Phosphodiesterase 1 Upregulation in Pulmonary Arterial Hypertension
Target for Reverse-Remodeling Therapy

Ralph Theo Schermuly, PhD*; Soni Savai Pullamsetti, PhD*; Grazyna Kwapiszewska, PhD; Rio Dumitrascu, MD, PhD; Xia Tian, MSc; Norbert Weismann, PhD; Hossein Ardeschir Ghofrani, MD; Christina Kaulen, MD; Torsten Dunkern, PhD; Christian Schudt, PhD; Robert Voswinckel, MD; Jiang Zhou, MSc; Arun Samidurai, PhD; Walter Klepetko, MD; Renate Paddenberg, PhD; Wolfgang Kummer, MD; Werner Seeger, MD; Friedrich Grimminger, MD, PhD

Background—Pulmonary arterial hypertension (PAH) is a life-threatening disease, characterized by vascular smooth muscle cell hyperproliferation. The calcium/calmodulin-dependent phosphodiesterase 1 (PDE1) may play a major role in vascular smooth muscle cell proliferation.

Methods and Results—We investigated the expression of PDE1 in explanted lungs from idiopathic PAH patients and animal models of PAH and undertook therapeutic intervention studies in the animal models. Strong upregulation of PDE1C in pulmonary arterial vessels in the idiopathic PAH lungs compared with healthy donor lungs was noted on the mRNA level by laser-assisted vessel microdissection and on the protein level by immunohistochemistry. In chronically hypoxic mouse lungs and lungs from monocrotaline-injected rats, PDE1A upregulation was detected in the structurally remodelled arterial muscular layer. Long-term infusion of the PDE1 inhibitor 8-methoxymethyl 3-isobutyl-1-methylxanthine in hypoxic mice and monocrotaline-injected rats with fully established pulmonary hypertension reversed the pulmonary artery pressure elevation, structural remodeling of the lung vasculature (nonmuscularized versus partially muscularized versus fully muscularized small pulmonary arteries), and right heart hypertrophy.

Conclusions—Strong upregulation of the PDE1 family in pulmonary artery smooth muscle cells is noted in human idiopathic PAH lungs and lungs from animal models of PAH. Inhibition of PDE1 reverses structural lung vascular remodeling and right heart hypertrophy in 2 animal models. The PDE1 family may thus offer a new target for therapeutic intervention in pulmonary hypertension. (Circulation. 2007;115:2331-2339.)

Key Words: cardiovascular diseases ■ hypertension, pulmonary ■ muscle, smooth ■ phosphodiesterases ■ pharmacology

Pulmonary arterial hypertension (PAH) is a severe disease with still largely unresolved pathogenesis. It is characterized by increased pulmonary vascular resistance and thus right ventricular (RV) afterload, which in the further course of the disease leads to RV failure and death. Both vasoconstriction and structural remodeling of the pulmonary vessels contribute to the progressive course of PAH, irrespective of different underlying causes.1,2 New treatment concepts in pulmonary hypertension include local and systemic administration of prostacyclin and its analogues, inhalation of nitric oxide (NO), and endothelin receptor antagonists.3,4 Recently, phosphodiesterase (PDE) 5 inhibitors have been demonstrated to be potent, selective pulmonary vasodilators.5-9

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PDEs hydrolyze the cyclic nucleotide second messengers cAMP and cGMP, which are known to play an important role in regulating vascular tone and smooth muscle cell (SMC) proliferation.10 Members of the PDE1 gene family are activated by calcium/calmodulin and are therefore termed “cal-
cium/calmodulin–dependent PDEs.” Three different PDE1 isoforms, namely, PDE1A, PDE1B, and PDE1C, have been reported thus far.\textsuperscript{11,12} Each of the calcium/calmodulin PDEs hydrolyzes both cAMP and cGMP but with different efficacy. PDE1A and PDE1B have higher affinity for cGMP than cAMP, whereas PDE1C hydrolyzes cAMP and cGMP with similar efficiency. Five different PDE1C splice variants, PDE1C1 to PDE1C5, have been reported in human and mouse species.\textsuperscript{13,14} Previous studies reported a high expression of PDE1C in proliferating human arterial SMCs,\textsuperscript{15} thereby linking PDE activity not only to regulation of vascular tone but also to control of proliferation. Furthermore, the inhibition of PDE1C in SMCs isolated from normal aorta or from atherosclerotic lesions with antisense oligonucleotides or the PDE1 inhibitor 8-methoxymethyl-isobutyl-1-methylxanthine (8MM-IBMX) resulted in suppression of SMC proliferation.\textsuperscript{16} Recently, it has been demonstrated that the addition of vinpocetine, another PDE1 inhibitor, enhanced pulmonary vasodilation and cGMP release induced by NO breathing without causing systemic vasodilation.\textsuperscript{17}

The present study is the first to address a putative role of the PDE1 family in chronic PAH. We investigated the expression and function of PDE1 in 2 established models of experimental pulmonary hypertension and studied the expression of the PDE1 family in human lung explant material.

Methods

Patient Characteristics and Measurements

Human lung tissue was obtained from 5 donors and 5 patients with idiopathic PAH (IPAH) undergoing lung transplantation. Patient characteristics are given in the online Data Supplement. Lung tissue was snap-frozen directly after explantation for mRNA and protein extraction. The study protocol for tissue donation was approved by the ethics committee (“Ethik Kommission am Fachbereich Humanmedizin der Justus Liebig Universität Giessen”) of the University Hospital Giessen (Giessen, Germany) in accordance with national law and with “Good Clinical Practice/International Conference on Harmonisation” guidelines. Written informed consent was obtained from each individual patient or the patient’s next of kin.

Cell Culture and PDE Activity

A description of cell culture techniques and measurements of PDE isoenzyme activities are provided in the online Data Supplement.

In Vivo Experiments

All experiments were performed according to institutional guidelines that complied with national and international regulations. Mice were exposed to chronic hypoxia (10% O\textsubscript{2}) in a ventilated chamber, as described previously.\textsuperscript{18} Rats were injected with 60 mg/kg monocrotaline (MCT) subcutaneously.\textsuperscript{18–20} A detailed description of the animal models is given in the online Data Supplement.

Hemodynamic Measurements

Animals were anesthetized with ketamine (6 mg/100 g IP) and xylazine (1 mg/100 g IP). The trachea was cannulated, and the lungs were ventilated with room air. Systemic arterial pressure was determined by catheterization of the carotid artery. For measurement of RV systolic pressure, a catheter was inserted into the RV via the right vena jugularis, as described previously.\textsuperscript{18}

Isolated Mouse Lung Experiments

The isolated perfused lung model has been described in detail previously.\textsuperscript{21} (See the online Data Supplement.)

Pharmacological Treatments

To investigate the effects of the PDE1 inhibitor 8MM-IBMX on acute hypoxic vasoconstriction, 4 groups of mice (6 in each group) were studied in isolated lung experiments. Two groups were normoxic animals in which the effect of increasing doses of 8MM-IBMX or placebo on acute hypoxic pulmonary vasoconstriction was investigated. In these experiments, repetitive hypoxic challenges were performed, and 8MM-IBMX or placebo was applied in the normoxic periods. The other 2 groups consisted of chronically hypoxic mice (21 days at 10% O\textsubscript{2}) in which identical experiments with 8MM-IBMX or placebos were performed.

The long-term AQ effects of PDE1 inhibition were assessed in mice exposed to hypoxia for 35 days and rats injected with MCT for 35 days. After 21 days of either hypoxia or MCT injection, animals were randomized to receive either 8MM-IBMX or placebo via continuous infusion by implantation of osmotic minipumps. As described previously, animals were anesthetized with ketamine/xylazine, and a catheter was inserted into the jugular vein.\textsuperscript{19,22} The animals received either 20 µg of 8MM-IBMX per kilogram per minute or placebo for 14 days.

Assessment of Right Heart Hypertrophy and Vascular Remodeling

The RV was dissected from the left ventricle and septum (LV+S), and these dissected samples were weighed to obtain the RV to LV+S ratio [RV/(LV+S)].\textsuperscript{18–20} A detailed description of the methods is given in the online Data Supplement.

Laser-Assisted Microdissection

Microdissection was performed as described in detail previously.\textsuperscript{23–25} In brief, cryosections (10 µm) from lung tissue were mounted on glass slides. After hemalum staining for 30 seconds, the sections were subsequently immersed in 70% and 96% ethanol and stored in 100% ethanol until use. No more than 10 sections were prepared at once to reduce the storage time. Intrapulmonary arteries at a size between 50 and 200 µm were selected and microdissected under optical control with the Laser Microbeam System (P.A.L.M., Bernried, Germany; Data Supplement Figure 1).

RNA Isolation, cDNA Synthesis, and Relative mRNA Quantification by Real-Time Polymerase Chain Reaction

RNA from laser-microdissected material, lung homogenate, and freshly isolated SMCs was isolated by RNeasy Micro and RNeasy Mini kits, respectively (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. For cDNA synthesis, reagents and incubation steps were applied as described previously.\textsuperscript{24} (See the online Data Supplement.)

Statistical Analyses

Data are mean±SEM. For comparison of the pharmacological effects of 8MM-IBMX, 1-way ANOVA with the Student-Newman-Keuls post hoc test was performed. For comparison of 2 groups, a Student \(t\) test was performed. Statistical significance was assumed when \(P<0.05\).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

PDE1A, PDE1C, and PDE5A Expression in Patients With IPAH

The expression of PDE1A, PDE1C, and PDE5A was investigated by real-time reverse-transcription polymerase chain reaction in lung homogenate, microdissected pulmonary arteries (30 to 100 µm in diameter), and isolated SMCs (Figure 1A). A strong upregulation of PDE1C and PDE5A was
evident in microdissected pulmonary arteries and SMCs of patients with IPAH. Furthermore, a stronger immunoreactivity of PDE1C and PDE5A in lung specimens from IPAH patients, along with smooth muscle actin colocalization, demonstrated that the site-specific changes of both PDE1C and PDE5A occur especially in the medial layer of pulmonary arteries (Figure 1B; supplemental Figure II). In contrast, only weak expression of PDE1C was detected in pulmonary vessels of healthy donor lung tissue. In addition, PDE1A expression was constitutively present in both donor and IPAH tissue, again located in the medial layer of pulmonary arteries. Immunoreactivity against PDE1A, PDE1C, and PDE5A was also noted for bronchial SMCs in small airways.

**PDE1C Activity Is the Major Activity in Human Pulmonary SMCs, and Inhibition of PDE1 Inhibits DNA Synthesis**

Human pulmonary artery SMCs obtained from PromoCell (Heidelberg, Germany) were processed to analyze the cAMP/cGMP hydrolysis activity of different PDE family members. As shown in Figure 2A, a hydrolytic activity of PDEs 3, 4, and 5 was detected, whereas no obvious PDE2 activity was observed. Incubation of the cellular lysate with calcium/calmodulin induced cAMP and cGMP hydrolysis strongly and to an equal degree, which must be attributed to PDE1C expression. In addition, the PDE1 inhibitor P179 dose dependently inhibited the DNA synthesis of human pulmonary artery SMCs (Figure 2B).
Expression of PDE1A, PDE1C, and PDE5A in Animal Model of Pulmonary Hypertension

The expression of PDE1A, PDE1C, and PDE5A was investigated in lung homogenate, microdissected pulmonary arteries, and freshly isolated SMCs from MCT-injected rats (Figure 3A) and chronically hypoxic mice (21 days, 10% O2; supplemental Figure IIIA) by quantitative reverse-transcription polymerase chain reaction. No significant upregulation of any of the 3 genes was detected in lung homogenate, whereas PDE1A was significantly upregulated in pulmonary arterial SMCs of MCT-treated rats and chronically hypoxic mice. These expression changes were specific for the pulmonary circulation, because no expression changes were found in aortic tissue from MCT rats (supplemental Figure IV). Immunohistological staining of PDE1A, PDE1C, PDE5A, and α-smooth muscle actin in serial sections is given in Figure 3B (MCT rats) and supplemental Figure IIIB (chronically hypoxic mice). Strong immunoreactivity of PDE1A and PDE5A, which colocalizes with smooth muscle actin, confirmed medial expression of these enzymes in experimental pulmonary hypertension. Similar data were obtained from chronically hypoxic mice.

8MM-IBMX Improves Hemodynamics, Right Heart Hypertrophy, and Vascular Remodeling in MCT-Treated Rats

The injection of MCT resulted in severe pulmonary hypertension within 21 days, which was sustained until day 35. RV systolic pressure was increased significantly compared with the saline-challenged group (Figure 4A). The PDE1 inhibitor 8MM-IBMX was infused continuously from day 21 to 35 and reversed chronic pulmonary hypertension. Mean systemic arterial pressure (SAP, in mm Hg; Figure 4B), total pulmonary resistance (TPR, in mm Hg · mL⁻¹ · min⁻¹ per 100 g of body weight; Figure 4C), and total systemic resistance (TSR, in mm Hg · mL⁻¹ · min⁻¹ per 100 g of body weight; Figure 4D) are given for the different experimental groups. 8MM-IBMX was infused intravenously by implanted osmotic minipumps at a dose of 20 μg · kg⁻¹ · min⁻¹. *P<0.05 vs control; †P<0.05 vs MCT at day 21; ‡P<0.05 vs MCT at day 35.
arterial pressure (Figure 4B), total systemic resistance (Figure 4D), and left ventricular pressures (not shown) did not change in any of the treatment groups. Compared with control animals, total pulmonary resistance (0.98±0.05 mm Hg · mL⁻¹ · min⁻¹ per 100 g of body weight) was increased in the MCT group at day 21 (2.14±0.26 mm Hg · mL⁻¹ · min⁻¹ per 100 g of body weight; \( P<0.05 \)) and 35 (3.23±0.35 mm Hg · mL⁻¹ · min⁻¹ per 100 g of body weight; \( P<0.05 \)). In the 8MM-IBMX–treated animals, total pulmonary resistance decreased significantly compared with sham treatment (1.32±0.33 mm Hg · mL⁻¹ · min⁻¹ per 100 g of body weight; \( P<0.05 \); Figure 4C). In addition to the hemodynamic changes, structural changes occurred that were characterized by right heart hypertrophy, measured as RV/(LV+S) (Figure 5A), and muscularization of normally nonmuscularized small pulmonary arteries (Figure 5B). The muscularization of pulmonary arteries at a size of 20 to 70 \( \mu \)m is depicted in an exemplary fashion in Figure 5C through 5E. The continuous infusion of 8MM-IBMX (20 \( \mu \)g · kg⁻¹ · min⁻¹) via implanted osmotic pumps from day 21 to day 35 reversed right heart hypertrophy (\( P<0.05 \) versus MCT at day 21 and day 35) and increased the portion of nonmuscularized pulmonary arteries (\( P<0.05 \) versus MCT at day 21 and day 35).

**8MM-IBMX Reverses Acute Hypoxia–Induced Vasocostriction in Isolated Mouse Lungs**

The PDE1 inhibitor 8MM-IBMX dose dependently reversed acute pulmonary vasocostriction in isolated lungs from mice that were kept under normoxic conditions (Figure 6, closed circles). Notably, when investigating lungs isolated from mice that were kept for 21 days under hypoxic conditions (10% O₂), we noted a significantly enhanced sensitivity to 8MM-IBMX inhibition, as is obvious from the leftward shift of the dose-response curve in these lungs (open circles). This leftward shift signaled higher PDE1 activity in the chronically hypoxic lungs.

**8MM-IBMX Improves Hemodynamics, Right Heart Hypertrophy, and Vascular Remodeling in Chronically Hypoxic Mice**

Mice kept at hypoxia developed severe pulmonary hypertension within 21 days, which was sustained until day 35. Consequently, RV systolic pressure was increased significantly compared with normoxic animals (Figure 7). The continuous infusion of 8MM-IBMX (20 \( \mu \)g · kg⁻¹ · min⁻¹) via implanted osmotic pumps from day 21 to day 35 reversed chronic pulmonary hypertension to near normal values (24.0±2.9 mm Hg, \( P<0.05 \) versus hypoxia at 21 and 35 days). In hypoxic animals, a significant RV hypertrophy developed as a consequence of increased pulmonary arterial pressures. RV/LV+S increased from 0.24±0.05 (controls) to 0.38±0.04 (21 days of hypoxia) and 0.42±0.06 (35 days of hypoxia), respectively (both \( P<0.05 \) versus controls). 8MM-IBMX
IBMX caused a reduction of this ratio to 0.33±0.02 (P<0.05 versus hypoxia for 35 days). We then quantitatively assessed the degree of muscularization of pulmonary arteries with a diameter between 20 and 70 μm. In controls, the majority of vessels of this diameter are nonmuscularized (54%), with lower percentages of partially muscularized (37%) and fully muscularized (9%) vessels (Figure 8). In hypoxic animals, both at day 21 and at day 35, a significant decrease in nonmuscularized pulmonary arteries occurred, with a concomitant increase in fully muscularized pulmonary arteries. Treatment with 8MM-IBMX resulted in a significant reduction of fully muscularized arteries compared with both hypoxia groups (21 days [ie, before start of 8MM-IBMX treatment] and 35 days), and increased the percentage of nonmuscularized pulmonary arteries.

**Discussion**

Abnormal media proliferation and de novo muscularization are characteristic features of PAH. In the present study, strong vascular upregulation of PDE1C was demonstrated by laser-assisted microdissection followed by quantitative reverse-transcription polymerase chain reaction in lung explants and isolated pulmonary SMCs from patients with IPAH. Immunostaining consistently demonstrated strong expression of PDE1C in the pulmonary arterial SMCs of the diseased but not the control lungs. In the present study, we also demonstrate that PDE1A is constitutively expressed in the vessel wall. Recently, Wharton et al26 demonstrated a significant upregulation of PDE5A in lungs from patients with pulmonary hypertension, and the present study confirms the strong pulmonary vascular upregulation of PDE5A in IPAH patients.

Chronic hypoxia and MCT injection are well-recognized stimuli for pulmonary vasoconstriction and structural remodeling of the precapillary lung resistance vessels. Hypertrophy and proliferation of vascular SMCs, with complete muscularization of physiologically partially muscularized vessels and de novo muscularization of physiologically nonmuscularized vessel, represent key features of the structural remodeling process. When PDE1A and PDE1C expression was assessed in such remodeled pulmonary vasculature, striking differences to the control rats and mouse vasculature became apparent. The PDE1A isoenzyme was strongly expressed in the pulmonary arteries of lungs from MCT-injected rats and
chronically hypoxic mice, with predominant localization in pulmonary arterial SMCs. This was clearly demonstrated by immunostaining in serial sections that showed colocalization with the SMC marker α-smooth muscle actin. Moreover, a functional role of this upregulated PDE1 in the control of vasoregulation was demonstrated in isolated lungs from chronically hypoxic mice: The 8MM-IBMX dose-effect curve was significantly shifted leftward, which indicates enhanced sensitivity to this inhibitor and thus a major contribution of PDE1 to the control of lung vascular tone in the chronically hypoxic lungs. A recent study from Evgenev et al demonstrated that selective inhibition of PDE1 augments the therapeutic efficacy of inhaled NO in an ovine model of acute thromboxane-induced pulmonary hypertension, which demonstrates the contribution of PDE1 to pulmonary vascular tone. On the selectivity of 8MM-IBMX for PDE1 versus other PDEs, previously published data from our group27 and from others40 suggest a 10- to 30-fold more selective inhibition of PDE1 versus PDE3 and PDE4, respectively. Thus, at the currently chosen dose, no relevant inhibition of other PDE isoforms is to be expected to contribute to the findings presented here.

In analogy with these findings in the experimental models, very low levels of PDE1C expression were noted in the “quiescent” vascular SMC of the healthy donor lungs, whereas this isoenzyme was strongly expressed at the mRNA and protein level in the IPAH lungs, confirmed to be localized predominantly in the media of the small pulmonary arteries by immunohistochemistry. In addition to contributing to the regulation of vascular tone in the IPAH lungs, this localization suggests a role of PDE1C in the structural remodeling process underlying the development of severe pulmonary hypertension. Both cAMP and cGMP, targeted by this PDE isoenzyme, have been found to counteract several pathways involved in SMC proliferation.28,29 For example, cAMP was shown to inhibit cyclin-dependent kinase 4, extracellular signal-regulated kinase activation,30 and upregulation of the cyclin-dependent kinase 2 inhibitor p27Kip1.31 In line with these observations, prostacyclin, a potent stimulus of vascular cAMP generation, can inhibit proliferation of aortic SMCs by blocking cell cycle progression from the G1 to the S phase, largely via occupancy of the cAMP response elements in the promoter region of the cyclin A gene, along with an increase in the level of cAMP response element-binding protein transcription factor.32,33

Because PDE1C, in contrast to PDE1A and PDE1B, is strongly upregulated during SMC proliferation,10 it may act as a major cAMP-hydrolyzing PDE in SMCs under these conditions. This does not cast doubt on the role of PDE3 and PDE4, which are also expressed in the medial layer of the pulmonary artery and which also hydrolyze cAMP.28 but the specific link of PDE1C to the proliferative state of the SMC renders this PDE a particularly interesting candidate for therapeutic intervention aimed at antiremodeling or reversal of remodeling. The PDE activity profile of human pulmonary SMCs demonstrated significant PDE1 activity, which supports the role of PDE 1 in cyclic nucleotide hydrolysis in vascular SMCs. The PDE5 inhibitor sildenafil does possess some inhibitory potency toward PDE1C, although less than PDE5, but some of the strong beneficial effects of sildenafil in PAH patients35–38 might also be linked to some extent to its effect on PDE1C. Sildenafil is a PDE 1/5/6 inhibitor, with IC50 values of 280, 3.5, and 37 nmol/L for the different PDE subtypes.39,40 The IC50 of sildenafil against PDE1 is ∼280 nmol/L, and these plasma levels are clearly reached after sildenafil application in men. Paul et al41 reported plasma levels in the range of 750 ng/mL after oral intake of a single dose of 100 mg of sildenafil in patients with PAH. In addition, plasma levels of 380 ng/mL of the active metabolite desmethylsildenafil were reached. These plasma levels translate into a concentration of ∼1.2 μmol/L, which clearly indicates the PDE1 inhibitory capacity of sildenafil. In line with these results, another study reported a plasma level of 1070 ng/mL after a single application of 100 mg of sildenafil,42 which corresponds to ∼1.3 μmol/L. In both studies, plasma levels above the IC50 value of sildenafil for PDE1 were maintained over a range of 4 hours. Taken together, we hereby highlight the evidence that in common clinical doses, sildenafil, in addition to its PDE5 inhibitory effects, concomitantly achieves plasma levels capable of inhibiting PDE1.

Along with the first proof of PDE1 upregulation in the pulmonary circulation provided in the present report, a strong rationale to explain potential antiproliferative effects via PDE1 inhibition is provided. Moreover, the present findings may add to the understanding of why certain hemodynamic differences between clinically available PDE5 inhibitors were found7 and explain the strong synergism between sildenafil and camp-increasing compounds, eg, inhaled iloprost, which has been demonstrated experimentally27 and clinically.43–45 Using another PDE1 inhibitor, PI79, we demonstrated the inhibition of DNA synthesis of pulmonary SMCs in a dose-dependent manner. Similar results were obtained by inhibition of PDE1C in aortic SMCs with antisense oligonucleotides or a PDE1 inhibitor.16 More recently it has been shown by Murray et al46 in pulmonary arterial SMCs from pulmonary hypertensive patients that PDE1C is upregulated on mRNA, protein, and activity levels. Intervention studies on the in vitro level nicely corroborate the present findings in the currently used in vivo disease models. To address the putative antiproliferative potential of PDE1 interference in the pulmonary vasculature in a direct fashion, continuous infusion of the PDE1 inhibitor 8-MM-IBMX via osmotic minipumps, implanted in rats injected with the plant alkaloid MCT and mice undergoing chronic hypoxia, was undertaken. The therapeutic intervention started after pulmonary hypertension, structural remodeling of the lung vasculature, and right heart hypertrophy had fully developed. Most impressively, animals treated intravenously with 8MM-IBMX, but not vehicle-treated controls, showed not only a stoppage of further progression but significant regression of all changes within the next 2 weeks. In particular, the pattern of small pulmonary artery muscularization (nonmuscularized compared with partially muscularized and fully muscularized vessels) nearly approached the profile of control lungs. Thus, reversal of pulmonary artery remodeling was achieved in the 14 day-treatment period, accompanied by regression of right heart hypertrophy.
In conclusion, strong upregulation of PDE1C in hyperproliferative pulmonary artery SMCs was noted in clinical PAH, both on the mRNA and protein level, and was corroborated on the functional level in the animal model. Long-term infusion of a PDE1 inhibitor reversed pulmonary hypertension, lung vascular remodeling, and right heart hypertrophy in MCT-injected rats and chronically hypoxic mice, in which PDE1A appears to be the responsible PDE1 variant. We suggest that upregulation of PDE1C plays an important role in the structural remodeling process underlying severe pulmonary hypertension, thus offering a target for therapeutic intervention aimed at reversing lung vascular remodeling and subsequent right heart hypertrophy.

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Disclosures

Dr Dunkern and Dr Schudt are employed by Altana Pharma AG. The remaining authors report no conflicts.

References


**CLINICAL PERSPECTIVE**

Pulmonary arterial hypertension (PAH), a life-threatening disease, is characterized by aberrant pulmonary vascular remodeling; at a cellular level, evidence exists of abnormal smooth muscle cell proliferation in the medial wall, neointima formation, and endothelial cell dysfunction. Cyclic nucleotide phosphodiesterases (PDEs) control the effects induced by several mediators such as nitric oxide and prostanoids via hydrolyzing their second messengers, cGMP and cAMP. PDE5 is known to be upregulated in the lung vasculature, and the PDE5 inhibitor sildenafil has been approved for the treatment of PAH. To date, little is known about the antiproliferative effects observed with sildenafil treatment. Acknowledging the considerable PDE1 inhibitory capacity of sildenafil, we hypothesized that PDE1 may play a role in pulmonary vascular smooth muscle cell proliferation. The expression of PDE1 in explanted lungs from patients with idiopathic PAH and animal models of pulmonary hypertension was investigated. Therapeutic application of a selective PDE1 inhibitor was performed in 2 animal models of PAH. In essence, strong upregulation of PDE1C in pulmonary arterial vessels from idiopathic PAH patients compared with healthy donor lungs was noted. Long-term PDE1 inhibitor treatment in the experimental models resulted in reduced pulmonary artery pressure, reversed remodeling of the lung vasculature, and reduction in right heart hypertrophy. Our results, therefore, could explain in part the antiremodeling effects of sildenafil by its fractional PDE1 inhibitory capacity. Furthermore, our results open speculation about the therapeutic efficacy of selective PDE1 inhibitors in pulmonary vascular disorders.
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Phosphodiesterase 1 upregulation in pulmonary arterial hypertension – target for reverse-remodeling therapy

Ralph Theo Schermuly 1,6, Soni Savai Pullamsetti 1,6, Grazyna Kwapiszewska 2, Rio Dumitrascu 1, Xia Tian1, Norbert Weissmann 1, Hossein Ardeschir Ghofrani 1, Christina Kaulen 1, Torsten Dunkern 3, Christian Schudt 3, Robert Voswinckel 1, Jiang Zhou 1, Arun Samidurai 1, Walter Klepetko 4, Renate Paddenberg 5, Wolfgang Kummer 5, Werner Seeger 1, and Friedrich Grimminger 1

1 University of Giessen Lung Centre (UGLC), Giessen, Germany.
2 Department of Pathology, Justus-Liebig-University Giessen, Germany
3 Department of Biochemistry 2 Inflammation, Altana Pharma, Konstanz, Germany.
4 Department of Cardiothoracic Surgery, University of Vienna, Vienna, Austria.
5 Department of Anatomy and Cell Biology, Justus-Liebig-University Giessen, Giessen, Germany.
METHODS

Inhibitors

The PDE1 inhibitor 8MM-IBMX was supplied from Calbiochem (Bad Soden, Germany). Motapizone was a generous gift from Aventis (formerly Rhone-Poulenc Rorer, Köln, Germany). PI79 is a nanomolar PDE1 selective inhibitor with selectivity up to x100 versus other PDE family members. This compound was provided by ALTANA Pharma AG. The selective PDE4 inhibitor piclamilast and PDE5 inhibitor sildenafil were prepared at the chemical facilities of ALTANA Pharma AG.

Preparation of pulmonary artery smooth muscle cells (PASMC) from patients and animals for PDE expression profiling.

Primary SMC were isolated from human and rat/mouse pulmonary artery by carefully preparing <1 mm3 pieces of media, devoid of adventitial tissue as assessed by microscopic control as described 1-3. The pieces of media were placed into 12-well cell culture plates with 500 µl culture medium (Dulbecco’s modified Eagle’s medium) at 37°C; 95% air; 5% CO2. SMC identity was verified by characteristic appearance in phase-contrast microscopy, indirect immunofluorescent antibody staining for smooth muscle-specific isoforms of α-actin and myosin (at least 95% of cells stained positive), and lack of staining for von Willebrand factor. The cells were studied in the third passage.

Cell culture for PDE activity assay and proliferation assay.

Human pulmonary smooth muscle cells were obtained from Promocell GmbH (Heideberg, Germany) and cultured for up to three passages in human smooth muscle cell medium II (Promocell GmbH, Heidelberg, Germany). These cells were used for determination of phosphodiesterase isoenzyme activities and proliferation assay.
Measurements of phosphodiesterase isoenzyme activities and preparation of cellular extracts

Cells (1-3x10^6) were washed twice in phosphate buffered saline (4°C) and resuspended in 1 ml homogenization buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, 10mM HEPES, 1 mM EGTA, 1 mM MgCl2, 1mM β-mercaptoethanol, 5 µM pepstatin A, 10 µM leupeptin, 50µmM phenylmethylsulfonyl fluoride, 10 µM soybean trypsin inhibitor, 2 mM benzamidine, pH 8.2). Cells were disrupted by sonication (Branson sonifier, 3 x 15 s) and lysates were immediately used for PDE activity measurements. PDE activities were assessed in cellular lysates as described 4 with some modifications 5. The assay mixture (final volume 200 µl) contained Tris HCl 30mM; pH 7.4, MgCl2 5mM, 0.5 µM either cyclic AMP or cyclic GMP as substrate including [3H]cAMP or [3H]cGMP (about 30,000 c.p.m. per well), 100 µM EGTA, PDE isoenzyme-specific activators and inhibitors as described and cellular lysates. Incubations were performed for 20 min at 37°C and reactions were terminated by adding 50 µl 0.2 M HCl per well. Assays were left on ice for 10 min and then 25 µg 5'-nucleotidase (Crotalus atrox) was added. Following incubation for 10 min at 37°C assay mixtures were loaded onto QAE-Sephadex A25 columns (1 ml bed volume). Columns were eluted with 2 ml 30 mM ammonium formiate (pH 6.0) and radioactivity in the eluate was counted. Results were corrected for blank values (measured in the presence of denatured protein) that were below 2% of total radioactivity. Cyclic AMP degradation did not exceed 25% of the amount of substrate added. The final DMSO concentration was 0.3% (v/v) in all assays. Selective inhibitors and activators of PDE isoenzymes were used to determine activities of PDE families as described previously 6 with modifications. Briefly, PDE4 was calculated as the difference of PDE activities at 0.5 µM cyclic AMP in the presence and absence of 1 µM Piclamilast. The difference between Piclamilast-inhibited cyclic AMP hydrolysis in the presence and absence of 10 µM Motapizone was defined as PDE3. The fraction of cyclic GMP (0.5 µM) hydrolysis in the presence of 10 µM Motapizone that was
inhibited by 100 nM Sildenafil reflected PDE5. At the concentrations used in the assay Piclamilast (1 µM), Motapizone (10 µM) and Sildenafil (100 nM) completely blocked PDE4, PDE3 and PDE5 activities without interfering with activities from other PDE families. PDE1 was defined as the increment of cyclic AMP hydrolysis (in the presence of 1 µM Piclamilast and 10 µM Motapizone) or cyclic GMP hydrolysis induced by 1 mM Ca\(^{2+}\) and 100 nM calmodulin.

**Exposure to Chronic Hypoxia**

All experiments were performed using adult male mice (8-week-old BALB/c) according to the institutional guidelines that comply with national and international regulations. Mice were exposed to chronic hypoxia (10% O\(_2\)) for 21 days in a ventilated chamber, as described previously \(^3\). The level of hypoxia was held constant by an auto regulatory control unit (model 4010, O\(_2\) controller, Labotect; Göttingen, Germany) supplying either nitrogen or oxygen. Excess humidity in the recirculating system was prevented by condensation in a cooling system. CO\(_2\) was continuously removed by soda lime. Cages were opened once a day for cleaning as well as for food and water supply. The chamber temperature was maintained at 22–24°C. Control mice were kept in identical chambers under normoxic condition.

**Hemodynamic measurements**

Mice were anaesthetized with ketamine (6 mg/100 g, intraperitoneally) and xylazine (1 mg/100 g, intraperitoneally). The trachea was cannulated, and the lungs were ventilated with room air at a tidal volume of 0.2 ml and a rate of 120 breaths per minute. Systemic arterial pressure was determined by catheterization of the carotid artery. For measurement of right ventricular systolic pressure (RVSP) a PE-80 tube was inserted into the right ventricle via the right vena jugularis, as described \(^3\).
**Isolated mouse lung experiments**

The isolated perfused lung model has previously been described in detail. Initially, male BALB/c mice (mean body weight ~ 26 g) were anticoagulated with heparin (1000 U/kg) and anaesthetized with intraperitoneal pentobarbital sodium (100 mg/kg). Tracheotomy was performed and the animals were ventilated with room air, using a piston pump (tidal volume, 250 µl; frequency, 90 breaths/min; positive end expiratory pressure, 2 cm H2O). After mid-sternal thoracotomy, catheters were placed into the pulmonary artery and the left atrium, and perfusion with Krebs-Henseleit buffer was started. The lungs were perfused with a constant flow of 2 ml/min (ISM834A, Ismatec, Germany). Left atrial pressure (P_{LA}) was set at 2 mmHg in all experiments. In parallel with the onset of artificial perfusion, room air supplemented with 4 % CO₂ was used for ventilation. Pressures in the pulmonary artery, the left atrium and the trachea were registered (zero referenced at the hilum). The whole system was heated to 37°C. For hypoxic ventilation, a gas mixture containing 1% O₂, 5.3% CO₂, balanced with N₂ was used. Ten minute periods of hypoxic ventilation (1% O₂) were alternated with 15 min normoxic periods (21% O₂).

**Pharmacological treatments**

To investigate the effects of the PDE1 inhibitor 8MM-IBMX on acute hypoxic vasoconstriction, four groups of mice (six in each group) were studied in isolated lung experiments. Two groups were normoxic animals in which the effect of increasing doses of 8MM-IBMX or placebo on acute hypoxic pulmonary vasoconstriction was investigated. In these experiments, repetitive hypoxic challenges were performed and 8MM-IBMX or placebo was applied in the normoxic periods. The other two groups consisted of chronically hypoxic mice (21 days at 10% O₂) in which identical experiments with 8MM-IBMX or placebos were performed.
The chronic effects of PDE1 inhibition were assessed in mice exposed to hypoxia for 35 days. Briefly, 20 animals were kept in hypoxic conditions to develop pulmonary hypertension. After 21 days, animals were randomized to receive either 8MM-IBMX or placebo via continuous infusion by implantation of osmotic minipumps. As described previously, animals were anaesthetized with ketamine/xylazine and a catheter was inserted into the jugular vein. The animals received either 20 µg 8MM-IBMX/kg/min or placebo for 14 days.

Assessment of right heart hypertrophy and vascular remodeling

Hemodynamics of mice exposed to hypoxia or room air for 3 or 5 weeks were recorded as described above. After recording systemic arterial and right ventricular pressure, the animals were exsanguinated and the lungs and heart were isolated. The right ventricle (RV) was dissected from the left ventricle + septum (LV + S) and these dissected samples were weighed to obtain the right to left ventricle plus septum ratio (RV/LV+S).

The lungs were perfused with a solution of 10% phosphate buffered formalin (pH 7.4). At the same time 10% phosphate buffered formalin (pH 7.4) was administered into the lungs via the tracheal tube at a pressure of 20 cm H₂O. These specimens were processed for light microscopy by routine paraffin embedding. The degree of muscularization of small peripheral pulmonary arteries was assessed by double-staining the 3 µm sections with an anti-α-smooth muscle actin antibody (dilution 1:900, clone 1A4, Sigma, Saint Louis, Missouri) and anti-human von Willebrand factor antibody (vWF, dilution 1:900, Dako, Hamburg, Germany) modified from a protocol described elsewhere. Polyclonal antibodies against human PDE1 a and C (FabGennix, Shreveport, USA) raised in rabbits was used for PDE1 staining. Dewaxed and rehydrated sections were subjected to proteolytic antigen retrieval with 0.1% trypsin in 0.1% calcium chloride (pH 7.6) at 37°C for 8 min and stained with the avidin-biotin-peroxidase complex (ABC Elite, Vector Laboratories, Burlingame, USA) method, with 3, 3-diaminobenzidine as substrate. Sections were counterstained with hematoxylin and examined
by light microscopy using a computerized morphometric system (Qwin, Leica, Wetzlar, Germany). At 40x magnification, 50–60 intraacinar vessels accompanying either alveolar ducts or alveoli were analyzed by an observer blinded to treatment in each mouse. As described, each vessel was categorized as nonmuscularized, partially muscularized or fully muscularized. The percentage of pulmonary vessels in each category was determined by dividing the number of vessels in that category by the total number counted in the same experimental group.

RNA isolation, cDNA synthesis and relative mRNA quantification by real-time PCR

RNA from lung homogenate, laser-microdissected material and pulmonary smooth muscle cells was isolated by RNeasy Micro kit and RNasy Mini kit, respectively (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For cDNA synthesis, reagents and incubation steps were applied as described previously. Briefly, total RNA was reverse transcribed using the SMART™ PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA). Complementary DNA was purified by the QIAquick™ PCR Purification Kit (Qiagen, Hilden, Germany) and eluted in 45 μl elution buffer (EB). From the eluted cDNA, 2 μl were separated for further determination of the amplification factor. For the PCR-based amplification, the reactions (final volume: 25 μl) were set up with the Platinum SYBRGreen qPCR SuperMix UDG (Invitrogen) using 2 μl of cDNA. The regulation of a subset of genes was analyzed by real-time quantitative PCR using the ΔΔCT method for the calculation of relative changes. Briefly, the regulation factor (RF) is calculated as follows: RF=2 -ΔΔCT. Real-time PCR was performed by the Sequence Detection System 7700 (PE Applied Biosystems).

Porphobilinogen deaminase (PBGD), an ubiquitously as well as consistently expressed gene that is free of pseudogenes was used as reference. The oligonucleotide primer pairs for human samples PBGD FP: 5’ CCC ACG CGA ATC ACT CTC AT 3’; RP: 5’ TGT CTG GTA ACG GCA ATG CG 3’; PDE1a FP: 5’ CTC AAA AGC CGA AAC TTC TTC ATG CG 3’, RP: 5’
CGT CTT AGT GCA TCA GCA ATG TG 3’; PDE1c FP: 5’ TGT GAGTCC ATT AAT CGA TGA AAC C 3’, RP: 5’ ACC TGA TCG CTT GGC ATC TG 3’; PDE5 FP: 5’ TGT CCC TGG AAC ACC AAC CA 3’, RP: 5’ CCT CAG AAT CCT TGA CAA CAA TGG 3’ (final concentration 200 nM). The oligonucleotide primer pairs for mouse samples PBGD FP: 5’ CAA GGT TTT CAG CAT CGC TAC CA 3’; RP: 5’ ATG TCC GGT AAC GGC GGC 3’;
PDE1a FP: 5’ ATC AGC CAC CCA GCC AAA 3’, RP: 5’ GGA GAA AAC GGA AGC CCT AAT 3’; PDE1c FP: 5’ TGT CCT GTC ATT TCC ACC AA 3’, RP: 5’ GAC TGA TGT CTG CTG TGT GC 3’; PDE5 FP: 5’ CTC TGA AAG CAG GCA AGA TTC 3’, RP: 5’ CGC TCC GCT GTA TGT ATG AGT 3’ (final concentration 200 nM). The oligonucleotide primer pairs for mouse samples PBGD: 5’ GGT ACA AGG CTT TCA GCA TCG C 3’; RP: 5’ ATG TCC GGT AAC GGC GGC 3’;
PDE1a: 5’ AAC AAT GGT TGC CCA GTC G 3’, RP: 5’ GTC TGC CCC GTA GTT TGA AG 3’; PDE1c: 5’ ATG CAG CCA CAG TGC TTG AA 3’, RP: 5’ ATC CCG AAC TTC TGA AGG CA 3’; PDE5: 5’ AGG TTG TTG GTG TAG CTC AGG C 3’, RP: 5’ AAC AAT ACC ACA GAA TGC CAG GT 3’ (final concentration 200 nM). Cycling conditions were 95°C for 6 min, followed by 45 cycles of 95°C for 5 s, 59°C for 5 s and 72°C for 10 s. Due to the non-selective dsDNA binding of the SYBR™Green I dye, melting curve analysis and gel electrophoresis were performed to confirm the exclusive amplification of the expected PCR product.
References


FIGURE LEGEND

Figure 1. Laser-assisted microdissection of a small intrapulmonary artery.
An intrapulmonary artery (PA) was selected and microdissected under optical control from hemalaun-stained cryo-sections (10 μm) using the Laser Microbeam System. Scale bar: 20 μm.

Figure 2. PDE1A, PDE1C and PDE5A expression in serial sections from patients with IPAH.
Figure 2 shows the PDE1A, PDE1C, PDE5A and α smooth muscle actin immunostaining in pulmonary arteries from healthy donors and from IPAH patients. Scale bar: 20 μm.

Figure 3. PDE1A, PDE1C and PDE5A expression in lung homogenate (LH), microdissected pulmonary arteries (PA) and isolated pulmonary arterial smooth muscle cells (PASMC)(A) and in serial sections from chronic hypoxic mice (B).
The regulation of PDE1A, PDE1C and PDE5A in lung homogenate (LH), microdissected pulmonary arteries (PA) and isolated pulmonary arterial smooth muscle cells (PASMC) was analyzed by real-time quantitative PCR using the ΔΔ CT method for the calculation of the regulation factor (RF) (A, *, p<0.05 versus control). Panel B shows the PDE1A, PDE1C, PDE5A and α smooth muscle actin immunostaining in pulmonary arteries from control and from chronic hypoxic mice. Scale bar: 20 μm.

Figure 4. PDE1A, PDE1C and PDE5A expression in aorta and heart from MCT-injected rats.
The regulation of PDE1A, PDE1C and PDE5A in aorta (A) and heart tissue (B) was analyzed by real-time quantitative PCR using the ΔΔ CT method for the calculation of the regulation factor (RF).
**Table 1: Patient characteristics**

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Figure 1
Figure 2

PDE1A  PDE1C  PDE5A  α-SMA

IPAH #3

IPAH #4

IPAH #5

IPAH #6

Donor  -Ve  IPAH
Figure 3

A

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B

Control

Hypoxia [35d]
Figure 4

Aorta

Regulation Factor (RF)

PDE1A  PDE1C  PDE5A
Figure 4

Heart

Regulation Factor (RF)

PDE1A  PDE1C  PDE5A