulmonary artery was dissected free from lung and cardiac tissue.
The central pulmonary artery was then separated, and the distal arterial tissue
were then cut into small pieces and washed with HBSS.
(A) The cells migrated from control rat pulmonary artery original magnification × 100.
(B) The cells migrated from MCT28d rat pulmonary artery original magnification × 100.
(C) The cells migrated from control rat pulmonary artery original magnification × 200.
(D) The cells migrated from MCT28d rat pulmonary artery original magnification × 200.

### 3.5 Immunocytochemistry

Characterization of PASMCs was done at the primary passage using immunocytochemical staining for α-smooth muscle actin (Sigma, Germany) and desmin (Neomarkers, USA). PASMCs cultured on 1×1 mm round coverslips were fixed with 4 % paraformaldehyde for 15 min and washed with three changes of 1×PBS at room temperature. All the immunostaining procedures were carried out directly on the coverslips at room temperature. The coverslips were first immersed in blocking solution containing 1% bovine serum albumin (BSA) and 1% goat serum in PBS (Sigma, Germany) for 30 minutes. After washing three times in PBS, cells were incubated with mouse monoclonal antibodies against α-smooth muscle (Sigma, Germany) (Figure 9A) desmin (Figure 9B) (Neomarker, U.S.A.) diluted in blocking solution for 1 h α-smooth muscle actins proteins are highly expressed in smooth muscle cells.

Desmin is an intermediate filament protein expressed in both smooth and striated muscles. Antibodies to desmin react with smooth muscle cells as well as striated (skeletal and cardiac) cells. The DAKO labeled streptavidian-biotin system was used to detect the signal and color development was performed by incubation with DAB substrate-chromogen (DAKO, Germany) for 5-10 min. After counterstaining the cell nuclei with hematoxylin, coverslips were mounted with the cell layer down, on glass slides. The staining protocol was performed according to the DakoCytomation LSAB2 System-HRP manufacturer's instructions as follows:

1 Peroxidase block: Hydrogen peroxide was applied to cover cells on the glass slide, which was incubated for 5 min. After that, slider was gently rinsed with distilled water and placed in fresh 1× PBS buffer.

2. Blocking: Solution containing 1% BSA and 1% goat serum in 1× PBS was applied for 30 min.

3 Primary antibody or negative control reagent: Primary antibody or negative control reagent were applied to cover the glass slide. After 1 h incubation, they were gently rinsed with 1× PBS buffer.

4 Biotinylated link: The link antibody was applied to cover the cells at the glass slide. After 30 min, slide was rinsed as in step 3.

5 Streptavidin-HRP: The streptavidin reagent was applied to cover the cells on the glass slide, and was incubated for 30 min, and rinsed as before.

6 Substrated-chromogen solution: The DAB substrate-chromogen solutions was removed from 2-8 °C storage. The DAB solution was prepared as follows: one drop (or 20 µl) of the DAB chromogen solution per ml of substrate buffer. After 2 min incubation, the brown colour development was performed, and slide was rinse gently with distilled water.

7 Hematoxylin counterstain: Slide was immersed in hematoxylin. Incubated for 2 or 5 min, depending on the strength of hematoxylin used. Slides was rinsing in a bath of distilled water for 2 min twice.

8 Mounting: Glass slide was mounted with an aqueous-based mounting medium.

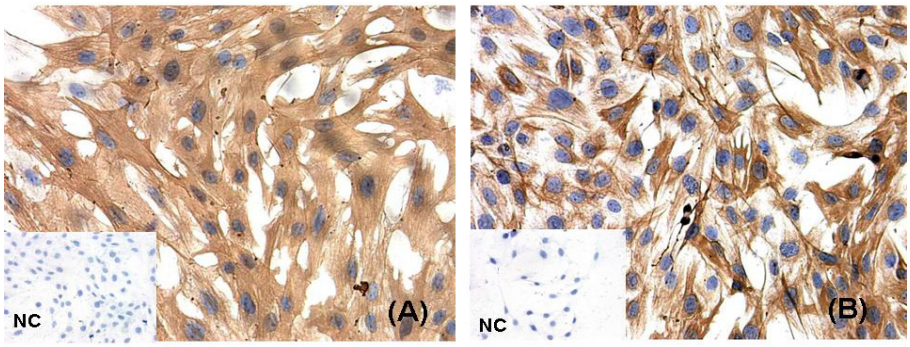


**Figure 9. Immunocytochemistry with cell-type specific markers in pulmonary arterial smooth muscle cells**

Characterization of PASMCs was done at the primary passage using immunocytochemical staining for α-smooth muscle actin (Sigma, Germany) and desmin (Neomakers, USA). (A) The α-smooth muscle actin and negative control (NC). The α-smooth muscle actin proteins are highly expressed in smooth muscle cells. The α-smooth muscle actin is found in muscle tissues and a major constituent of the contractile apparatus (B) Desmin and negative control (NC). Desmin is an intermediate filament protein expressed in both smooth and striated muscles. The anti-desmin antibody is useful in identification of vascular smooth muscle cells.

### 3.6 mRNA extraction

Total RNA was isolated from PASMCs at the primary passage with Trizol reagent (Life Technologies, USA), following a determination of the RNA concentration by spectrophotometer, and quality by electrophoresis on agarose gels as well as spectrophotometry.

The procedure of whole mRNA extraction was as follows:

1. Homogenisation: Lung tissue samples in were homogenised Trizol reagent (about 50 mg tissue in 1 ml). In cell samples, cells were lysed directly in the culture dish, using 1 ml of the reagent for 5-10×$10^6$ PASMCs
2. Phase separation: Samples were kept for 5 min at room temperature, and then 0.2 ml chloroform was added per 1 ml of Trizol which were then, shaken slightly for 15 s, and kept on ice for 15 min following centrifugation at 12,000 g for 20 min at 4 °C. After centrifugation, the mixture separated in to two phase: a lower red phenol-chloroform phase, and an upper aqueous phase.
3. RNA precipitation: The suspension was gently transferred to a new tube and the RNA was precipitated by adding by 0.5 ml isopropanol per 1ml Trizol reagent used in the first step. The sample was kept at room temperature for 10 min following centrifugative at 12,000 g for 20 min at 4 °C, after which, the pellet of RNA had precipitated at the bottom of tube.
4. RNA wash: The supernatant was removed, and the pellet was washed with 75% ethanol. Samples were then centrifuged at 7,500 g for 5 min at 4 °C.
5. RNA solubilisation: The 75% ethanol was gently removed, and the RNA was dried at room temperature. After that, samples of RNA add RNase-free water (100 μl per 10 cm dish) by diethylpyrocarbonate (DEPC) treatment. The quality and quantify of RNA measure the concentration by spectrophotometer.

### 3.7 Reverse transcription - polymerase chain reaction

Reverse transcription-polymerase chain reaction is a very sensitive technique for the detection and quantity of target gene messenger RNA (mRNA). This method consists of two parts: 1) the synthesis of cDNA (complementary DNA) from mRNA by reverse transcription and 2) the amplification of a specific cDNA by the polymerase chain reaction (PCR). The first-strand cDNA was synthesized with the ImProm-II™ reverse transcription system (Promega, USA), using oligo (dT) primers according to the manufacturer's instructions. Subsequently, 0.5 μg of cDNA product was used as a template in polymerase chain reaction (PCR) amplifications together with the primers, following the manufacturer's recommendations. Primers for PCR were designed with the Primer3 program (http://fokker.wi.mit.edu/primer3/input.htm). After an initial PCR activation step for 10 min at 95 °C, the following thermal profile was used: 1 min 94 °C, 1 min 55 °C annealing, 2 min elongation at 72 °C (30 cycles). The final products were electrophoresed in a 1.5% agarose gel and detected by ethidium bromide staining. The expression levels of glyceraldehyde dehydrogenase (GAPDH) were monitored as a loading control and quantified by densitometry.

The RT reaction was performed according to the manufacturer's instructions as follows:

1. The RT reaction mixture was prepared by combining the reagent of the ImProm-II™ reverse transcription system in the sterile tube on ice, as described below:

| RT Reaction | Volume |
|---|---|
| Nuclease-free water (to final volume of 15 μl) | X μl |
| ImProm-II™ 5× Reaction buffer | 4.0 μl |
| $MgCl_2$ (final concentration 1.5-8.0 mM) | 1.2-6.4 μl |
| dNTP Mix (final concentration 0.5 mM each dNTP ) | 1.0 μl |
| Recombinant RNasin® ribonuclease inhibitor | 2.0 μl |
| ImProm-II™ reverse transcriptase | 1.0 μl |
| **Final volume** | **15 μl** |

2. 15 µl RT reaction mix reagent 5 µl RNA and oligo (dT) primer mix for the final volume of 20 µl per tube
3. Anneal: The tubes were placed in a temperature-controlled heating block equilibrated at 25 °C for 5 min.
4. Extension: The tubes were incubated a inactivation in a temperature–controlled heating block at 42°C for one hr.
5. Inactivate reverse transcriptase: The RT samples were placed in the heat block at 70 °C for 15 min, and then stored in the fridge for PCR amplification.

PCR amplification was performed according to the manufacturer's instructions as follows:

1. Prepare the PCR reaction mix by combining the following reagents in the tube.

| PCR Reaction | Volume per 25 µl reaction |
|---|---|
| Nuclease-free water | 16.025 µl |
| 10× reaction buffer (without $MgCl_2$) | 2.475 µl |
| $MgCl_2$ 25mM (final concentration 2 mM) | 1.95 µl |
| PCR nucleotide Mix, 10mM (0.2 mM final) | 0.5 µl |
| Primer ( Final concentration 1 mM) | 3.3 µl |
| Taq DNA polymerase (5.0 units) | 0.25 µl |
| **PCR Mix** | **24.5 µl** |
| Volume of RT reaction | 0.5 µl |

2. Place the PCR reactions in the thermal cycler that has been preheated to 94 °C. The PCR program was set as follows:
3. After the cycle was complete, store the sampled at 4 °C.

| | |
|---|---|
| **Denaturation** | 95 °C**.** for 2 min |
| | 30 cycles: |
| **Denaturation** | 95 °C. for 1 min |
| **Annealing** | 55 °C for 1min |
| **elongation** | 72 °C for 2 min |
| | |
| **Final extension** | 72 °C for 5 min |
| **Hold** | 4 °C |

### 3.8 SDS-PAGE

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) is a technique widely used to separate proteins according to their electrophoretic mobility. The 10% protein separating gel has two parts: The lower part is a separating gel, and the upper is a stacking gel.

| **Separating gel** | **Volume per 6 ml** |
|---|---|
| deionized distilled water (dd water) | 2.5 ml |
| Acrylamide/Bis | 2.95 ml |
| Tris 1.5M buffer pH=8.8 | 1.875ml |
| 10% SDS | 75 µl |
| 10% ammonium persulfate | 75 µl |
| TEMED | 7.5 µl |
| **Stacking gel** | **Volume per 5 ml** |
| deionized distilled water (dd water) | 2.9 ml |
| Acrylamide/Bis | 0.75 ml |
| Tris 1.5M buffer pH=6.8 | 1.25ml |
| 10% SDS | 50 µl |
| 10% ammonium persulfate | 50 µl |
| TEMED | 5 µl |

After removing the medium, the PASMCs were washed with HBSS and lysed in 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% V/V Nonidet P-40, 0.05% W/V sodium deoxycholate, 0.025% W/V SDS, and 0.1% V/V Triton X-100 supplemented with PMSF (0.1 mg/ml), leupeptin (10 µg/ml), and aprotinin (25 µg/ml)( Sigma, Germany) [Clarke et al., 2005]. Insoluble proteins were removed by centrifugation at 10,000 rpm for 3 min. For nuclear protein extraction, the PASMC was subjected to nuclear protein isolation with the CellLytic NuCLEAR extraction kit (Sigma-Aldrich, Germany) performed according to the manufacturer's instructions.

The supernatants were assessed for protein content using Dye Reagent Concentrate (Bio-Rad, Germany). Extracts containing equal amounts of protein were denatured by boiling for 5 min in Laemmli's buffer containing β–mercaptoethanol and separated in 10 or 12% SDS-polyacrylamide gels at 130 V for 60min together with a rainbow molecular marker (Amersham, Germany).

### 3.9 Immunoblotting

Proteins resolved by SDS-PAGE were transferred to nitrocellulose membranes (PALL life sciences, Germany). For this propose, the trans-blot electrophortic transfer cell was used. The gel and nitrocellulose membrane were rinsed with blotting buffer in 20% methanol (Sigma, Germany). The gel and membrane were covered with three layers of paper in blotting buffer, and electrical current was applied for 1 h. After that, membranes were washed with 1× PBS on the rotational shaker for 5 min, and were blocked with 5% non-fat milk powder for 30 min. Membranes were then immunoblotted with rabbit polyclonal antibody to the IP receptor (Cayman, USA) at 1:500 dilution, the EP4 receptor (Sigma, Germany) at 1:500 dilution, PCNA (Neomarker, USA) at 1:1000. Peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Sigma, Germany) were used as secondary antibody. Blots were visualized using the enhanced chemiluminescence detection system (Amersham, Germany). Samples were normalized to GAPDH and quantified by densitometry.

### 3.10 Proliferation assay

The PASMCs were isolated, and cultures were maintained at 37 °C in a humidified 5%$CO_2$/95%$O_2$ atmosphere [Schermuly et al., 2005a]. To investigate the appropriate dose *in vitro* and the effects of iloprost or treprostinil on PASMC proliferation, rat PASMC from passage 1 were seeded in 12 well plates at a density of $4 \times 10^4$ cells/well in 10% FBS/DMEM. Cells were rendered quiescent by incubation in serum-free DMEM for 2 h, followed by serum deprivation (DMEM containing 0.1%FBS) for 48 h. Subsequently, cells were stimulated with 10% FBS/DMEM to induce cell cycle reentry. After treatment with 0, 10, 100, 500, 1000 nM iloprost or treprostinil during the last 12 h and throughout the stimulation period, 1.5 μCi [$^3$H] thymidine (Amersham, Germany) was added to each well. The [$^3$H] thymidine content of cell lysates was determined by scintillation counting, and normalized for protein concentration.

### 3.11 Determination of cAMP accumulation

The principle of radioimmunoassay (RIA) for cyclic AMP is a competition experiment. The samples were incubated in monoclonal antibody-coated tubes in the presence of $^{125}$I-labeled cAMP. Following incubation, the contents of the tubes were aspirated, and bound radioactivity was measured in a gamma counter. A calibration curve was established and values for samples were interpolated from the standard curve. The effects of the EP4 receptor antagonist (AH23848) or EP2 receptor antagonist (AH6809) (Sigma, Germany) on cAMP accumulation mediated by iloprost was measured by a commercial RIA cyclic AMP ($^{125}$I) kit (Immunotech, France) following the manufacturer's protocol. The PASMCs were grown to 90% confluence in 12-well plates, as described [Schermuly et al., 2007a;Lai et al., 2008]. After preincubation in 500 μM IBMX (Sigma, Germany) for 30 min at 37 °C, PASMCs were incubated with AH23848 or AH6809 (1, 10, 100 μM) for 15 min at 37 °C. Cells were then stimulated with iloprost (100 nM) for 15 min. 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 radioimmunoassay. Protein determination was performed according to the method of Bradford. The 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.

The assay procedure followed to the manufacturer's instructions, as follows:

1. Preparation of reagents: Reagents were brought come to room temperature, the 50 ml of the concentrated solution was diluted with 450 ml of distilled water. The content of each vial of calibrator was reconstituted with 1 ml of diluent.
2. Assay procedure
   - 2.1 Immunological step: A 100 µl of sample or calibrator was added to to antibody coated tubes, followed by 500 µl of tracer diluent solution. Tubes were maintained at 2-8 °C for 18 h.
   - 2.2 Wash step: The tracer diluent solution was removed from the tubes, except the "total cpm".
   - 2.3 The coated tubes were counted in a gamma-ray scintillation counter.

The results were obtained from the standard curve by interpolation. The curve serves for the determination of cAMP concentration in the samples measured at the same time as the calibrator. The mean was calculated from triplicates, and statistical analysis was carried out with a Student's *t*-test.

### 3.12 Statistical analysis

Data from multiple experiments are expressed as the mean and standard error (SE). All statistical analysis was carried out with Student's *t*-test. Differences between groups were considered significant when p was less than 0.05.

# 4. Results

## 4.1 Immunoblottig of the IP and EP4 receptor in human donor and idiopathic pulmonary arterial hypertensive lung tissue

The expression of IP and EP4 receptor protein was detected in human donor and IPAH lung tissue. As shown in the western blots of Figure 10 A, the IP receptor band was detected at 52 kDa. The ratio of the IP receptor to GAPDH exhibited a decreased expression of the IP receptor in IPAH lungs compared to human donors ($P<0.001$), while the EP4 receptor was detected at 78 kDa and displayed a similar level of expression between the human donor and IPAH lung samples (Figure 10 B). The results reveal that the expression of the IP receptor protein was decreased, but the expression of the EP4 receptor was stable in IPAH patient lung tissue compared to donor lung tissue. Because of the limitation related to the paraffin human lung samples and IP receptor antibody, the immunoblotting was applied. Immunohistrochemistry was utilized to detecting the IP and EP4 receptor in the lungs of rats with MCT-induced pulmonary hypertension.

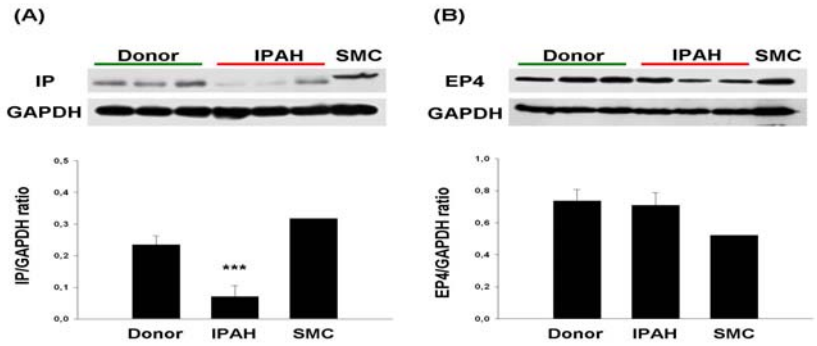


**Figure 10. The IP and EP4 receptor protein level in human donor and IPAH lung tissue**
(A) Levels of the IP receptor protein were decreased in IPAH lung tissues, compared to donor lung tissue. (B) The EP4 receptor protein exhibited stable expression in lungs from patients, IPAH as compared to those from donors. The bars represent the mean ± SEM of three samples in each group, with human PASMC as a positive control. *** $P<0.001$, compared to donor.

## 4.2 Immunohistochemistry of the IP and EP4 receptor in control and pulmonary hypertensive rats lung sections

In monocrotaline challenged rats, prominent medial wall hypertrophy is evident in the muscular pulmonary arteries. The thick medial layer displays smooth muscle proliferation. The pulmonary arteries from control rat lung sections demonstrated IP and EP4 receptor positive staining (A and D) in the medial smooth muscle wall (Figure 11). The MCT28d rat lung section exhibited only scant IP receptor positive staining (B), but stable EP4 receptor positive staining (E). No labeling was seen in negative controls in immunohistochemstry experiments (C and F).

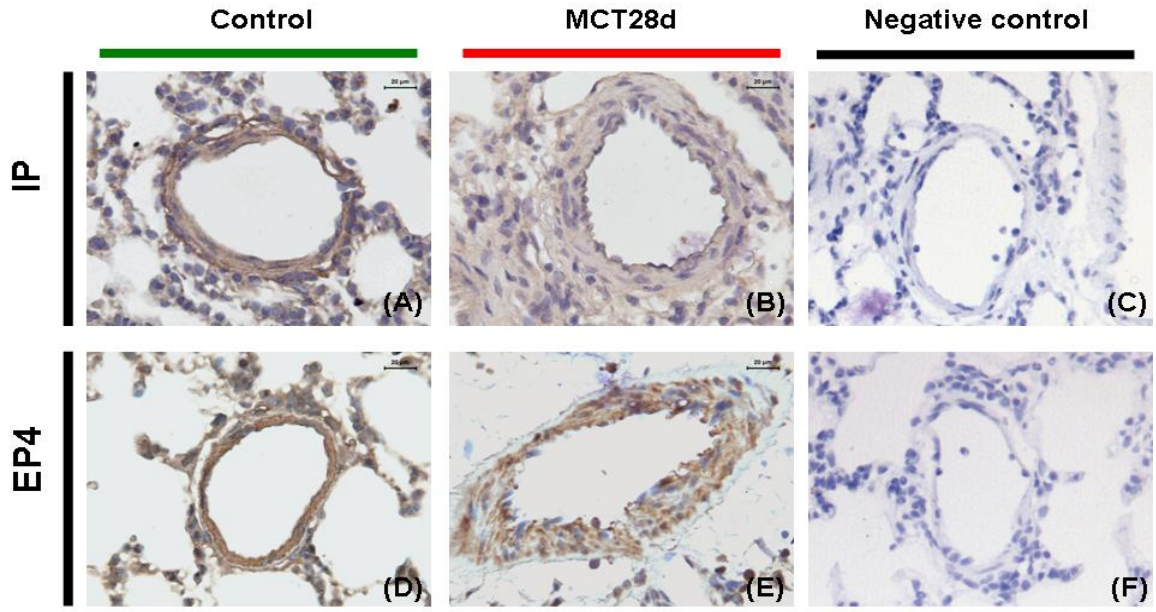


**Figure 11. Immunohistochemical localization of IP and EP4 receptors in control and pulmonary hypertensive rats lung section**

Bar=20mm, original magnification×400.

### 4.3 Gene expression of prostanoid receptors changes at passage two in PASMCs

Expression analysis by RT-PCR was used to survey the relative gene expression of prostanoid receptors and from primary passage to passage five of PASMCs from control rats (Figure 12). The PASMCs were isolated from the distal pulmonary artery regions and cultured in the presence of 10% FBS. To characterize PASMCs, we used the smooth muscle cell-specific gene markers α-SM-actin and desmin. Desmin was downregulated at passage three. The primers and product sizes of the prostanoid receptors and related genes are listed in the Material and Methods section. The IP, EP2, EP3, and FP receptor were down-regulated at passage two. Therefore, the PASMCs were used before passage two for all of the in vitro experiments.

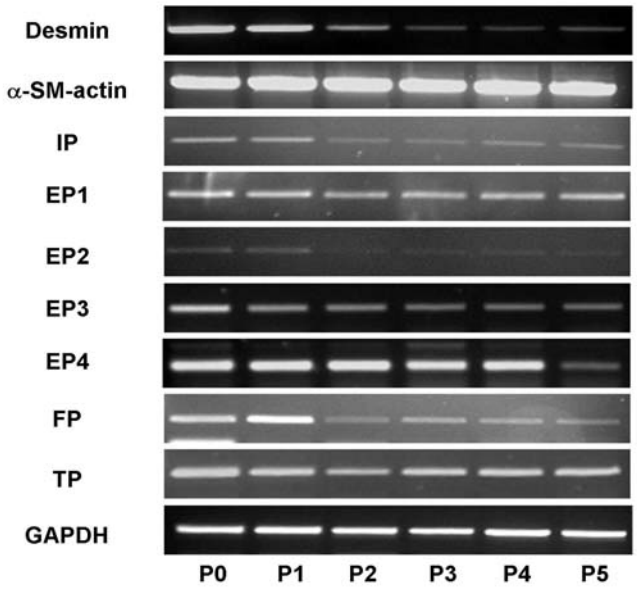


**Figure 12. Smooth muscle-specific and prostanoid receptor gene profile of control rat PASMCs are the first five passages in culture..** Representative RT-PCR analysis. After passage two, the mRNA expression levels of the IP, EP2, EP3 and FP receptors were reduced in rat PASMCs.

### 4.4 Gene expression profiling of the prostanoid receptors and the related genes in distal and proximal PASMCs from control and MCT28d rats

The PASMCs were isolated from MCT28d and control rats. To obtain proximal and distal PASMC, 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). In the distal and proximal PASMCs from control and MCT28d rats: The mRNA expression was analyzed separately in PASMCs from five individual rats per group: (four groups: control rat proximal and distal PASMCs and MCT28d rat proximal and distal PASMCs), and this revealed the variability in the pattern of gene expression and the pattern associated with the pulmonary artery hypertrophy.

In the distal and proximal PASMCs from control and MCT28d rats, the data were shown as the mean ± SEM for the same group of five 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 MCT28d groups (Figure 13). In primary or secondary pulmonary hypertension, because of the characteristic changes in vascular structure, the muscular arteries and arterioles exhibit smooth muscle cell proliferation leading to further medial hypertrophy in the distal musculature [Wharton et al., 2000]. Within the four PASMCs groups: under study, the MCT28d rat proximal or distal PASMCs and control rat proximal or distal PASMCs, COX-2 expression was unchanged. The IP receptor was down-regulated in both the proximal and distal PASMC groups of MCT28d compared to the control groups. The EP1 and TP receptor were down-regulated in the MCT28d distal group. The EP2 and EP4 receptors were not significantly changed in any groups. The EP3 and FP receptors were down-regulated in the proximal and distal groups of MCT28d, and also in the distal group of the control. To the best of our knowledge these findings are the first to demonstrate that the prostanoid receptor genes presenting in the pulmonary hypertension animal model exhibit different expression patterns in the distal and proximal PASMCs.

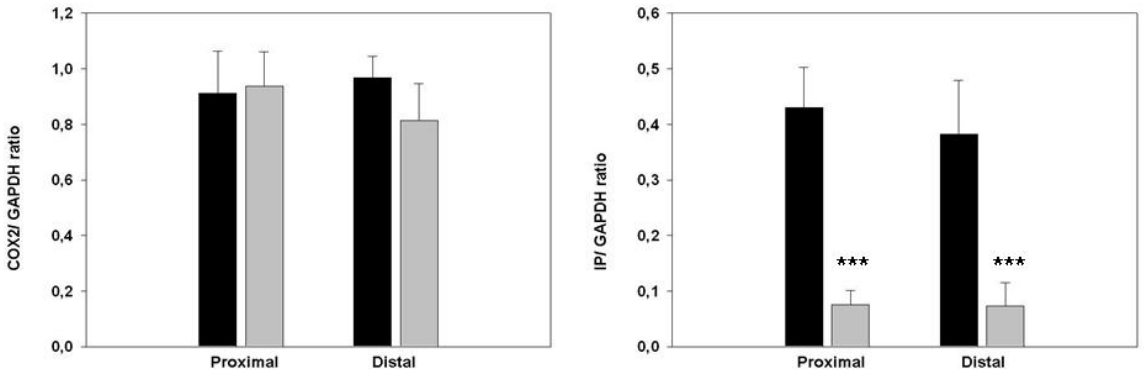


**Figure 13-A. Densitometric quantification of COX-2 and IP receptor in distal and proximal PASMCs** Densitometric quantification of COX-2 and IP receptor gene expression in four groups of PASMC. Data are shown as the mean ± SEM (n=5). The black bars represent the proximal or distal PASMCs of the control groups. The gray bars represent the proximal or distal PASMCs of the MCT28d groups, *** P<0.001, compared to control groups..

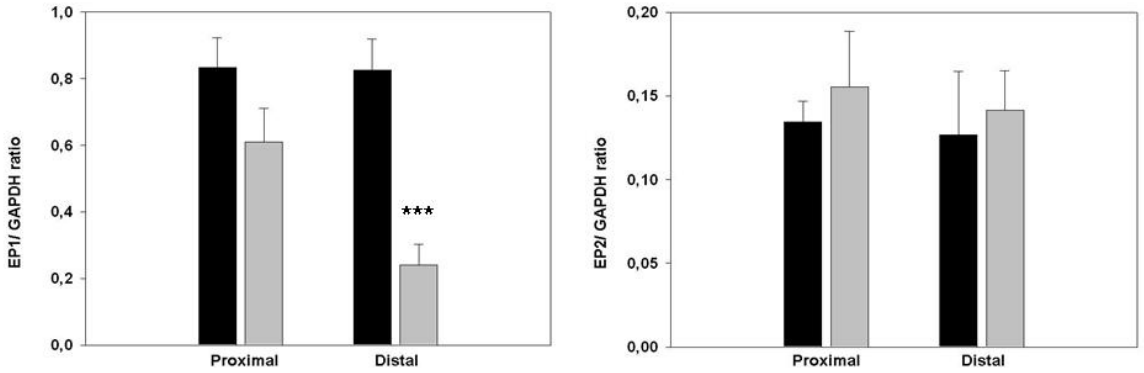


**Figure 13-B. Densitometric quantification of EP1 and EP2 receptor in distal and proximal PASMCs** Densitometric quantification of EP1 and EP2 receptor gene expression in four groups of PASMC. Data are shown as the mean ± SEM (n=5). The black bars represent the proximal or distal PASMCs of the control groups. The gray bars represent the proximal or distal PASMCs of the MCT28d groups. *** P<0.001, compared to control groups.

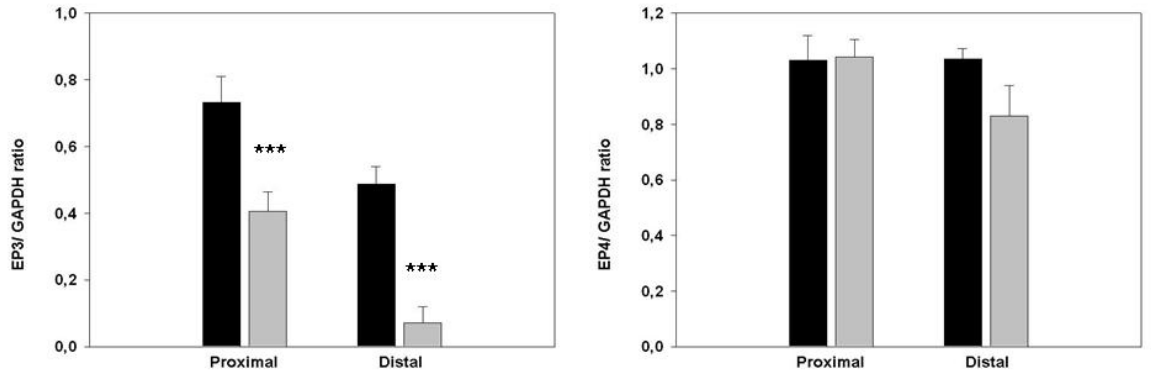


**Figure 13-C. Densitometric quantification of EP3 and EP4 receptor expression in distal and proximal PASMCs** Densitometric quantification of EP3 and EP4 receptor gene expression in four groups of PASMC. Data are shown as the mean ± SEM (n=5). The black bars represent the proximal or distal PASMCs of the control groups. The gray bars represent the proximal or distal PASMCs of the MCT28d groups. *** P<0.001, compared to control groups.

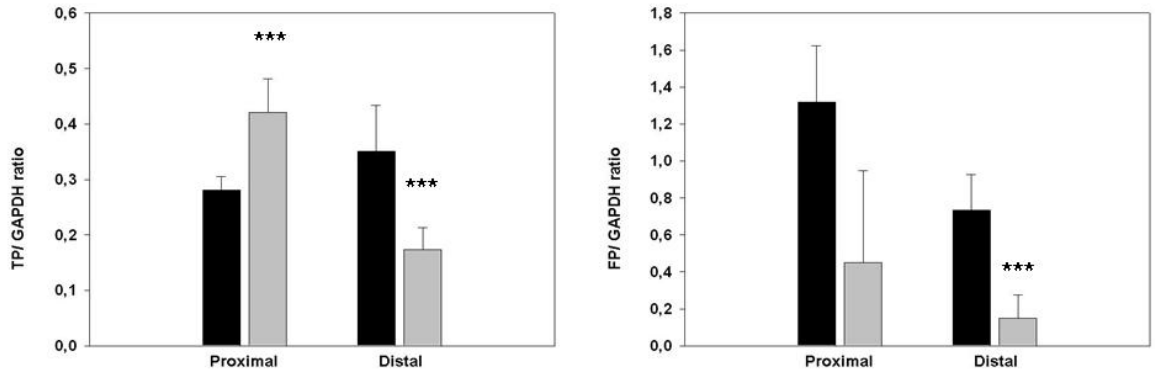


**Figure 13-D. Densitometric quantification of TP and FP receptor expression in distal and proximal PASMCs** Densitometric quantification of TP and FP receptors in terms of the gene expression in four groups of PASMC. Data are shown as the mean ± SEM in the same group of five individual PASMCs. The black bars represent the proximal or distal PASMCs of the control groups. The gray bars represent the proximal or distal PASMCs of the MCT28d groups. *** P<0.001, compared to control groups.

### 4.5 Immunoblotting of IP and EP4 receptor expression in distal PASMCs of control and pulmonary hypertensive rats

At the mRNA level, low IP receptor gene expression and stable EP4 receptor gene expression was observed in MCT28d PASMC. 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 14A), the IP receptor protein band was detected at 52 kDa. The ratio of IP receptor to GAPDH was shown to have decreased IP receptor expression in MCT28d compared to control PASMCs ($P<0.05$). However, the EP4 receptor was detected at 78 kDa, indicating stable expression in the control and MCT28d rats (Figure 14B). There is evidently reduced IP receptor protein expression in the remodeled vessels in pulmonary hypertension patients [Hoshikawa et al., 2001]. Taken together, the results indicate that the expression IP receptor protein was decreased but EP4 receptor protein expression was stable in both the pulmonary hypertension animal model (MCT28d) and in IPAH lung samples. Thus, we used the MCT28d PASMCs with slight IP receptor protein expression to test the hypothesis that iloprost mediates vasodilatory functions via the EP4 receptor associated with PAH when the IP signaling fails.

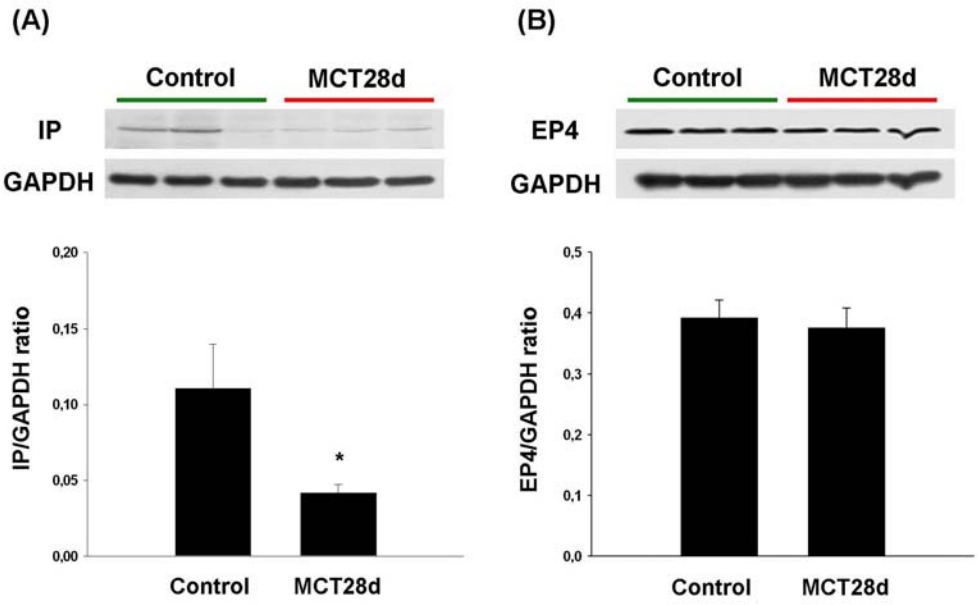


**Figure 14. Immunoblotting for IP and EP4 receptor in primary PASMCs from control and MCT28d rats.**

**(A)** Densitometric analysis from three different experiments in each group. The IP receptor was identified as a 52 kDa immunoreactive band that was decreased in MCT28d rat PASMCs compared to control PASMCs. Data represent the mean±SEM, n=3 in each group. *P<0.05 compared with controls.

**(B)** The EP4 receptor was identified as a 78 kDa immunoreactive band and was stably expressed in both MCT28d and control PASMCs.

## 4.6 The effect of the EP4 receptor antagonist (AH23848) or the EP2 receptor antagonist (AH6809) on cAMP accumulation by pulmonary hypertensive rat PASMC

The PASMCs from MCT28d rats exhibited scant IP receptor, but stable EP4 receptor and EP2 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 [Fullerton et al., 1994;Gilman, 1990;Narumiya et al., 1999], leading to a mediation of vasodilatory functions. The EP2 and EP4 receptors are both coupled via Gs to induce elevations in intracellular cAMP leading to smooth muscle relaxation. In our experiment, MCT28d rat PASMCs exhibited scant IP receptor, but stable EP4 and EP2 receptor expression. We applied EP2 antagonist (AH6809) to demonstrate that the EP4 receptor specificity may play an important role in generating cAMP during iloprost treatment. Pre-incubation with AH23848 was used to block the EP4 receptor while AH6809 was used to block the EP2 receptor. Pre-incubation with IBMX [Pang et al., 1998] excluded a role for phosphodiesterases in these experiments. The two negative controls with or without IBMX (500 μM) were not significantly different during the 30 min stimulation. The MCT 28d rat PASMCs were stimulated for 30 min at various AH23848 or AH6809 concentrations (0, 1, 10, 100 μM), with or without IBMX (500 μM) and, then incubated with or without iloprost (100 nM) for 15 min. Iloprost-induced intracellular cAMP accumulation was inhibited in a dose-dependent manner by AH23848 (the EP4 receptor antagonist) (Figure 16 A) but not by AH6809 (the EP2 receptor antagonist) (Figure 16 B) to show that iloprost-induced intracellar cAMP accumulation was inhibited in a dose-dependent manner by AH23848 but not inhibited significantly by AH6809. Our results indicated that iloprost may mediate vasodilatory functions via the EP4 receptor in place of the IP receptor signal transduction in MCT28d rat PASMC.

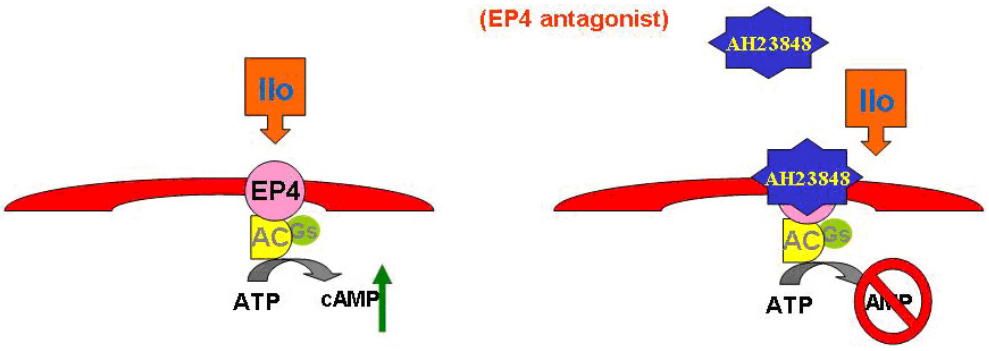


**Figure 15. A scheme of the hypothesis that $EP_4$ receptors may take over the function of the IP receptor in the MCT28d rat PASMCs.**

Iloprost-induced intracellar cAMP accumulation was inhibited in a dose-dependent manner by AH23848 (EP4 receptor antagonist). Ilo=Iloprost; EP4=prostanoid EP4 receptor; AC=adenylate cyclase, Gs= the stimulatory G-protein.

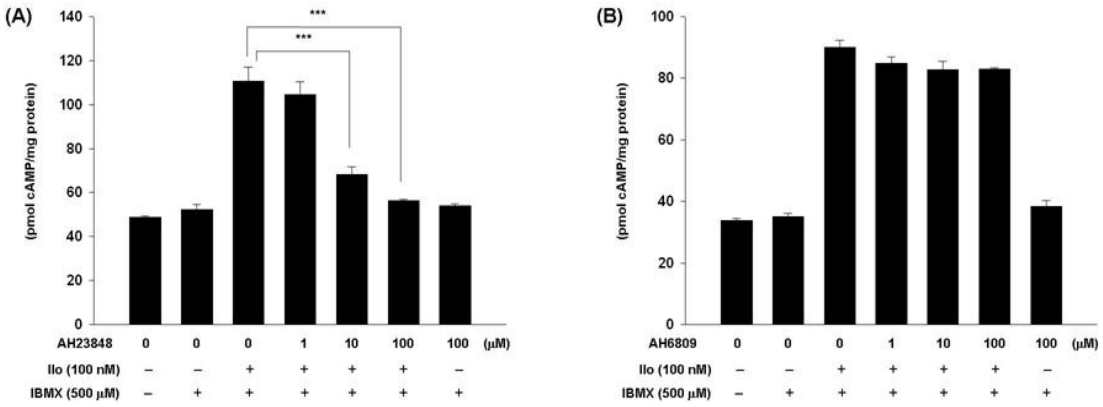


**Figure 16. EP4 antagonist AH23848 blocks the cAMP accumulation mediated by iloprost in MCT28d rat PASMCs.**

The intracellular cAMP accumulation induced by iloprost was inhibited by AH23848 but not AH6809. The MCT28d rat PASMCs, which exhibit low IP but stable EP4 receptor expression, were stimulated for 30 min at several concentrations (0, 1, 10, 100 μM) of AH23848 (A) or the EP2 antagonist AH6809 (B), with or without iloprost (100 nM) for 15 min. Data are the mean±SEM of three different experiments. *** $p<0.001$, as compared to iloprost treatment alone.

## 4.7 Prostacyclin inhibits pulmonary artery smooth muscle cells proliferation

Iloprost inhalation has clear advantages for the treatment of PAH but also certain drawbacks. Inhaled Iloprost lasts only 30 to 90 min, and six to nine inhalations are needed to achieve good clinical results. Currently, treprostinil is one of the long-acting stable $PGI_2$ analogs, with a duration of action up to three to four hours. The effect of Trep on proliferation of PASMC stimulated by 10% serum/DMEM was investigated in MCT 28d PASMC. Under control conditions, 10% serum increased the cell proliferation rate, which was set at 100%. Incubation with iloprost (Figure 17 A) or treprostinil (Figure 17 B) (10, 100, 500 and 1000 nM) inhibited MCT 28d PASMC proliferation stimulated by 10% serum in a dose-dependent manner. Treprostinil displayed marked antiproliferative activity starting at 100 nM (***P<0.01) as assessed by [$^3$H]thymidine incorporation assay. In addition, treprostinil possessed a more potent antiproliferative efficiency than Ilo.

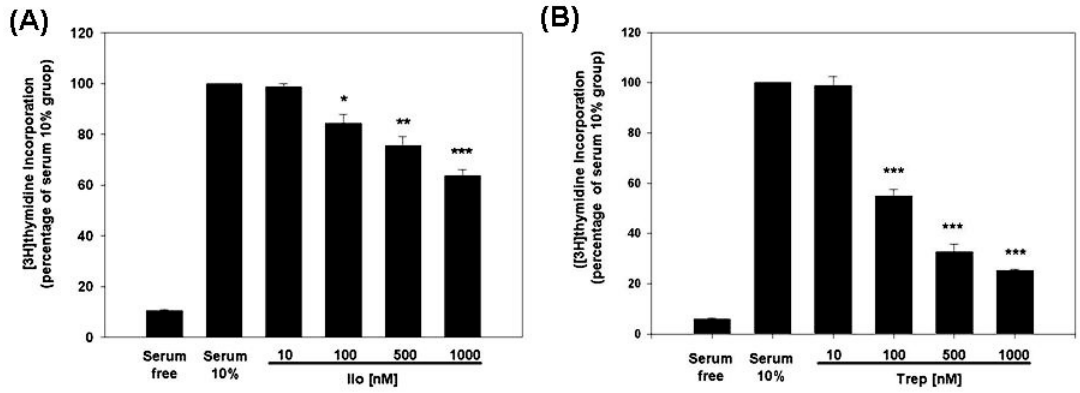


**Figure 17. Treprostinil significantly inhibited MCT 28d rat PASMC proliferation in a dose-dependent manner.** Iloprost (Ilo) inhibited the thymidine incorporation into the PASMCs 99%, 84%, 75% and 63% at the final concentrations of 10, 100, 500 and 1000 nM (A). Treprostinil (Trep) inhibited the thymidine incorporation into the PASMCs 99%, 55%, 33% and 25% at the final concentration 10, 100, 500 and 1000 nM (B), respectively, as determined by [$^3$H]thymidine incorporation assay. (*p<0.05, **p<0.01, ***p<0.001, compared with the 10% serum group)

### 4.8 Treprostinil inhibited the nuclear translocation of ERK

Treprostinil has been shown to inhibit smooth muscle cell growth. The extracellular-regulated kinases (ERK1/2) is also known as p42/44 mitogen-activated protein (MAP) kinase, and is implicated in the regulation of proliferation of PASMC, since phosphorylated ERK must translocate into the nucleus for proliferation to occur. To investigate whether the effect of Trep can inhibit proliferation of PASMC from MCT-treated rats via anti-nuclear tranlocation of ERK, we utilized immunocytochemistry to examine whether Trep can inhibit ERK nuclear translocation. After serum free treatment for 24 h, ERK signaling cascade of MCT 28d PASMCs was activated by serum stimulation at 10% serum concentration in the medium, and immunocytochemistry was performed with an antibody to phosphorylated ERK (Santa Cruz, USA). The cellular distribution of phosphorylated ERK in PASMCs from MCT-treated rats is illustrated in Figure 18. Cells were serum-free for 24 h (Figure 18 A). After 24 h serum-free conditions, ERK nuclear translocation was activated by 10 % serum (Figure 18 B). After serum-free condition for 24 h, cells were incubated with treprostinil (100 nM) for 30 min, and subsequently activated by 10% serum (Figure 18 C). The results shown in Figure 18C demonstrated that Treprostinil can inhibit nuclear tranlocation of the ERK 1/2, which is related to the regulation of cell proliferation.

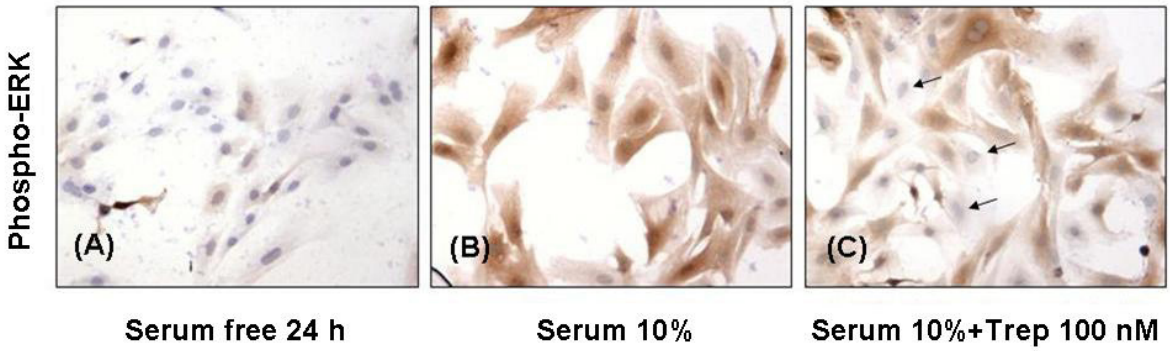


**Figure 18. Nuclear translocation of ERK in pulmonary hypertension rat PASMCs after treatment with treprostinil.**

The immunocytochemical analysis of ERK nuclear translocation is illustrated (A) Cells were kept serum-free for 24 h. (B) after 24 h serum-free condition, cells were activated by 10% serum. (C) After serum-free condition for 24 h, cells were incubated in treprostinil (Trep) (100 nM) for 30 min, following activation by 10% serum. Cells exhibited brown staining was considered positive for the expression of Phospho-ERK. Treprostinil can inhibit Phospho-ERK nuclear translocation in PASMCs (arrowheads).

### 4.9 The effect of the EP4 receptor antagonist on cAMP accumulation induced by iloprost or treprostinil in PASMCs from rats with monocrotaline-induced pulmonary hypertension

Expression profiling of IP and EP receptor in MCT-treated rat PASMCs revealed scant expression of the IP receptor but stable expression of the EP4 receptor compared to controls. Iloprost-induced elevations in intracellular cAMP levels in PASMCs was dose-dependently reduced by the EP4 receptor antagonist (AH23848). As a result, iloprost can mediate vasodilatory functions via the EP4 receptor in the case of the low prostacyclin receptor expression associated with pulmonary hypertension. It is established that $PGI_2$ agonists can mediate vasdilatory functions via the EP4 receptor to increase cAMP levels. To test the function of treprostinil, we aimed to assess whether treprostinil activation of the EP4 receptor activation involved the cAMP pathway.

Pre-incubation with AH23848 was used to block the EP4 receptor. Pre-incubation with IBMX [Pang et al., 1998] excluded a role for the phosphodiesterases in these experiments. The two negative controls with or without IBMX (500 μM) were not significantly different during the 30 min stimulation. The MCT 28d rat PASMCs were stimulated for 30 min at various AH23848 concentrations (0, 1, 10, 100 μM), while IBMX (500 μM) was applied, and cells were then incubated with or without iloprost (100 nM) for 15 min. Iloprost-induced intracellular cAMP accumulation was inhibited in a dose-dependent manner by AH23848 (an EP4 receptor antagonist) (Figure 19 A) but not treprostinil (Figure 19 B). of the iloprost- or treprostinil- treated groups were set at 100%, and it is evident that iloprost-induced intracellar cAMP accumulation was inhibited in a dose-dependent manner by AH23848. However, treprostinil induced intracellar cAMP accumulation in MCT-induced PASMCs, an effect ($p<0.05$) an effect that was not sensitive to AH23848, except at the highest dose employed (100μM). Our results indicate that the effects of treprostinil may not be mediated via the EP4 receptor/cAMP signal transduction in MCT28d rat PASMCs.

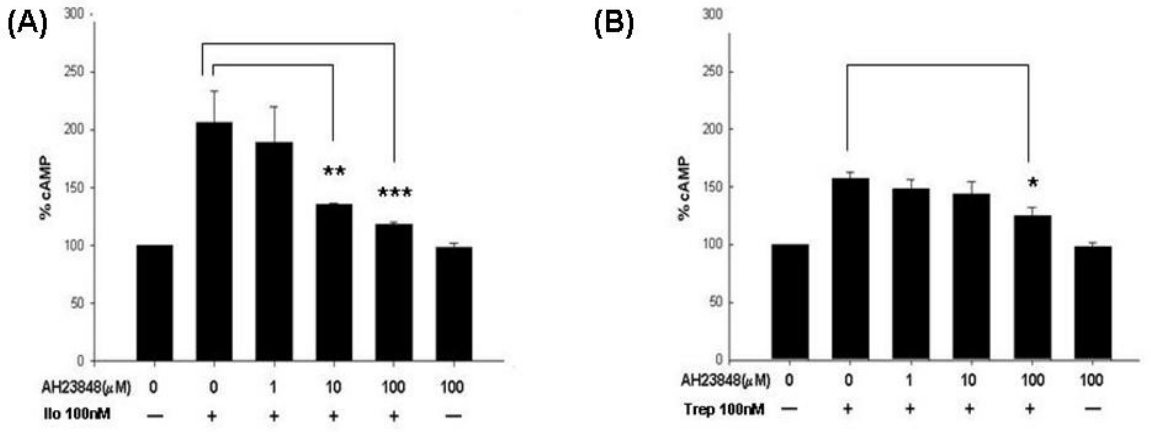


**Figure 19. The EP4 antagonist AH23848 blocks cAMP accumulation mediated by iloprost or by treprostinil in MCT28d rat PASMCs.**

The intracellular cAMP accumulation induced by iloprost (Ilo) (A) or by treprostinil (Trep) (B) was inhibited by AH23848. The MCT28d rat PASMCs, with scant IP but stable EP4 receptors expression, were stimulated for 30 min at various concentrations (0, 1, 10, 100 μM) of AH23848 and then with or without iloprost (Ilo) 100 nM or treprostinil (Trep) 100 nM for 15 min. Data are the mean±SEM of three different experiments. *p<0.05, **p<0.01, ***p<0.001, as compared to iloprost or treprostinil treatment alone.

## 4.10 Scant expression of PPAR protein in idiopathic pulmonary arterial hypertensive human lung tissue

A stable prostacyclin agonist can signal through by ligand binding to nuclear peroxisome proliferator-activated receptor (PPAR): 1)PPAR-α, 2)PPAR-β/δ, and 3)PPAR-γ. The PPARs are a family of nuclear transcription factors that bind to the specific peroxisome proliferator response elements (PPREs) to regulate target gene expression [Lim and Dey, 2002]. To determine if the PPARs might underlie the effects of treprostinil, the expression of PPARs in both human donor and IPAH lung tissue was analyzed. The expression of PPAR protein was detected in both human donor and IPAH lung tissue. As shown on the immunoblots, the PPAR-α protein band was detected at 52 kDa (Figure 20 A), PPAR-β/δ protein band was detected at 50 kDa (Figure 20 B), and PPAR-δ protein band was detected at 67 kDa (Figure 20 C). The ratio of the PPAR protein to GAPDH revealed decreased expression of the PPARs in IPAH lungs compared to human donor lungs. The results reveal that the expression of the three PPAR proteins are decreased in IPAH patient lung tissue compared to donor lung tissue.

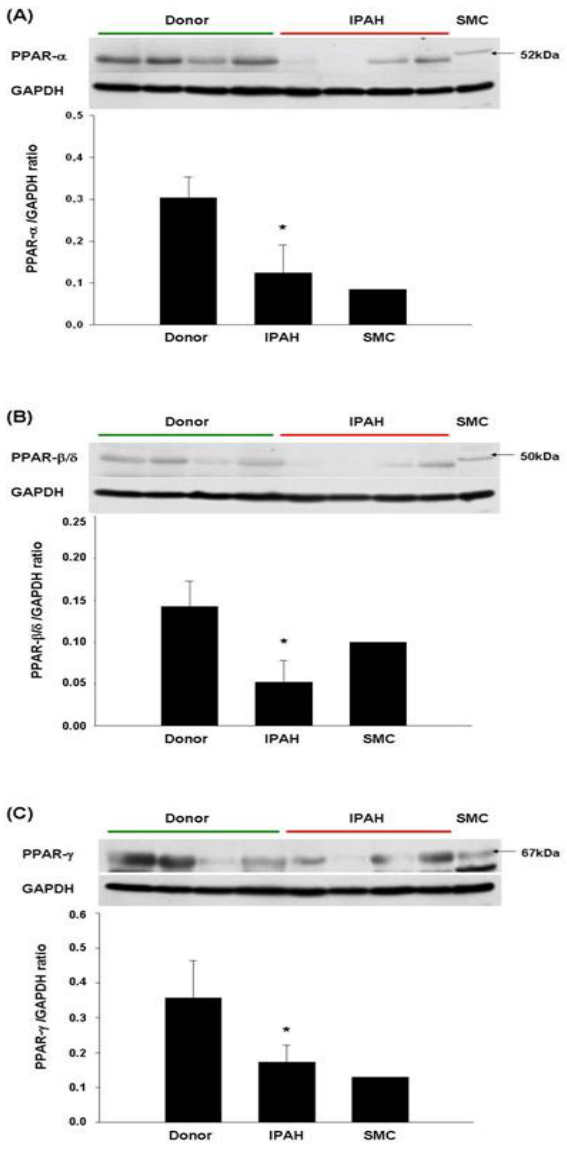


**Figure 20. the PPAR protein expression levels in human donor and IPAH lung tissue.**

(A) The PPAR-α protein was detected in lung tissues as a 52 kDa band, and band intensity was decreased in IPAH lung tissue compared to donor lung tissue. (B) The PPAR- β/δ protein was detected in lung tissues as a 50 kDa band, and band intensity was decreased in IPAH lung tissues compared to donor lung tissue. (C) The PPAR-γ protein was detected in lung tissues as a 67 kDa band, and band intensity was decreased in IPAH lung tissues compared to donor lung tissue. The bars represent mean ± SEM of four samples in each group, with human PASMC incuded as a positive control. * $P<0.05$, compared to donor.

## 4.11 Scant expression of PPAR gene in the distal PASMCs of pulmonary hypertensive rats

The results revealed that the three PPARs protein expression levels of these PPARs were decreased in IPAH patient lung tissue. To determine whether the MCT-induced pulmonary hypertension animal model possessed similar PPARs expression pattern, the PASMCs were isolated from MCT-treated rats with pulmonary hypertension (MCT 28d) and control rats. Proximal PASMCs were obtained from the trunk and lobar arteries (>2 mm external diameter), and distal PASMCs were isolated from peripheral arteries (<1 mm external diameter). In the distal and proximal PASMC from control and MCT28d rats, mRNA expression was separately analyzed in three individual rats per experimental group (four groups: control rat proximal and distal portions, MCT28d rat proximal and distal portions). Within the distal portion of the MCT28d rat and control rat, the PPAR expression was down-regulated in the distal PASMC from MCT28d, compared to control group (Figure 21). These findings are the first to identify that the PPARs genes presenting in the pulmonary hypertension animal model exhibit different expression levels in distal and proximal PASMCs.

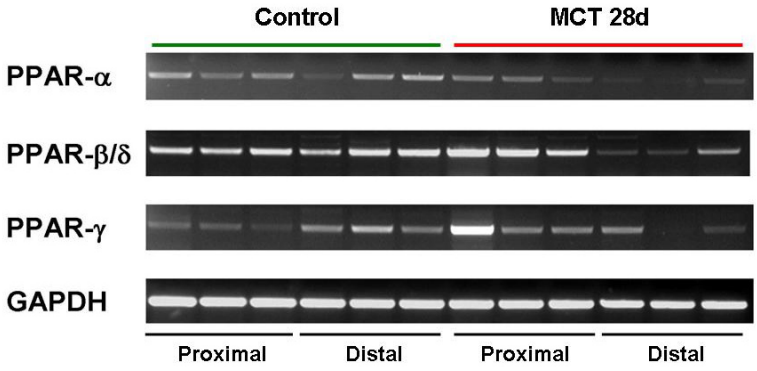


**Figure 21. The PPAR-α, PPAR-β/δ, PPAR-γ gene expression profile of distal and proximal PASMC.**

A representative RT-PCR analysis is illustrated, documenting the mRNA expression of PPARs in the proximal and distal PASMCs that were isolated from either control or MCT28d rat pulmonary arteries. The expression differences were compared with GAPDH as a loading control (n=3). Smooth muscle cells were harvested for RNA isolation in the primary passage.

## 4.12 Scant PPAR protein expression in distal PASMCs from pulmonary hypertensive rats

At the mRNA level, low PPAR gene expression was detected in MCT28d PASMCs. To evaluate the protein expression of the PPARs, protein extracts were prepared from the distal PASMCs of control and MCT28d rats. As is evident in the immunoblots (Figure 22), the protein expression of three PPARs were decreased in MCT28d rats. Reduced PPAR-γ protein expression was been reported in the remodeled vessels of patients with pulmonary hypertension [Hansmann et al., 2007], but protein expression levels of PPAR-α and β/δ have not been addressed. The results indicate that the expression of PPAR protein was decreased in both the pulmonary hypertension animal model (MCT28d) and IPAH lung samples. Thus, we used the MCT28d PASMCs with low PPARs protein expression to test whether treprostinil, a stable prostacyclin agonist, might regulate PPAR protein expression.

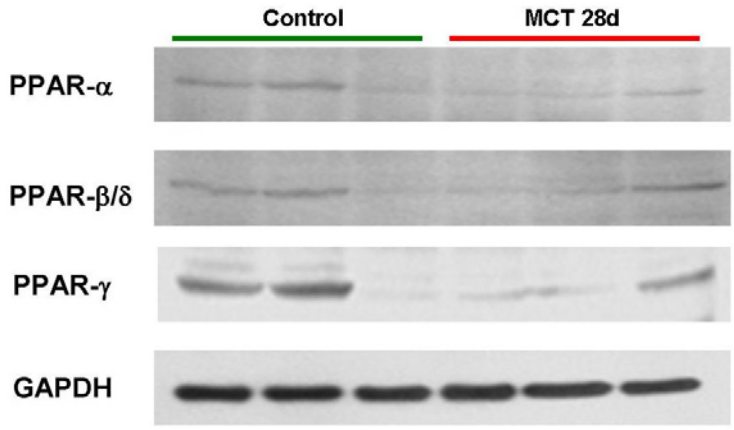


**Figure 22. Immunoblotting for PPARs in primary PASMCs from control and pulmonary hypertensive rats.**

Immunoblotting analysis from three different experiments in each group. The PPAR-α receptor was identified as a 50 kDa immunoreactive band, the PPAR-β/δ protein was identified as a 52 kDa immunoreactive band, the PPAR-γ protein was identified as a 67 kDa immunoreactive band. The expression of all three PPARs was decreased at the protein levels in MCT28d rat PASMCs compared to control PASMCs.

## 4.13 PPAR-α and PPAR-γ protein expression is induced in PASMC of pulmonary hypertensive rats after treprostinil treatment

The resultsso far have demonstrated low PPAR expression in both the pulmonary hypertension animal model (MCT28d) and IPAH lung samples. To test whether treprostinil, a stable prostacyclin agonist, might regulate PPAR protein expression, PASMCs were incubated with 100 nM treprostinil for 0, 3, 6, 12, and 24 h. Time-course data are given as the mean fold-increase in protein expression on immunoblot (Figure 23 A), where the PPAR-α protein band was detected at 50 kDa. The ratio of PPAR-α to GAPDH increased in PASMC from pulmonary hypertensive rats, which was observed as early as 3 h after treprostinil (100 nM) treatment. The PPAR-β/δ protein band was detected at 52 kDa. The ratio of PPAR-β/δ to GAPDH was not significantly increased in PASMCs after treatment (Figure 23 B). The ratio percentage of PPAR-γ to GAPDH demonstrated increased the expression in the pulmonary hypertensive rat PASMCs, which was observed as early as 3 h after treatment (Figure 23 C). The results indicate that treprostinil, a stable prostacyclin agonist, induces the PPAR-α and PPAR-γ protein expression, but not PPAR-β/δ protein expression.

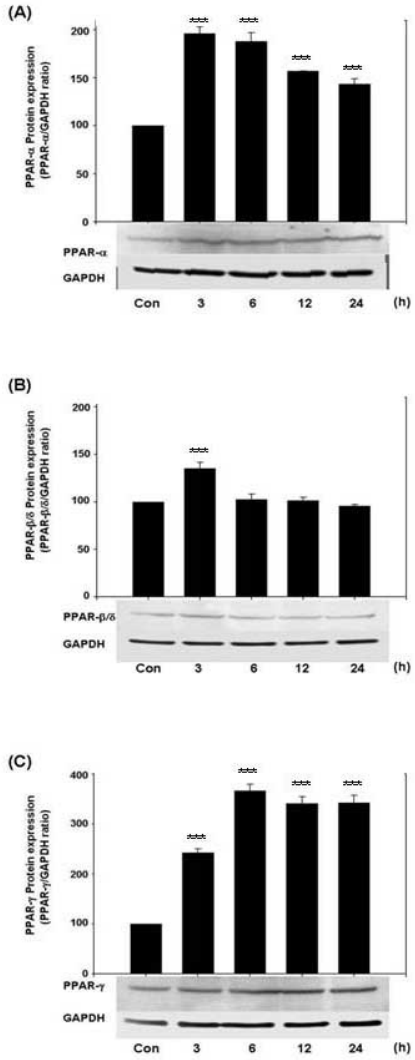


**Figure 23. Time course of PPAR protein expression induced in PASMCs from pulmonary hypertensive rats after treprostinil treatment** The PASMCs were incubated with 100 nM treprostinil (Trep) for 0, 3, 6, 12, and 24 h. Western blot analysis for PPAR-α protein (A), PPAR-β/δ protein (B), and PPAR-γ protein (C) expression increased in the pulmonary hypertensive rat PASMCs which was observed as early as 3 h after Treprostinil 100 nM treatment. The results indicate that treprostinil, a stable prostacyclin agonist, induces the PPAR-α and PPAR-γ protein expression, but not PPAR-β/δ protein expression. Con, control ; Data represent the mean±SEM, n=2 in each group. ***P<0.001 compared with controls.

## 4.14 Summary of results

This first part of these results demonstrates that IP receptor expression was reduced in PAH patient lung samples and MCT-treated rat lungs, compared to controls. Reverse transcriptase-polymerase chain reaction (RT-PCR) and immunoblotting from MCT-treated rat PASMC extracts revealed low expression of the IP receptor, but stable expression of the EP4 receptor compared to controls. Iloprost-induced elevations in intracellular cAMP levels in PASMCs was dose-dependently reduced by AH23848, but not by AH6809. In summary, iloprost mediates vasodilatory functions via the EP4 receptor in the case of the low prostacyclin receptor expression associated with pulmonary hypertension. The first part of these results were published in *Am J Respir Crit Care Med.* 2008 Jul 15;178(2):188-96.

Treprostinil is another novel long-acting stable $PGI_2$ analog, the half-life of which is approximately 3-4 h, and is Food and Drug Administration (FDA) approved for subcutaneous infusion. In the second part of these results, treprostinil was shown to inhibit smooth muscle cell growth, in an ERK 1/2 (is also known as p42/44 mitogen-activated protein kinase)-dependent manner. For the regulation of cell proliferation, phosphorylated ERK must translocate into the nucleus. Treprostinil can inhibit pulmonary hypertensive rat PASMC proliferation by blocked nuclear translocation of ERK. In vascular smooth muscle cells, prostaglandin stimulates adenylate cyclase, which converts adenosine triphosphate to cAMP. Treprostinil exhibited a stronger antiproliferative effect than did iloprost, and prevented the nuclear translocation of phosphorylated ERK. However, the intracellular cAMP levels elevated by iloprost were dose-dependently reduced by AH23848 treatment, but not by treprostinil suggesting a role for the EP4 receptor in iloprost-induced cAMP generation. Immunoblotting demonstrated a downregulation of PPAR expression in IPAH patients and MCT28d PASMCs. Moreover, treprostinil was found to activate PPAR-$\alpha$ and PPAR-$\gamma$, but not PPAR $\beta/\delta$. These results indicate that IPAH patients lack PPARs, and a similar expression pattern was observed in MCT-induced PAH. Trep might be a ligand for the nuclear receptor PPARs, and mediate antiremodeling effects via the PPAR–$\alpha$ and PPAR-$\gamma$ associated with PAH.

# 5. Discussion

In this thesis project, an effort has been made to elucidate the prostacyclin signaling pathway from the cell surface to the nucleus, by PASMC from MCT-induced pulmonary hypertension in rats. In preclinical and clinical studies, it has been shown that prostacyclin, iloprost or treprostinil reduce pulmonary arterial pressure, increase cardiac output and increase exercise capacity. Prostacyclin and its analogs work mainly by binding to the prostacyclin receptor, which belongs to the family of G-protein coupled receptors. Activation of the receptor leads to an elevation of intracellular cAMP by activation of adenylate cylase. In the first part of my thesis, the expression of the different prostanoid receptors was investigated in lungs and smooth muscle cells of pulmonary hypertensive rats and in lungs from patients undergoing lung transplantation due to IPAH. Interestingly, the expression of the IP was markedly reduced under the conditions of both experimental and clinical pulmonary hypertension, while other prostaglandin receptors, such as the prostanoid EP4 receptor, were unchanged in their expression. In the second part, functional experiments were performed which show that iloprost and treprostinil reduce serum-induced proliferation of rat PASMC. In addition, the iloprost-induced cAMP production by PASMCs, but not that of treprostinil, could be blocked by the EP4 receptor antagonist AH23848, suggesting that iloprost, at least in part, acts via the EP4 receptor. An investigation was carried out on treprostinil, which is known to activate nuclear PPARs in addition to the prostanoid receptors. Interestingly, the PPARs were downregulated in experimental and clinical pulmonary hypertension but treprostinil induced PPAR-$\alpha$ and PPAR-$\gamma$, suggesting a potential role for a prostanoid receptor-independent mechanism of treprostinil. The discussion is divided into two major sections. The first section covers the cell surface prostanoid receptor: the specific contribution of EP4 in mediating the effects of iloprost in the case of the low IP receptor expression associated with PAH is considered. The next section covers the role of prostacyclin versus nuclear receptor: peroxisome proliferator-activated receptors in prostacyclin sensing.

## 5.1 The specific contribution of EP4 in mediating the effects of iloprost in the case of low IP receptor expression associated with pulmonary arterial hypertension

One of the key pathways that is altered in PAH is the prostacyclin signaling pathway. It is known that disturbances to prostacyclin synthesis [Tuder et al., 1999;Christman et al., 1992], as well as polymorphisms in genes encoding $PGI_2$ synthase (PGIS) [Iwai et al., 1999] contribute to severe pulmonary hypertension. Substitution of prostacyclin, either by overexpression of PGIS [Geraci et al., 1999] in experimental pulmonary hypertension, or application of the stable prostacyclin analogs iloprost [Schermuly et al., 2004;Schermuly et al., 2005c] or beraprost [Itoh et al., 2004], decreased pulmonary arterial pressure and vascular remodeling. Prostacyclin is a product of cyclooxygenases and mediates potent anti-platelet, vasodilator, and anti-inflammatory actions by activating the IP receptor [Vane and Botting, 1995]. However, there is evidence that the lungs of PAH patients have decreased expression of the IP receptor [Hoshikawa et al., 2001]. In this study, the question of how iloprost may work under conditions of low IP receptor expression was
addressed.

Considering that the entire prostacyclin system is altered (for example, decreased levels of the prostacyclin metabolite 6-keto-PGF1α in urine [Christman et al., 1992], decreased expression of prostacyclin synthase [Tuder et al., 1999] and polymorphisms of the PGIS gene [Iwai et al., 1999], the decreased expression of the receptor is important evident. Nevertheless, therapeutic application of prostanoids results in the improvement of survival and hemodynamics in PAH patients, as has been shown to be of benefit in several clinical trials [Olschewski et al., 1996;Barst et al., 1996;Barst et al., 2003;Rubin et al., 1990]. These effects of prostanoids on clinical improvement may be related to non-receptor-mediated effects in the pulmonary vessels (for example, anti-thrombotic effects) or the vasodilation of the less heavily remodeled pulmonary arteries, which may have preserved prostacyclin receptor signaling [Cowan et al., 2000;Tuder and Zaiman, 2002]. Alternatively, receptors other than the prostacyclin receptor could be involved in the mediation of these vasodilatory and vasculoprotective effects[Narumiya et al., 1999;Wilson et al., 2004]. Based on the prostanoid signaling pathway,

the prostanoid receptors can be subdivided into three categories. The relaxant receptors, including the $EP_2$, $EP_4$, and IP receptors, generally cause increases in intracellular cAMP levels and mediate vasodilation [Breyer et al., 2001;Narumiya et al., 1999]. The TP, $EP_1$ and FP receptors are coupled to $Ca^{2+}$ mobilization, while the $EP_3$ receptor is an alternatively spliced gene, with at least eight isoforms identified to date. Depending on the subtype, this receptor can be negatively or positively coupled to Gs [Hata and Breyer, 2004;Narumiya et al., 1999].

This receptor and all other prostanoid receptors are members of the GPCR superfamily and coupled to adenylate cyclase and phospholipase C [Boie et al., 1994;Coleman et al., 1994b;Namba et al., 1994]. To investigate the expression profile of prostanoid receptors and to perform functional experiments, the proximal (vessels >2 mm external diameter) and distal (vessels <1 mm external diameter) PASMC were isolated from MCT-treated rats. This animal model of pulmonary hypertension is characterized by remodeling of the precapillary vessels (medial thickening, and de novo muscularization of small pulmonary arterioles). Due to this mimicry of clinical PAH, the rat MCT model has repeatedly been employed for investigating the acute hemodynamic effects of vasodilators and the chronic anti-remodeling effects of pharmacologically active agents [Schermuly et al., 2005;Schermuly et al., 2007]. As expected, the expression of the differentiation marker desminis decreased during the passage of the cells, while expression of α smooth muscle actin remained constant. Along these lines, certain receptors (for example, IP, $EP_2$, $EP_3$, and FP) have been shown to be regulated, while others stay constant in their expression profile. Previous *in vitro* studies have already suggested the substantial antiproliferative potency of prostacyclin analogs in human PASMCs [Clapp et al., 2002]. 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 are PASMCs from proximal pulmonary arteries (>8 mm external diameter) [Wharton et al., 2000]. Addressing this issue in distal and proximal PASMCs, IP, $EP_3$, FP and TP was decreased in MCT-treated administered rats compared to control.

### Excluding the contribution of EP1 and EP3 receptors in mediating the effects of iloprost

Based on the prostanoid signal pathway, the prostanoid receptors can be subdivided into three categories. Prostanoid receptor gene expression was profiled, and the 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 levels or to elevate $Ca^{2+}$ levels, and are involved primarily in vascular contraction via the $Ca^{2+}$/phospholipase C pathway [Breyer et al., 2001;Narumiya et al., 1999]. Thus, the role of EP1 and EP3 receptor in the iloprost-induced increases in intracellular cAMP level in MCT28d rat PASMCs was excluded.

### Excluding the contribution of EP2 to demonstrate the contribution of the EP4 receptor in mediating the effects of iloprost

The EP2 and EP4 receptors both couple via Gs to induce elevations in intracellular cAMP levels leading to smooth muscle relaxation. The 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 the IP receptor expression is reduced in MCT28d rat PASMCs. Furthermore, prostacyclin, cicaprost and iloprost are generally accepted as selective IP receptor agonists, and they have all been observed to be agonists in the $EP_4$ receptor-expressing cell line (HEK-h$EP_4$) with varying $EP_4$ affinity [Wilson et al., 2004]. In addition, it has been shown that in the piglet saphenous vein which has high levels of the $EP_4$ receptor, iloprost acts as a potent agonist of the porcine $EP_4$ receptor [Wilson and Giles, 2005].

To delineate the contribution of the EP2 and EP4 receptors to iloprost-induced intracellular cAMP accumulation when the IP receptor expression levels are low, the additional functional experiments in MCT28d rat PASMC used AH6809 (a selective EP2 receptor antagonist) and AH23848 (a selective EP4 receptor antagonist) in combination with iloprost. The $EP_4$ antagonist AH23848 potently inhibited the iloprost-induced cAMP level increase in PASMC. This compound is widely used to inhibit the $EP_4$ receptor and to investigate its role [Davis et al., 2004;Lin et al., 2006]. 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 and excluded the contribution of the EP2 receptor in mediating the effects of iloprost. Interestingly, the EP4 receptor is stably expressed in both human PAH and MCT-induced pulmonary
hypertension in rats. On the other hand, we have now demonstrated that the IP receptor is downregulated in human PAH and this fact is in accordance with a previous report that describes the decreased expression of the prostacyclin receptor in PAH [Hoshikawa et al., 2001]. Inhalation of aerosolized iloprost has been shown to cause selective pulmonary vasodilation in pulmonary hypertension [Hoeper et al., 2000;Olschewski et al., 1996;Olschewski et al., 2002]. The major signaling mechanism of iloprost acts via prostacyclin receptors (the IP receptor). However, there is evidence that the lungs of PAH patients have decreased expression of the IP receptor [Hoshikawa et al., 2001]. The question of how iloprost may work under conditions of low IP receptor expression in IPAH?

The EP4 receptor is stably expressed in both human PAH and MCT-induced pulmonary hypertension in rat lungs, suggesting that the EP4 receptor may be an interesting therapeutic target. The signaling mechanism is similar to the IP receptor and involves the well-known cAMP-PKA axis, which results in vasodilation and antiproliferation. Interestingly, iloprost has been documented as an EP4 receptor agonist [Wilson et al., 2004;Wilson and Giles, 2005]. Apart from the IP receptor, iloprost activates the EP4 receptor, which may overcome the effects of downregulation of the IP receptor under disease conditions. The functional experiments revealed that iloprost-induced intracellular cAMP accumulation was inhibited in a dose-dependent manner by AH23848. These results suggest that iloprost mediates vasodilatory functions via EP4 receptor in the case of low IP receptor expression associated with PAH. Our findings suggest a previously-unrecognized mechanism for iloprost and indicate that the EP4 receptor and its pathways may be a potentially novel therapeutic target for the treatment of PAH.

### On the relative importantance of the $PGI_2$ receptor compared to other vasodilators

The regulation of pulmonary vascular tone under physiological conditions is mainly controlled by prostacyclin and nitric oxide and to a minor extent by mediators like arterial and brain natriuretic peptides, vasoactive intestinal peptide (VIP), endothelin or thromboxane [Christman et al., 1992;Tuder et al., 1999].

Disturbances to prostacyclin synthesis, as well as polymorphisms of PGIS [Iwai et al., 1999] have been related to pulmonary hypertension. Prostacyclin and its analogs (iloprost, beraprost, treprostinil) has been shown to improve hemodynamics, clinical status, and survival of patients displaying severe PAH [Olschewski et al., 1996;Olschewski et al., 2002]. The antiproliferative pathways mediated by the IP receptor which upregulate cAMP levels are directly correlated with a regression of smooth muscle cell proliferation. In addition, there is evidence that the nitric oxide (NO) system is dysfunctional as well, either by decreased expression of NOS [Giaid and Saleh, 1995] or low NO bioavailability due to increased oxidative stress [Coggins and Bloch, 2007]. Nitric Oxide synthesized in endothelial cells by endothelial NO synthase (eNOS or NOS3), is an endogenous modulator of pulmonary vasodilator tone and an inhibitor of smooth muscle cell proliferation. This pathway is currently targeted by phosphodiesterase 5 inhibition, which amplifies the NO signal by stabilization of the downstream second messenger cGMP [Ghofrani et al., 2006]. New pharmacological activators of soluble guanylate cyclase may thus further amplify the NO signaling cascade[Dumitrascu et al., 2006]. However, there are no data demonstrating improved survival with long-term inhaled NO treatment, and there is evidence that NO possesses lower vasodilator potency than do the prostanoids in pulmonary hypertension patients [Hoeper et al., 2000;Pepke-Zaba et al., 1991].

Alternatively, peptides including the natriuretic peptides or VIP counteract vasoconstriction, and substitution of these vasodilative and anti-proliferative peptides is currently under clinical development. The VIP acts as a potent systemic and pulmonary vasodilator. However, the clinical application of VIP is limited for two major reasons. First, VIP is susceptible to rapid chemical and biochemical degradation following systemic administration, resulting in low potency and a short duration of action in clinical applications[Onoue et al., 2007;Takubo et al.,

1991]. Second, systemic administration of VIP and its analogs cause cardiovascular side effects [Sergejeva et al., 2004].

Since PAH is a complex disease, targeting a single pathway can not be expected to be uniformly successful. Prostacyclin and its analogs (iloprost, beraprost, treprostinil) have offered beneficial effects in PAH and iloprost is now the first-line drug of PAH therapy, therefore, it defines the more important vasodilator-antiproliferative pathways compared to others.

## 5.2 Prostacyclin analog signal transduction may trigger PPAR-α and PPAR-γ to inhibit nuclear translocation of phosphorylated ERK in anti-proliferative effect on PASMC from rats with pulmonary hypertension

There are multiple signaling options for prostacyclin. Stimulation by the prostanoid pathway is cell-specific, depending not only on the ability of prostacyclin to activate the cell-surface prostacyclin receptor, but also on its ability to act intracellularly via the nuclear PPARs. The aim of the second direction of this study is an investigation of prostacyclin analog activity via PPARs, a non-prostanoid receptor pathway, in PASMC of MCT-induced pulmonary hypertension.

Inhaled iloprost has been shown to be effective for the treatment of PAH, and to provide potent pulmonary vasodilation with minimal systemic side effects and no risk of catheter-related complications. However, there are certain drawbacks, such as the fact that inhaled iloprost lasts only 30 to 90 min, and that six to nine inhalations are needed to achieve good clinical results. Treprostinil is another long-acting stable $PGI_2$ analog, with a duration of action up to three to four hours, and is FDA approved for subcutaneous infusion. The safety and effectiveness of treprostinil were demonstrated in several small clinical trials and one large randomized, controlled trial with 470 patients [Simonneau et al., 2002]. Improvement in exercise capacity, improved indices of dyspnea, a reduction in signs and symptoms of pulmonary hypertension, and improved hemodynamics were noted in the patients who received subcutaneous treprostinil [Simonneau et al., 2002]. In addition, the patients experienced improved functional classification and exercise tolerance, without reported adverse effects [Voswinckel et al., 2006].

Treprostinl is growing in importance in the treatment of pulmonary hypertension, but the signaling mechanism is still not clear. Therefore, in the second part of this thesis, I first established a PASMC proliferation assay with treprostinil. The results showed that treprostinil can more potently inhibit PASMC proliferation than iloprost. For the regulation of cell proliferation, phosphorylated ERK must translocate to the nucleus. The ERK nuclear translocation can be suppressed via cAMP-mediated arrest of cell proliferation [Li et al.,2004]. In this study, the result showed that treprostinil can inhibit pulmonary hypertensive rat

PASMC proliferation by blocking nuclear tranlocation of phopho-ERK. In a previous study, it was shown that the $EP_4$ receptor may take over the function of the IP receptor in the remodeled vessels of pulmonary hypertensive subjects. The prostacyclin analog iloprost increases cAMP levels in smooth muscle cells by binding to the $EP_4$ receptor [Lai et al., 2008]. To investigate whether treprostinil may act on the EP4 receptor to increase cAMP generation, I then used the EP4 antagonist AH23848 in a study of treprostinil-mediated cAMP accumulation. Interestingly, the intracellular cAMP levels elevated by iloprost were dose-dependently reduced by AH23848 treatment, but not with treprostinil, suggesting a role for the EP4 receptor in iloprost-induced cAMP generation. Recently, Clapp and coworkers utilized HEK-293 cells stably expressing the IP receptor to show treprostinil potently inhibited proliferation of PASMC via a cAMP-independent pathway, and that PPAR-γ was activated through the IP receptor via a cyclic AMP-independent mechanism and contributed to the antiproliferative effect of prostacyclin analog [Clapp et al., 2002]. However, in a previous study, the expression of the IP receptor was markedly reduced under conditions of both experimental and clinical pulmonary hypertension. The results of this study etamine whether prostacyclin analogs exert thus effects via non-prostanoid receptor pathways.

Currently, there is a growing body of evidence indicating that prostacyclin analogs function via non-prostanoid receptor pathways. Prostacyclin and it agonists, such as iloprost and treprostinil, have potent vasodilatory and anti-proliferative effects in the cardiovascular system. A stable prostacyclin agonist also can also act as the binding ligand to nuclear PPARs [Falcetti et al., 2007;Hatae et al., 2001]. To investigate prostacyclin analog activity exerted via non-prostanoid receptor pathways associated with pulmonary hypertension, immunoblotting was performed to investigate the downregulation of PPAR expression in IPAH patients and PASMC from MCT-treated rats. Treprostinil had a stronger antiproliferative effect than did iloprost, and prevented the nuclear translocation of phosphorylated ERK. However, the intracellular cAMP levels elevated by iloprost were dose-dependently reduced with AH23848 treatment but not treprostinil, suggesting a role for the EP4 receptor in iloprost-induced cAMP generation. Moreover, treprostinil activated PPAR-α and PPAR-γ, but not PPAR β/δ.

Several reports have shown a reduced lung tissue PPARγ gene and protein expression in lungs from patients with severe PH, and a loss of PPARγ expression in the complex vascular lesions

characteristic of PAH [Hansmann et al., 2008]. In addition, the total PPARγ mRNA has been reported to be decreased in patients with severe pulmonary hypertension when compared with normal lung tissue or tissue from patients with emphysema, suggesting that a lack of PPARγ expression is a marker of an abnormal endothelial cell phenotype, and that a lack of PPARγ expression inhibits apoptosis and facilitates endothelial cell growth and angiogenesis [Ameshima et al., 2003;Hansmann et al., 2008]. Taken together, the work presented here is important because it showed for the first time that the downregulation of PPAR expression in IPAH patients and PASMC from MCT-treated rats. In addition, the results suggest that treprostinil activates PPAR-α and PPAR-γ to reverse smooth muscle cell proliferation.

The prostacyclin signaling interaction between the cell surface receptor and nuclea proteins is still not clear. There are multiple signaling options for prostacyclin. Following stimulation by prostanoids, the receptor of the cell are cell-specific, depending not only on the ability of prostacyclin to activate the cell surface prostacyclin receptor, but also on its ability to act intracellularly via the nuclear PPARs. The PPAR proteins may play an important role in the regulation of cell differentiation and growth, particularly in the lung [Becker et al., 2006]. Various groups have established that PPARs are expressed to varying degrees in endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and fibroblasts [Ali et al., 2006;Falcetti et al., 2007;Hansmann et al., 2007;Hansmann et al., 2008]. Further work is required to define which genes in these cellular settings are regulated through PPARs, through what mechanisms, and whether such actions are at work *in vivo*, either in endogenous signaling or in response to pharmacologic agents.

## 5.3 Conclusion

The major part of this investigation has shown that the $EP_4$ 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 levels in smooth muscle cells by binding to the $EP_4$ receptor. The thesis reports research findings on prostacyclin receptor modulation of pulmonary vascular remodeling in clinical and experimental PAH with an important melding of clinical data from human samples and data from animal models of disease. This finding provides a previously unrecognized mechanism for iloprost and the prospect that the EP4 receptor may be a novel therapeutic approach for the treatment of PAH, and has been published in the *Am.J.Respir.Crit Care Med* [Lai et al., 2008]. In addition, these results also indicate that IPAH patients lack PPARs and a similar expression pattern was observed in MCT-induced PAH. Treprostinil might be a ligand for the nuclear receptor PPARs and mediate antiremodeling effects via the PPAR-α and PPAR-γ associated with PAH.

Pulmonary arterial hypertension is a multifactorial disease based on various molecular and cellular disturbances. Prostacyclin and its analogs have been shown to extend the survival of patients with PAH. However, it is not yet clear if prostacyclin analogs exert effects only via a single prostanoid receptor pathway, or also operate via various prostanoid receptors or non-prostanoid receptor pathways.

Future research on the pathobiology of PAH and the strategy for the treatment of PAH should focus on the definition of the relative importance and on the interactions between the different pathways. Additionally, the intermediate steps involved in prostacyclin transduction signals from membrane to the cytoplasm to the nuclear are going to be explored in order to better understand how regressed prostacyclin signaling prevent in hypertensive pulmonary vascular disease.

## 6. Summary

Chronic pulmonary hypertension is characterized by vascular remodeling and perivascular inflammation. In clinical and experimental studies with inhaled or systemically-administered prostanoids, it has been shown that prostacyclin, iloprost or treprostinil reduce pulmonary arterial pressure, increase cardiac output and increase the exercise capacity. Prostacyclin and its analogs work mainly by binding to the prostacyclin receptor (IP), which belongs to the family of G-protein coupled receptors. Activation of the receptor leads to an elevation of intracellular cAMP by activation of adenylate cylase. In the first part of my thesis, the expression of the different prostanoid receptors was investigated in lungs and smooth muscle cells of pulmonary hypertensive rats and lungs from patients undergoing lung transplantation due to idiopathic pulmonary arterial hypertension. Interestingly, the expression of the prostacyclin receptor was markedly reduced under the conditions of both experimental and clinical pulmonary hypertension, while other prostaglandin receptors, such as the EP4 receptor, were unchanged in their expression. In the second part, functional experiments were performed which show that iloprost and treprostinil reduce serum-induced proliferation of rat pulmonary arterial smooth muscle cells (PASMC). In addition, the iloprost-induced cAMP-production of PASMCs, but not that of treprostinil, could be blocked by the EP4 receptor antagonist AH23848, suggesting that iloprost, at least in part, acts via the EP4 receptor. An investigation was carried out on treprostinil, which is known to activate nuclear peroxisome proliferator-activated receptors (PPARs) in addition to the prostanoid receptors. Interestingly, the PPARs were downregulated in experimental and clinical pulmonary hypertension but treprostinil induced PPAR-α and PPAR-γ suggesting a potential role for a prostanoid receptor-independent mechanism of treprostinil. Taken together, the prostacyclin receptor is downregulated in experimental and clinical pulmonary hypertension and a novel role is indicated for the EP4 receptor in the signaling of iloprost, a clinically-approved prostacyclin analog. The major results of thesis were published in *Am J Respir Crit Care Med.* in July 2008.

## 7. Zusammenfassung

Die chronische pulmonale Hypertonie ist eine eigenständige Erkrankung oder stellt die gemeinsame Endstrecke einer Vielzahl von degenerativen und inflammatorischen Lungenerkrankungen, dar. In klinischen und experimentellen Studien konnte gezeigt werden, dass gefäßerweiternde Substanzen, wie zum Beispiel Prostanoide, hochwirksame pulmonal drucksenkende Wirkstoffe sind. Dies führte nach erfolgreich durchgeführten präklinischen und klinischen Studien zur Zulassung von intravenös verabreichtem Prostazyklin und inhalativem Iloprost zur Therapie verschiedener Formen der chronischen PH. Prostazyklin sowie dessen Analoga wirken vornehmlich über den Gs-Protein gekoppelten Prostazyklinrezeptor, der nach Bindung des Liganden zu einer Erhöhung des Botenstoffes cAMP führt. Im ersten Teil der vorliegenden Arbeit wurde die Expression verschiedener Prostanoid rezeptoren in Lungen pulmonalhypertensiver Ratten und humanem Lungengewebe untersucht. Als wesentlicher Befund konnte gezeigt werden, dass der Prostazyklin Rezeptor in diesen Geweben herunter reguliert ist, während andere Rezeptoren, wie der Prostaglandin E4 Rezeptor (EP4 Rezeptor) stabil exprimiert werden. Im zweiten Teil der Arbeit wurden funktionelle Untersuchungen mit den klinisch verfügbaren Prostazyklin Analoga Iloprost und Treprostinil durchgeführt. Beide Substanzen senkten die Serum-induzierte Proliferation von glatten pulmonalarteriellen Muskelzellen. Darüber hinaus konnte im Gegensatz zu Treprostinil der Iloprost-induzierte cAMP Anstieg durch den EP4 Rezeptorantagonisten AH23848 unterdrückt werden, was auf eine Rolle des EP4 Rezeptors in der Signaltransduktion von Iloprost hinweist. Im dritten Teil der Arbeit wurde die Wirkungsweise von Treprostinil weiter untersucht, das bekannterweise ein Ligand der Peroxisome Proliferator-Activated Receptors (PPARs) ist, welche unter Bedingungen der pulmonalen Hypertonie niedriger exprimiert sind. Dennoch führt Treprostinil zu einer Translokation von PPAR-α and PPAR-γ in den Zellkern, was auf einen Prostanoid-Rezeptor unabhängigen Signalweg hinweist. Zusammengefasst konnte gezeigt werden, dass der Prostazyklin Rezeptor in der pulmonalen Hypertonie herunter reguliert ist und dass das klinisch zugelassene Iloprost möglicherweise neben dem IP Rezeptor auch über den EP4 Rezeptor wirkt, was die zukünftige Entwicklung neuer Prostanoide beeinflussen kann.

## 8. References


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# 9. Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

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**Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.**

**The curriculum vitae was removed from the electronic version of the paper.**

## Publications

1. **Protective Effect of Propylthiouracil Independent of Its Hypothyroid Effect on Atherogensis in Cholesterol-Fed Rabbits: PTEN Induction and Inhibition of Vascular Smooth Muscle Cell Proliferation and Migration**
   WJ Chen, KH Lin, **YJ Lai,** SH Yang, and JH S Pang . *Circulation*. **2004** Sep 7; 110(10):1313-9. (Impact Factor: 12.56 )
2. **Reversal of experimental pulmonary hypertension by PDGF inhibition.**
   Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, Sydykov A, **Lai YJ**, Weissmann N, Seeger W, Grimminger F. *J Clin Invest*. **2005** Oct;115(10):2811-21. (Impact factor 15.754)
3. **Partial reversal of experimental pulmonary hypertension by phosphodiesterase 3/4 inhibition.**
   Dony E, **Lai YJ**, Dumitrascu R, Pullamsetti SS, Savai R, Ghofrani HA, Weissmann N, Schudt C, Flockerzi D, Seeger W, Grimminger F, Schermuly RT. *Eur Respir J.* **2008** Mar;31(3):599-610. (Impact factor 5.076)
4. **Role of the prostanoid EP4 receptor in iloprostprost-mediated vasodilatation in pulmonary hypertension**
   **Lai YJ,** Pullamsetti SS, Dony E, Weissmann N, Butrous G, Banat GA, Ghofrani HA, Seeger W, Grimminger F, Schermuly RT. *Am J Respir Crit Care Med.* **2008** Jul 15;178(2):188-96. (Impact factor 9.091)

## Poster Presentation

1. ***Bacteroides forsythus* Lipopolysaccharide Induces Expression of Cell Adhesion Molecules in Human Vascular Endothelial Cell**
   **YJ Lai,** MY Wong, KL Lou, YY Shiau, WJ Chen, CJ Chang and JH S. Pang
   International Association for Dental Research Meeting 2002 Hong Kong, China
2. **Intracelluar Studies of a Tamoxifen Based Targeting Delivery System**
   **YJ Lai**, SD Li , AJ Wang. World Congress for Chinese Biomedical Engineers Conference 15-17 Oct 2004, Taipei ,Taiwan
3. **Role of prostanoid EP4 receptor in iloprostprost mediated vasodilation in pulmonary hypertension**
   **YJ Lai,** SS Pullamsetti, E Dony, N Weissmann, HA Ghofrani, W Seeger , F Grimminger, RT Schermuly. American Thoracic Society, 18-23 May 2007, San Francisco USA

PhD programme 2005

**The total credit hours of lectures, seminars and practical sessions are**

**491 credit hours**

| College/Institute | Type of training | From (mth/yr) | To (mth/yr) |
|---|---|---|---|
| Graduate program " molecular biology and medicine of lung" the Justus Liebig University of Giessen, Germany | molecular biology and medicine of lung | WS 2004 | SS 2005 |
| Graduate program " molecular biology and medicine of lung" the Justus Liebig University of Giessen, Germany | Pharmacology of the lung, practical course in Dr RT Schermuly's lab | WS 2004 | SS 2005 |
| Graduate program: Vascular medicine, Physiologisches Institut, the Justus Liebig University of Giessen, Germany | The seminar "Anatomy and Physiology of vascular systems" | WS 2005 | WS 2006 |
| Graduate program: Vascular medicine, Physiologisches Institut, the Justus Liebig University of Giessen, Germany | Practical method in cell biology research II | 20, Feb, 2005 | 24, Feb 2005 |
| Graduate program: Vascular medicine, Physiologisches Institut, the Justus Liebig University of Giessen, Germany | Stem cell culture, in Dr H Sauer's Lab | 05,Dem,2005 | 09,Dem, 2005 |
| Graduate program: Vascular medicine, Physiologisches Institut, the Justus Liebig University of Giessen, Germany | The seminar "Vascular biology" | 19,Apr, 2006 | 12,July, 2006 |
| Institute of Anatomy and Cell Biology, Medical School, the Justus Liebig University of Giessen, Germany | The seminar"Tissues and Organs" | 20, Oct, 2006 | 15, Feb, 2007 |
| Graduate program: Vascular medicine, Physiologisches Institut, the Justus Liebig University of Giessen, Germany | The seminar " Cell biology of the vascular systems" | 01,Nov,2006 | 10, Jan, 2007 |
| Research training group 543 on biological basis of vascular medicine of the german research foundation, Physiologisches Institut, | Statistical course/ Statistical consulting practical course held by Dr Gerrit Eichner | WS 2006 | WS 2007 |
| Research training group 543 on biological basis of vascular medicine of the german research foundation, Physiologisches Institut, the Justus Liebig University of Giessen, Germany | The seminar "Pathology and clinic of vascular diseases" | 18,Apr 2007 | 18,July, 2007 |

# A.1 Materials

## A.1.1 Enzymes, antibodies and inhibitors

### A.1.1.1 Enzymes for molecular biology

| | |
|---|---|
| Taq DNA-polymerase | Promega (Germany) |
| Protease cocktail inhibitor | Roche (Germany) |

### A.1.1.2 Antibodies

**Primary antibodies:**

| | |
|---|---|
| α-smooth muscle actin | Sigma (Germany) |
| Desmin | Neomarkers (USA) |
| EP4 receptor | Sigma (Germany) |
| GAPDH | Novus (USA) |
| IP receptor | Acris (Germany) |
| IP receptor | Sigma (Germany) |
| P-ERK | Santa Cruz (USA) |
| PPAR-α | Abcam (Germany) |
| PPAR-β/ δ | Abcam (Germany) |
| PPAR-γ | Santa Cruz (USA) |

**Secondary antibodies:**

| | |
|---|---|
| Anti-mouse IgG, peroxidase-conjugated | Sigma (Germany) |
| Anti-rabbit IgG, peroxidase-conjugated | Sigma (Germany) |

### A.1.1.3 Inhibitors and drugs

| | |
|---|---|
| AH23848 | Sigma (Germany) |
| AH6809 | Sigma (Germany ) |
| Ammonium persulfate | Sigma (Germany) |
| Aprotinin | Sigma (Germany) |
| Diethyl pyrocarbonate (DEPC) | Sigma (Germany) |
| Leupeptin | Sigma (Germany) |

| | |
|---|---|
| Penicillin/ streptomycin | PAN biotech (Germany) |
| Pepstatin | Sigma (Germany) |
| Trypsin/EDTA | PAN Biotech (Germany) |

### A.1.2 Reagents

| | |
|---|---|
| 100 bp DNA ladder marker | MBI Fermentas (Germany) |
| Acetone | Sigma (Germany) |
| Agarose (electrophoresis grade) | Sigma (Germany) |
| β-mercaptoethanol | Sigma (Germany) |
| BSA (bovine serum albumin) | Roth (Germany) |
| Boric acid | Sigma (Germany) |
| Calcium chloride | Sigma (Germany) |
| Chloroform | Sigma (Germany) |
| Coomassie-brilliant-blue R250 | Sigma (Germany) |
| Dakocytomation Faramount mounting medium | Dako (Germany) |
| Diethyl pyrocarbonate (DEPC) water | Roth (Germany) |
| Deoxy nucleotide mix (dNTPs) | Promega (Germany) |
| Dimethyl sulfoxide (DMSO) | Sigma (Germany) |
| 1, 4-Dithiothreitol (DTT) | Fluka (Germany) |
| Dulbecco's modified Eagle's-Ham's F-12 medium (DMEM/F-12) | GIBCO (Germany) |
| Dulbecco's phosphate. buffered saline (D-PBS) | PAN (Germany) |
| Earle’s Balanced Salt Solution (EBSS) | Gibco (Germany) |
| ethylene-bis (oxyethylenenitriloprost) tetraacetic acid (EGTA) | Sigma (Germany) |
| Ethanol | Fluka (Germany) |
| Ethidium bromide | Sigma (Germany) |
| Ethylenedinitriloprost tetraacetic acid (EDTA) | Sigma (Germany) |
| Fetal bovine serum (FBS) | Bio west (France) |
| Formamide | Sigma (Germany) |

| | |
|---|---|
| Formaldehyde | Sigma (Germany) |
| Glacial acetic acid | Sigma (Germany) |
| Glycine | Sigma (Germany) |
| Glycerol | Sigma (Germany) |
| Goat serum | Sigma (Germany) |
| Hanks' Balanced Salt Solution (HBSS) 1× | Gibco (Germany) |
| Hematoxylin, Mayer's | DAKO (Germany) |
| $^{3}$H-Thymidine | Amersham (Germany) |
| Hydrogen peroxide (30% solution) | Merck (Germany) |
| 3-isobutyl-1-methylxanthine (IBMX) | Sigma (Germany) |
| Isopropanol | Merck (Germany) |
| L-glutamine | PAN (Germany) |
| Methanol | Fluka (Germany) |
| NP-40 | Sigma (Germany) |
| Paraformaldehyde | Sigma (Germany) |
| Phenylmethylsulfonylfluoride (PMSF) | Fluka (Germany) |
| Rainbow Molecular Weight Markers | Amersham (Germany) |
| Scintillation liquid (Rotiszint Eco Plus) | Roth (Germany) |
| Sodium acetate | Sigma (Germany) |
| Sodium chloride | Sigma (Germany) |
| Sodium dodecylsulfate (SDS solution, 10% W/V) | Promega (USA) |
| Sodium hydroxide (NaOH) | Merck (Germany) |
| Sodium pyrophosphate | Sigma (Germany) |
| TEMED (tetramethylethylenediamine) | Sigma (Germany) |
| Trichloroacetic acid (TCA) | Sigma (Germany) |
| Tris-base | Sigma (Germany) |
| Tris-HCl 1.5M PH8.8 | Amresco (USA) |
| Tris-HCl 0.5M PH6.8 | Amresco (USA) |
| Tris HCl | Sigma (Germany) |
| Triton X-100 | Merck (Germany) |

| | |
|---|---|
| Trizol® | Invitrogen (Germany) |
| Trizma base | Sigma (Germany) |
| Tween 20 | Sigma (Germany) |
| Ventavis® (Iloprost) | Schering (Germany) |
| Xylol | Merck (Germany) |

### A.1.3 Kits

| | |
|---|---|
| DAKO labeled streptavidin-biotin system | Dako (Germany) |
| ECL Western Blotting System | Amersham (Germany) |
| ImProm-II ™ Reverse Transcription System | Promega (USA). |
| RIA cyclic AMP ($^{125}$I) kit | Immunotech (France) |
| Smooth muscle cell growth medium 2 kit | PromoCell (Germany) |
| Substrate-chromogen kit | Dako (Germany) |

### A.1.4 Host species

Homo sapiens (Human)

Sprague-Dawley rat (SD rat)

### A.1.5 Eukaryotic cells

Human vascular smooth muscle cell

SD rat vascular smooth muscle cell

### A.1.6 Oligonucleotides

All the oligonucleotides for PCR (polymerase chain reaction) were purchased from Metabion (Martinsried) Germany. The following are the primer sequences used to evaluate the expression of the respective genes.

**Rat smooth muscle alpha-actin (SM α--actin） (NM_031004)**

sense 5'- CGA TAG AAC ACG GCA TCA TC -3'

antisense 5'- CAT CAG GCA GTT CGT AGC TC -3'

Annealing temperature: 57.5 °C

Size of the PCR product: 525 bp

**Rat desmin (NM_022531)**

sense 5'- ACC TGC GAG ATT GAT GCT CT -3'

antisense 5'- CGG GTC TCA ATG GTC TTG AT -3'

Annealing temperature: 57.5 °C

Size of the PCR product: 368 bp

**Rat prostaglandin-endoperoxide synthase 2 (Cox-2) (NM_017232)**

sense 5'- ACT GTA CCG GAC TGG ATT CTA -3'

antisense 5'- CCA TCC TGG AAA AGT CGA AG -3'

Annealing temperature: 55.0 °C

Size of the PCR product: 580 bp

**Rat prostacyclin receptor (IP receptor)**

**(NM_001077644)**

sense 5'- TCA CGA TCA GAG GAT TCA CG -3'

antisense 5'- ATT CCC ACA GAA CAG CCA TC -3'

Annealing temperature: 57.5 °C

Size of the PCR product: 358 bp

**Rat prostaglandin E receptor 2 (EP2 receptor) (NM_013100)**

sense 5'- ACT GCC ACC TTC CTG TTG TT -3'

antisense 5'- GCC CAA GGC TAA TGA AAC AC -3'

Annealing temperature: 55.0 °C

Size of the PCR product: 373 bp

**Rattus prostaglandin E receptor 3 (EP3 receptor) (NM_012704)**

sense 5'- TAT GCC AGC CAC ATG AAG AC -3'

antisense 5'- CAC ATG ATC CCC ATA AGC TG -3'

Annealing temperature: 55°C

Size of the PCR product: 374 bp

**Rattus prostaglandin E receptor 4 (EP4 receptor) (NM_032076)**

sense 5'- AGT GAC CAT CGC CAG ATA CA -3'

antisense 5'- ATG TAA GAG AAG GCG GCG TA -3'

Annealing temperature: 57.5°C

Size of the PCR product: 339 bp

**Rattus thromboxane A2 receptor (TP receptor) (NM_017054)**

sense 5'-TGT GAG GTG GAG ATG ATG GT -3'

antisense 5'-AGG TCG TTA GCA GTC ACC AA -3'

Annealing temperature: 55.0 °C

Size of the PCR product: 369 bp

**Rattus prostaglandin F receptor (FP receptor) (NM_013115)**

sense 5'-TCA CGG GAG TCA CAT TTT G -3

antisense 5'- TGA GTT CCC AGA TGT GCA AG -3'

Annealing temperature: 55.0 °C

Size of the PCR product: 342 bp

**Rattus peroxisome proliferator-activated receptor alpha (PPAR α)** (**NM_013196**)

sense 5'-TCACACAATGCAATCCGTTT-3'

antisense 5'-ACTGGCAGCAGTGGAAGAAT-3'

Annealing temperature: 57°C

Size of the PCR product: 358 bp

**Rattus peroxisome proliferator-activated receptor beta/delta (PPAR β/δ)** (**NM_013121**)

sense 5'-TGTCAACAAAGACGGACTGC-3'

antisense 5'-TCTTCAGCCACTGCATCATC-3'

Annealing temperature: 55 °C

Size of the PCR product: 374 bp

**Rattus peroxisome proliferator activated receptor gamma (PPAR γ) (NM_013124)**

sense 5'-TTCAGAAGTGCCTTGCTGTG-3'

antisense 5'-ACTGGCACCCTTGAAAAA-3'

Annealing temperature: 57 °C

Size of the PCR product: 361 bp

**Rattuse glyceraldehyde-3-phosphate dehydrogenase** (**GAPDH) (NM_017008)**

sense 5'-TTCAT TGACC TCAAC TACAT-3'

antisense 5'-GAGGG GCCAT CCACA GTCTT-3'

Annealing temperature: 57.5 °C

Size of the PCR product: 469 bp

### A.1.7 Buffers, Media and other solutions

**Preparation of RNase-free glass bottles**

Measure water into RNase-free glass bottles.

Add 0.01% (v/v) diethylpyrocarbonate (DEPC)

Autoclave and allow to stand overnight

**10× TBS (pH=7.6)**

| | |
|---|---|
| Tris-base (100 mM) | 12.11 g |
| NaCl (150 mM) | 87.66 g |

Make up the volume to 1 l with $ddH_2O$

**1× TBST (wash buffer)**

| | |
|---|---|
| Tris | 4.48 g |
| NaCl | 7.70 g |
| EDTA | 3.6 g |
| Tween (0.05%) | 1 ml |
| $ddH_2O$ | 1 liter |

**SDS-page gel (10% Separating gel)**

**Stacking gel**

| | |
|---|---|
| Acrylamide/Bis | 0.75 ml |
| $ddH_2O$ | 2.9 ml |
| Stacking gel buffer (Tris 0.5 M, pH=6.8) | 1.25 ml |
| 10% SDS (W/V) | 0.05 ml |
| 10% ammonium persulfate | 0.05 ml |
| TEMED | 5 µl |

**Separating gel**

| | |
|---|---|
| A-B | 2.5 ml |
| $ddH_2O$ | 2.95 ml |
| Separating gel buffer (Tris 1.5 M, pH=8.8) | 1.875 ml |
| 10% SDS | 0.075 ml |
| 10% ammonium persulfate | 0.075 ml |
| TEMED | 7.5 µl |

**5× SDS-loading butter**

| | |
|---|---|
| Tris HCl, PH=6.8 | 2.5 ml |
| 10% SDS | 4.0 ml |
| Glycerol | 2.0 ml |
| β-mercaptoethanol | 1.0 ml (14.3 M) |
| 10% bromophenol blue | 200 µl |

**10× SDS-Page running butter**

| | |
|---|---|
| Tris | 6.0 g |
| Glycine | 28.8 g |
| SDS 10% | 20 ml |

Make up the volume to 2 l with $ddH_2O$

**1× SDS-page blotting butter**

| | |
|---|---|
| Tris | 6.0 g |
| Glycine | 3.0 g |
| Methanol | 200 ml |

Make up the volume to 1 l with $ddH_2O$

**Blocking buffer**

| | |
|---|---|
| TBS-T | 100 ml |
| Milk powder | 5 g |

**10% BAS blocking buffer**

| | |
|---|---|
| BSA powder | 20 g |
| Make up the volume to 200 ml with 1×PBS | |

**50× TAE butter (Tris-Acetate-EDTA)**

| | |
|---|---|
| Tris base | 242 g |
| Glacial acetic acid | 57.1 ml |
| EDTA | 18.6 g |
| Make up the volume to 1 l with $ddH_2O$ | |

**PASMCs culture media**

| | |
|---|---|
| DMEM-F12 | 450 ml |
| FCS | 50 ml |
| Glutamine | 5 ml |
| PEN STREP | 5 ml |

**Membrane protein lysis buffer**

| | |
|---|---|
| Tris-HCl (pH=7.4) | 20 mM |
| NaCl | 100 mM |
| EDTA | 1 mM |
| Nonidet P-40 | 0.1% V/V |
| sodium deoxycholate | 0.05% W/V |
| SDS | 0.025% W/V |
| Triton X-100 | 0.1% V/V |
| PMSF | 0.1 mg/ ml |
| Leupeptin | 10 μg/ml |
| Aprotinin | 25 μg/ml |

### A.1.8 Equipments

| | |
|---|---|
| 10 cm cell culture dish | Falcon BD GmbH (Germany) |
| 6 well cell culture dish | Falcon BD GmbH (Germany) |
| pure Nitrocellulose blotting membrane | PALL life sciences (Germany) |
| 10 ml pipettes | Falcon (Germany) |
| 5 ml pipettes | Falcon (Germany) |
| PCR 200 µl Eppendorf tube | Eppendorf (Germany) |
| 1.5 ml Eppendorf tube | Greiner (Germany) |
| 2.0 ml Eppendorf tube | Greiner (Germany) |
| 5 ml pipettes | Falcon (Germany) |
| 15 ml tube, type 2095 | Falcon (Germany) |
| 50 ml tube, type 2070 | Falcon (Germany) |
| Carbogen gas (95% O2/5% CO2) | Air Liquide (Germany) |
| Hyperfilm ECL | Amersham (Germany) |
| Filter tips (10 µl/ 100 µl/ 1000 µl) | Nerbe plus (Germany) |
| Liquid nitrogen | Air Liquide (Germany) |
| X-Ray film | AGFA (Germany) |
| Uvette | Eppendorf AG (Germany) |

### A.1.9 Instruments

| | |
|---|---|
| Anatomy microscope (SM 22-S12) | Hund (Germany) |
| Aqua-stabil | Julabo (Germany) |
| BioDocAnalyze | Biometra (Germany) |
| Bio-Photometer | Eppendof (Germany) |
| Centrifuge (Biofuge fresco) | Heraeus (Germany) |
| Cold light source FLQ 150 M | Hund (Germany) |
| Curix HX 530 U X-ray developer | AGFA (Germany) |
| Direct-Q Water Purification System (Q3) | Millipore (Germany) |
| DNA/ RNA Electrophoreis unit | Biometra (Germany) |
| Hemocytometer | Labor Optik (Germany) |
| Homogenizator (DIAX 900) | Heidolph (Germany) |
| Hood (Hera safe 9) | Heraeus (Germany) |
| Incubator (Hera cell 240) | Heraeus (Germany) |
| Incubator Lab-Therm | Heraeus (Germany) |
| Light Microscope | Hund (Germany) |
| ND-1000 UV-Vis spectrophotometer | Peqlab (Germany) |
| Net electrotransfer unit | BioRad (Germany) |
| PCR-thermocycler | Biometra (Germany) |
| pH-meter 766 | Knick (Germany) |
| SDS-PAGE Electrophoresis unit | BioRad (Germany) |
| -Mini-protein 3 tetra cell | |
| -Mini-trans Blot electrophortic transfer cell | |
| -PowerPac | |
| Shaker WT17 | Biometra (Germany) |
| Spectrophotometer Nano ND-1000 | Peqlab (Germany) |
| Tank Arpege 40 (for liquid nitrogen) | Air Liquide (Germany) |
| Tissue processor Leica TP 1050 | Leica (Germany) |
| Votex | VWR lab (Germany) |

# 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

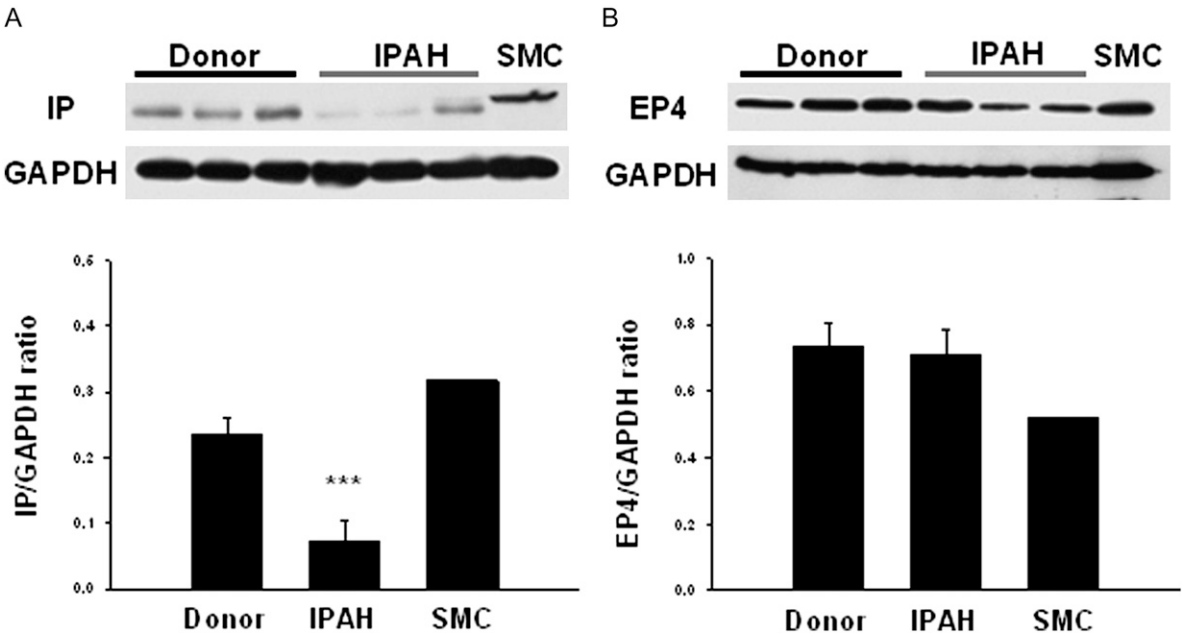


*Figure 1.* IP and EP4 receptor protein level in human donor and idiopathic pulmonary arterial hypertension (IPAH) lung. (*A*) The IP receptor protein was detected in lung tissues as a 52-kD band, and was decreased in IPAH lung tissues as compared with donor lung tissue. (*B*) The EP4 receptor protein 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.

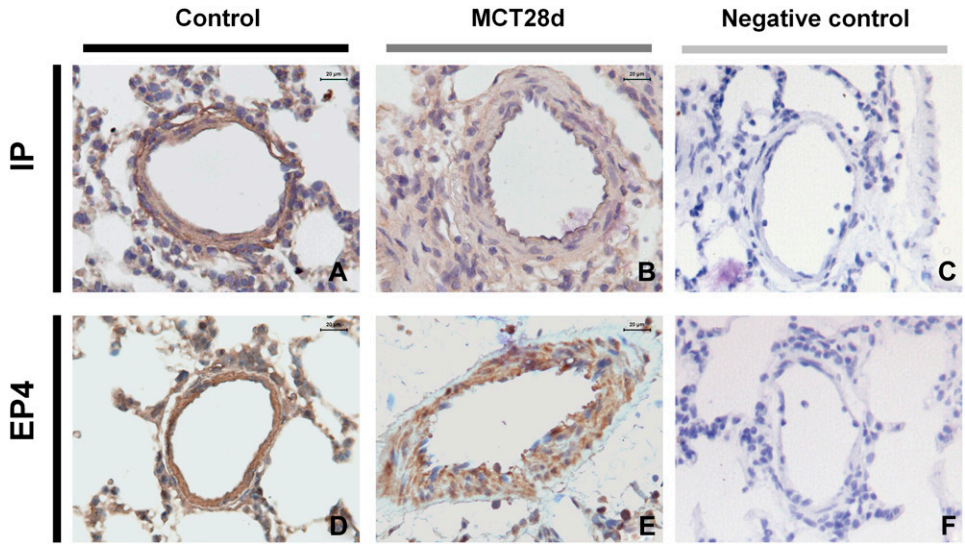


*Figure 2.* Immunohistochemical localization of IP and EP4 receptor in control and MCT28d rat lungs An analysis by immunohistochemistry of the IP and EP4 receptor was performed on lung sections of control and MCT28d rats. The IP receptor was expressed in the pulmonary arteries of control lungs (*A*). 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.

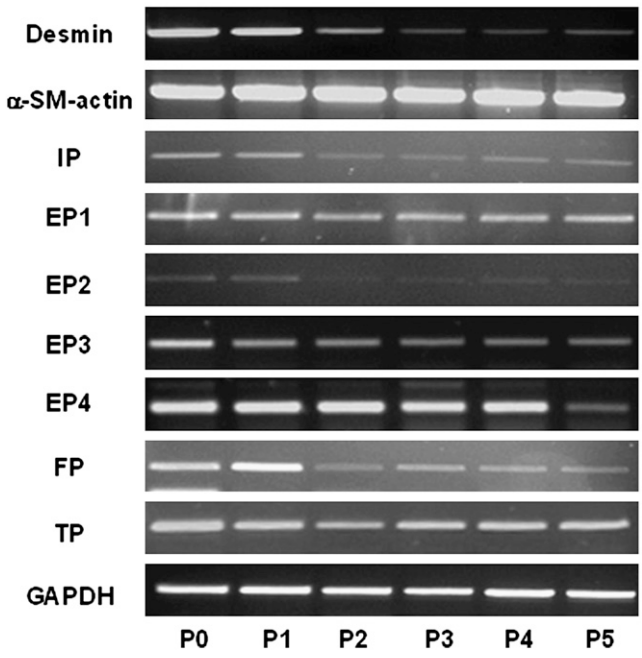


*Figure 3.* Prostanoid receptor gene profile of control rat pulmonary arterial smooth muscle cells (PASMCs) in different passages. Representative reverse transcriptase–polymerase chain reaction analysis. After passage 2, the mRNA expression levels of the IP, EP2, EP3, and FP receptors were reduced in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

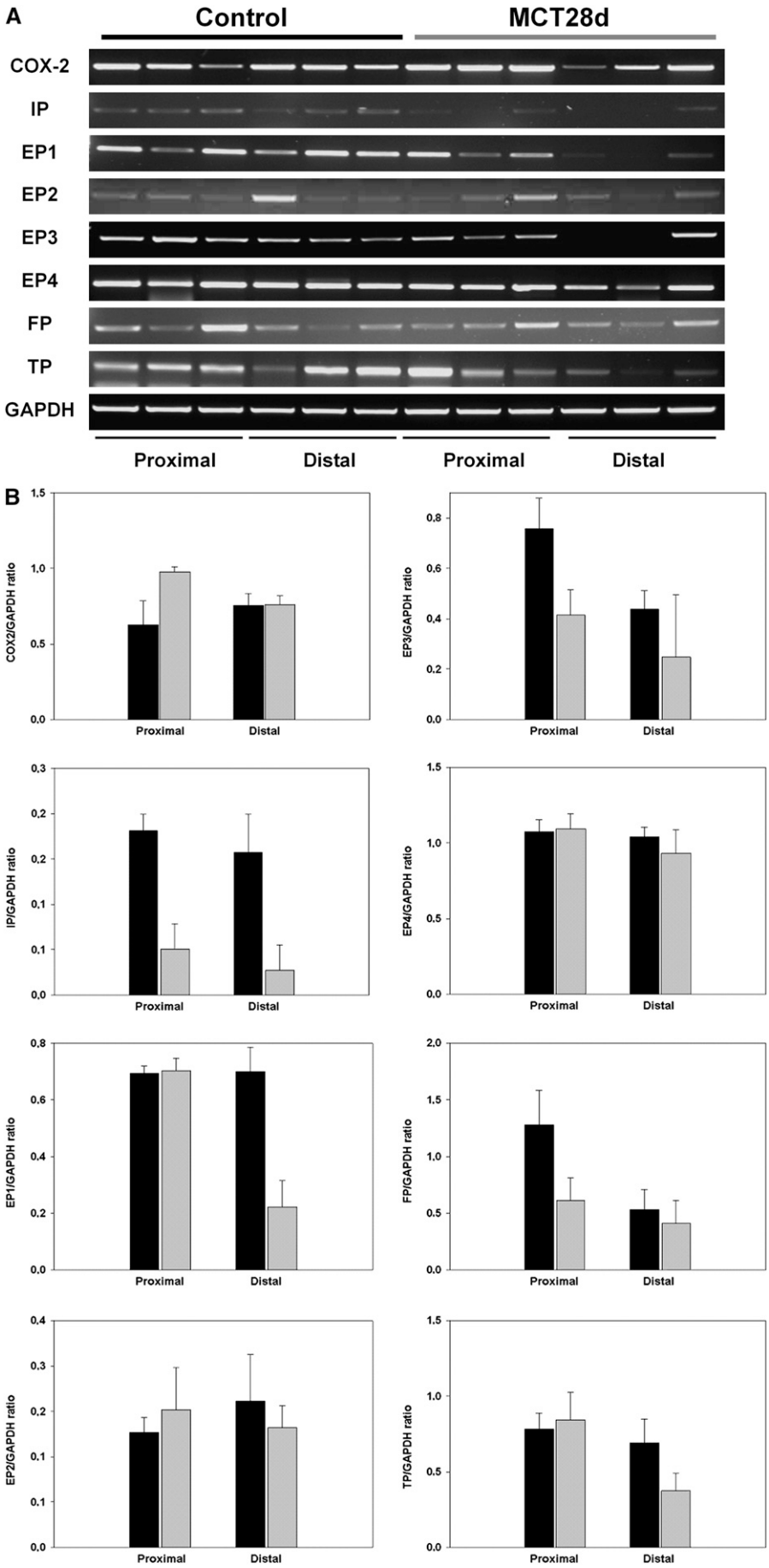


***Figure 4.*** The prostanoid receptor gene profile of distal and proximal pulmonary arterial smooth muscle cells (PASMCs). (*A*) Representative reverse transcriptase–polymerase chain reaction analysis. The mRNA expression of prostanoid receptors in the proximal and distal portion of PASMCs that were 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.

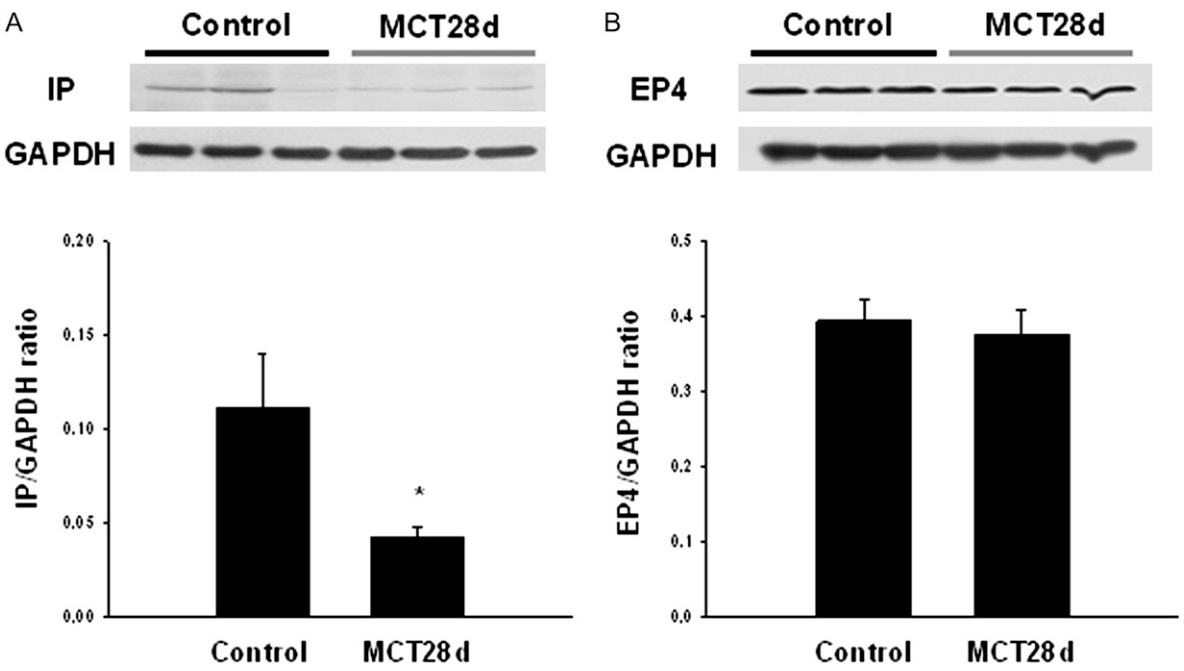


*Figure 5.* Immunoblotting for IP and EP4 receptors in primary pulmonary arterial smooth muscle cells (PASMCs) from control and MCT28d rats. (*A*) Densitometric analysis from three different experiments in each group. The IP receptor was identified as a 52-kD immunoreactive band that was decreased in 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).

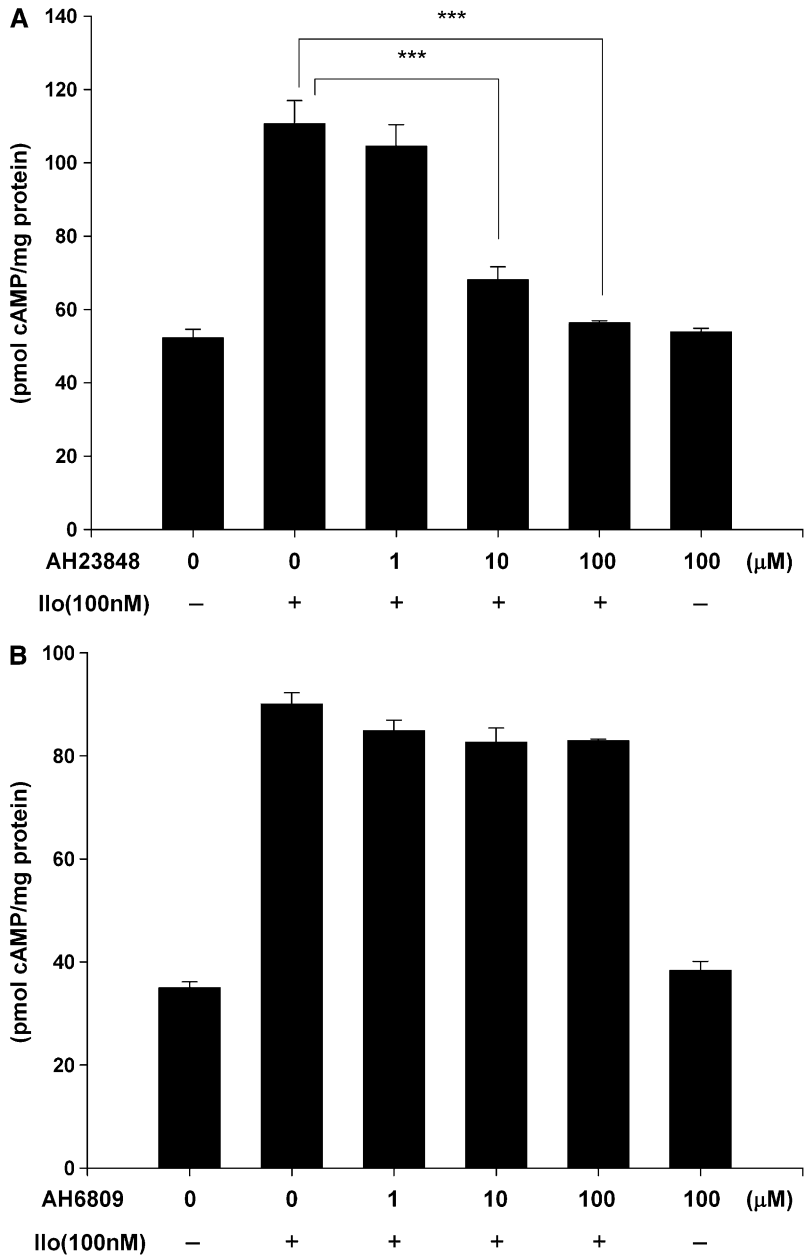


*Figure 6.* The EP4 antagonist AH23848 blocks the cAMP accumulation mediated by iloprost in MCT28d rat pulmonary arterial smooth muscle cells (PASMCs). The intracellular cAMP accumulation induced by iloprost was inhibited by AH23848 but not AH6809. The MCT28d rat PASMCs, which exhibit scant IP receptor 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.

***Conflict of Interest Statement***: Y.-J.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.S.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. N.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. G.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. G.-A.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H.A.G. received $4,000 in 2002–2004 for advisory board activity for Pfizer GmbH, $5,557 in 2002–2004 for Schering AG, $5,852 in 2003–2004 for Altana Pharma AG. He received research grants in 2003–2004 from Pfizer GmbH ($50,000). W.S. received/receives grant and contract support by the following companies: Schering AG, Pfizer Ltd., Altana Pharma AG, Lung Rx, Myogen/Gilead Colorado, Encysive. F.G. received grant and contract support from the following companies: Schering AG, Pfizer Ltd., Altana Pharma AG, Bayer AG, Novartis Pharma AG, GlaxoSmithKline, Actelion Pharma AG. R.T.S. participated as a speaker in scientific meetings or courses organized and financed by various pharmaceutical companies (Bayer-Schering, Encysive). He received research grants in 2006 and 2007 from Bayer-Schering ($160,000), Novartis Pharma ($150,000), Pfizer ($100,000), Actelion ($90,000), and Encysive ($40,000).

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