Pulmonary arterial hypertension (PAH) is a life-threatening disease characterized by progressive pulmonary vascular remodeling leading to right ventricular (RV) failure and death.1 The pathogenesis of PAH is complex and arises from a combination of pulmonary vasoconstriction, vascular wall remodeling, and thrombosis, resulting in severe loss of vessel cross-sectional area.2 Intimal changes in the vessel walls include endothelial injury, endothelial cell (EC) proliferation, invasion of the intima by (myo)fibroblast-like cells, enhanced matrix deposition, and in some cases obstruction of the vascular lumen by unique plexiform lesions.3 Despite the uncertainty of exact mechanisms causing pulmonary arteriolar obstructions, a growing body of evidence implicates EC apoptosis as the initiator of microvascular degeneration or apoptosis-resistant ECs.4 Thus, regeneration of the pulmonary vascular bed could be a novel therapeutic approach for reversal of pulmonary vascular disease in PAH patients.

Background—Pulmonary arterial hypertension is characterized by a progressive increase in pulmonary vascular resistance caused by endothelial dysfunction, inward vascular remodeling, and severe loss of precapillary pulmonary vessel cross-sectional area. Asymmetrical dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor, and its metabolizing enzyme dimethylarginine dimethylaminohydrolase (DDAH) play important roles in endothelial dysfunction. We investigated whether combined phosphodiesterase (PDE) 3 and 4 inhibition ameliorates endothelial function by regulating the ADMA-DDAH axis.

Methods and Results—We investigated the effects of the PDE3/4 inhibitor tolafentrine in vitro on endothelial cell survival, proliferation, and apoptosis. Effects of tolafentrine on the endothelial nitric oxide synthase/nitric oxide pathway, DDAH expression, DDAH promoter activity, and cytokine release from endothelial cells and their subsequent influence on DDAH expression were investigated. In monocrotaline-induced pulmonary arterial hypertension in rats, the effects of inhaled tolafentrine on DDAH expression and activity were investigated. Real-time-polymerase chain reaction, immunocytochemistry, and PDE activity assays suggested high expression of PDE3 and PDE4 isoforms in endothelial cells. Treatment of endothelial cells with PDE3/4 inhibitor significantly decreased ADMA-induced apoptosis via a cAMP/PKA-dependent pathway by induction of DDAH2. Chronic nebulization of PDE3/4 inhibitor significantly attenuated monocrotaline-induced hemodynamic, gas exchange abnormalities, vascular remodeling, and right heart hypertrophy. Interestingly, PDE3/4 inhibitor treatment reduced ADMA and elevated nitric oxide/cGMP levels. Mechanistically, this could be attributed to direct modulatory effects of cAMP on the promoter region of DDAH2, which was consequently found to be increased in expression and activity. Furthermore, PDE3/4 inhibitor suppressed apoptosis in endothelial cells and increased vascularization in the lung.

Conclusion—Combined inhibition of PDE3 and 4 regresses development of pulmonary hypertension and promotes endothelial regeneration by modulating the ADMA-DDAH axis. (Circulation. 2011;123:1194-1204.)

Key Words: dimethylarginine dimethylaminohydrolase (DDAH) • endothelial regeneration • hypertension, pulmonary • nitric oxide synthase • phosphodiesterase (PDE)
Nitrergic oxide (NO) synthesized by endothelial NO synthase (eNOS) is a potent vasodilator and plays an important role in regulating pulmonary vascular tone. In addition, NO is a potent stimulator of EC proliferation and migration, and eNOS is a critical mediator of angiogenesis. Reduced bioavailability of NO has long been suspected to play a pathogenic role in PAH. Although several mechanisms have been postulated, impairment of NOS activity by endogenous NOS inhibitors such as asymmetrical dimethylarginine (ADMA) in endothelial dysfunction–associated diseases has gained substantial interest in recent years. Recently, we reported that ADMA levels are increased in plasma from PAH patients and in monocrotaline (MCT)-induced pulmonary hypertensive (PH) rats, a preclinical model that mimics severe human PAH. This is linked to downregulation of the ADMA-metabolizing enzyme dimethylarginine dimethylaminohydrolase (DDAH) 2. Furthermore, several lines of evidence have revealed that the ADMA-DDAH pathway plays a crucial role in EC apoptosis, migration, and angiogenesis by regulating NO signaling. Recent studies have demonstrated that DDAH can activate cAMP-dependent signaling via a protein-protein interaction with protein kinase A (PKA). Hasegawa et al showed that PKA plays a stimulatory role in DDAH2-induced vascular endothelial growth factor (VEGF) transcription via an enhanced protein-protein interaction between DDAH2 and PKA. In addition, Tokou et al reported interaction among PKA, DDAH1, and neurofibromin, suggesting a cross-talk between DDAH and cAMP signaling cascades. However, the effect of cAMP signaling on the DDAH signaling axis has not been explored so far.

Phosphodiesterase (PDE) isoenzymes, predominantly PDE3 and 4, are essential coregulators of cAMP catabolism in many organs, including the lung, and are upregulated in experimental PAH models. In addition, cAMP-elevating agents have been shown to enhance various EC functions, including angiogenesis. On the other hand, direct activation of PKA by cAMP was shown to inhibit EC survival and angiogenesis. However, there is scant information on the molecular mechanisms and signaling pathways by which cAMP-elevating agents or a combined PDE3/4 inhibitor regulates endothelial degeneration. Furthermore, the effect of cAMP-elevating agents or a combined PDE3/4 inhibitor on endothelial degeneration and ADMA-DDAH signaling pathway has not been delineated.

We postulated that cAMP production induced by inhibition of PDE3 and PDE4 leads to microvascular regeneration by regulating ADMA and DDAH. In particular, we studied the influence of combined PDE3/4 inhibitor tolafentrine on (1) EC survival and proliferation; (2) serum starvation– and ADMA-mediated apoptosis in vitro followed by deciphering the signaling events involved; (3) the eNOS/NO pathway, DDAH expression, and promoter activity; (4) cytokine release from ECs and their subsequent influence on DDAH expression; (5) hemodynamics and structural remodeling associated with MCT-induced PH; and (6) the regulation of ADMA, DDAH, apoptosis, proliferation, and vascularization in vivo.

Methods

For details of the experimental setup, see the online-only Data Supplement.

EC Isolation and Culture

Primary human umbilical vein ECs (HUVECs) were isolated from umbilical veins. Human pulmonary arterial ECs and ECV304 cells were obtained from Lonza (Basel, Switzerland) and ATCC (Manassas, VA).

Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA was reverse transcribed with ImProm-II Reverse Transcription System, followed by real-time polymerase chain reaction analysis of PDE3, PDE4, and DDAH isoforms using the primers described in Table I in the online-only Data Supplement.

Assessment of Cell Viability, Apoptosis, and Proliferation of ECs

Influence of PDE3/4 inhibitor on EC viability, apoptosis, and proliferation was assessed with MTT, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL), and BrdU incorporation assays.

PDE Activity, DDAH Activity, and Intracellular NO/NOS Activity Measurements

PDE activity was determined via radioimmunoassay, DDAH activity by colorimetric assay, and NO/NOS activity by the DAF-2DA method.

DDAH Promoter Activity Assay

ECV304 cells were transfected with the DDAH2 promoter/reporter gene construct in the absence/presence of PDE3/4 inhibitor, 8-Br-cAMP, and KT5720 and measured for luciferase activity.

Measurement of ADMA, l-Arginine, Nitrite and Nitrate, and cGMP

Plasma ADMA and l-arginine were measured with high-performance liquid chromatography/fluorescence detection, nitrate and nitrate (NOx) with colorimetry, and cGMP with radioimmunoassay.

MCT Treatment, Nebulization of Tolafentrine, and Hemodynamic Measurements

Two weeks after a single MCT injection, rats were subjected to inhalation of tolafentrine or sham nebulization in an unrestrained, whole-body aerosol exposure system. After 4 weeks, hemodynamics, cardiac output, and RV hypertrophy were assessed.

Assessment of Vascular Remodeling, Microvessel Density, Apoptosis, and Proliferation In Vivo

Paraffin-embedded lung sections double-stained with α-smooth muscle actin and von Willebrand factor antibodies for assessing the degree of muscularization of peripheral pulmonary arteries. In addition, lung sections were stained for Elastic–Nuclear Fast Red to assess medial wall thickness. Vessels were detected by staining for von Willebrand factor, proliferation by staining for proliferating cell nuclear antigen, and apoptosis by staining with an in situ cell death detection kit.

Statistical Analysis

Data are presented as mean±SEM. Unless otherwise stated, statistical comparisons of samples were performed by ANOVA followed by the Dunnett posthoc test. Residuals were checked for normal distribution by normal quantile-quantile plots and Shapiro-Wilk tests; homogeneity of variances was checked with the Bartlett test. Dose dependency was checked by the Spearman correlation. Statistical comparisons for real-time polymerase chain reaction experiments are based on ΔCT values. Samples were compared by use of Student t tests with pooled variances. P values were corrected for multiple testing with the Holm procedure.
Results

PDE Isoform Expression in ECs

Using real-time polymerase chain reaction, we found that the PDE isoforms 3A, 3B, 4C, and 4D but not 4A and 4B are expressed in HUVECs (Figure 1A). These results were confirmed by immunostainings (Figure 1B and 1C). Furthermore, we found that PDE3 and PDE4 were the major contributors to total cAMP-PDE activity in HUVECs (Figure I in the online-only Data Supplement). Similar expression profiles of PDE3 and PDE4 isoforms were also observed in human pulmonary arterial ECs and in HUVECs (Figure II in the online-only Data Supplement).

Effect of a PDE3/4 Inhibitor on EC Survival and Proliferation

To determine whether PDE3/4 inhibitor has a general effect on cell survival and proliferation, we stimulated quiescent HUVECs with 0.1% FCS, 10% FCS, or VEGF in the absence/presence of PDE3/4 inhibitor. We first examined the effects of PDE3/4 inhibitor on EC survival. Both 10% FCS and VEGF increased the viability and proliferation of HUVECs compared with serum-starved cells. However, viability of 0.1% FCS– or 10% FCS–stimulated HUVECs was not significantly affected by various concentrations of PDE3/4 inhibitor (0.1, 0.5, or 1 μmol/L; Figure 2A and 2C). In contrast, PDE3/4 inhibitor significantly increased the viability of VEGF-stimulated HUVECs (Figure 2B). Notably, PDE3/4 inhibitor significantly increased DNA synthesis of both 10% FCS– and VEGF-stimulated HUVECs in a dose-dependent manner (P<0.0001; Figure 2D and 2E).

Effect of PDE3/4 Inhibitor on EC Apoptosis

To address apoptosis, cell death was induced in quiescent HUVECs by exposure to 0.1% FCS medium or ADMA (NOS inhibitor), and the effect of PDE3/4 inhibitor (0.1, 0.5, or 1 μmol/L) was investigated. Exposure of HUVECs to 0.1% FCS or ADMA (10 or 20 μmol/L) for 24 hours dose-dependently increased the number of TUNEL-positive cells (P=0.004; Figure 3A through 3C). Incubation with various concentrations of PDE3/4 inhibitor did not influence apoptosis induced by 0.1% FCS (Figure 3A). However, PDE3/4 inhibitor treatment dose-dependently inhibited apoptosis induced by ADMA in ECs (P<0.0001; Figure 3B and 3C).

cAMP Analogs and cAMP-Elevating Agents Mimic the Effect of PDE3/4 Inhibition on ADMA-Induced Apoptosis

We hypothesized that PDE3/4 inhibitors would be able to inhibit ADMA-induced EC apoptosis by increasing intracellular cAMP level. For this purpose, we examined the effects of forskolin, a potent and unique activator of adenylyl cyclase that elevates the intracellular cAMP levels, and 8-Br-cAMP, a cell-permeable and nonhydrolyzable cAMP analog, on ADMA-induced EC apoptosis. Similar to PDE3/4 inhibitor, both forskolin (10 or 50 μmol/L) and 8-Br-cAMP (0.01, 0.1, or 1 mmol/L) decreased ADMA-induced apoptosis in a concentration-dependent manner (P<0.0001; Figure 3D).

PDE3/4 Inhibitor Attenuates ADMA-Induced Apoptosis in a PKA-Dependent Manner

To demonstrate cAMP-mediated downstream signaling,15 we examined the effect of the PKA inhibitor KT5720 on ADMA-induced apoptosis. Interestingly, cotreatment of KT5720 (5 μmol/L) with PDE3/4 inhibitor did not alter ADMA-induced EC apoptosis (Figure 3E).

Since peroxisome proliferator–activated receptor-γ was shown to antagonize ADMA levels by inducing DDAH expression16 and cAMP-elevating agents were shown to be involved in peroxisome proliferator–activated receptor activation,17 we treated ADMA-stimulated HUVECs with GW9662, a potent antagonist of PPARγ (10 or 20 μmol/L). Treatment with GW9662 did not alter ADMA-induced EC apoptosis. In contrast, cotreatment with PDE3/4 inhibitor reduced ADMA-induced EC apoptosis (Figure 3F).
Effect of PDE3/4 Inhibitor on eNOS/NO Pathway

To examine whether the attenuation of effects of ADMA by PDE3/4 inhibitor is via regulation of eNOS/NO pathway, we evaluated intracellular NO production/NOS activity, eNOS expression, and eNOS phosphorylation. Direct measurement of intracellular NO levels/NOS activity by the DAF-2/DA method showed elevated NO production/NOS activity by treatment of HUVECs with PDE3/4 inhibitor (Figure 4A and Figure 3.

Effect of phosphodiesterase (PDE) 3/4 inhibitor, cAMP-elevating agents, cAMP analogs, protein kinase A (PKA) inhibitors, and peroxisome proliferator–activated receptor-γ (PPARγ) antagonists on apoptosis mediated by serum starvation or asymmetrical dimethylarginine (ADMA) in endothelial cells. A through C, Human umbilical vein endothelial cells (HUVECs) were serum starved (0.1% FCS) or treated with 10 or 20 μmol/L ADMA for 24 hours followed by treatment with PDE3/4 inhibitor (0.1, 0.5, or 1 μmol/L). HUVECs were treated with 10 μmol/L ADMA followed by treatment with (D) PDE3/4 inhibitor (0.5 or 1 μmol/L), forskolin (10 or 50 μmol/L), or 8-BrcAMP (0.01, 0.1, or 1) or with (E) the PKA inhibitor KT5720 (5 μmol/L) or (F) the PPARγ antagonist GW9662 (10 or 20 μmol/L) in the absence/presence of the PDE3/4 inhibitor (0.5 or 1 μmol/L). After 24 hours, apoptotic death was estimated by the terminal deoxynucleotidyl transferase–mediated diUTP nick-end labeling assay and is shown as values of absorbance at 405 nm. All values are mean±SEM (n=24). *P<0.05 vs ADMA-treated group; †P<0.05 vs GW9662-treated group.

Figure 2. Effect of phosphodiesterase (PDE) 3/4 inhibitor on vascular endothelial growth factor (VEGF)– or serum-induced endothelial cell survival and proliferation. Serum-starved human umbilical vein endothelial cells (HUVECs) were stimulated with 10% FCS, VEGF, or 0.1% FCS in the absence/presence of PDE3/4 inhibitor (0.1, 0.5, or 1 μmol/L) for 24 hours. A through C, The number of viable HUVECs was determined by MTT assay and shown as values of absorbance at 490 nm. D and E, HUVEC cell proliferation was assessed by BrdU incorporation assay and shown as values of absorbance at 370 nm. All values are mean±SEM (n=24). *P<0.05 vs 0.1% FCS; †P<0.05 vs 10% FCS– or VEGF-treated group.
4B). However, Western blot analysis revealed that PDE3/4 inhibitor did not influence either eNOS expression or eNOS phosphorylation (Figure 4C and 4D).

**Effect of PDE3/4 Inhibitor on DDAH Expression and DDAH Promoter Activity**

To investigate whether the attenuation of the effects of ADMA by PDE3/4 inhibitor is via regulation of DDAH, we evaluated changes in the expression of DDAH isoforms. As shown in Figure 4E and 4F, PDE3/4 inhibitor caused a 3-fold induction of DDAH2 but not DDAH1 mRNA compared with nontreated cells. Western blotting likewise disclosed increased expression of DDAH2 protein in HUVECs exposed to PDE3/4 inhibitor (Figure 4G and 4H).

Based on the presence of 6 putative cAMP response element binding (CREB) response element sequences in the DDAH2 promoter (Figure III in the online-only Data Supplement), we examined whether PDE3/4 inhibitor regulates DDAH2 promoter activity by transient transfection of DDAH2 promoter/reporter plasmid containing human DDAH2 promoter sequences in ECV304 cells. PDE3/4 inhibitor treatment significantly increased luciferase activity (âˆ¼60.7±8.7%) compared with the controls, which was significantly inhibited by cotreatment with the PKA inhibitor KT5720. Similarly, treatment with 8-Br-cAMP significantly increased DDAH2 promoter activity (Figure 4I).

**Effect of Cytokines on DDAH Expression and the Effect of PDE3/4 Inhibitor on Cytokine Release From ECs**

To determine which growth factors/cytokines induce DDAH2 expression, HUVECs were stimulated with different concentrations of platelet-derived growth factor, transforming growth factor-β1, tumor necrosis factor-α, interferon-γ, interleukin (IL)-1β, IL-4, IL-6, and IL-8. We found that tumor necrosis factor-α and interferon-γ stimulation significantly reduced DDAH2 expression, whereas IL-1β and IL-4 induced DDAH2 expression (Figure 5A through 5D). On the other hand, PDE3/4 inhibitor caused a decrease in tumor necrosis factor-α–stimulated IL-1β and IL-8 secretion from
HUVECs (Figure IVA through IVD in the online-only Data Supplement).

Effect of PDE3/4 Inhibitor on Hemodynamics and Gas Exchange

To assess therapeutic potential of PDE3/4 inhibitor in MCT-induced PH (MCT-PH), MCT-injected rats were treated with aerosolized PDE3/4 inhibitor or saline for 2 weeks. MCT-PH rats treated with saline have increased RV systolic pressure (66.5 ± 3.2 versus 25.9 ± 4.0 mm Hg) and RV hypertrophy (0.53 ± 0.04 versus 0.29 ± 0.02) compared with control rats (Figure 6A). In contrast, MCT-PH rats treated with saline have reduced cardiac index (data not shown) and body weight but have no significant changes in systemic arterial pressure compared with control rats (Figure 6B and 6C). Aerosolized PDE3/4 inhibitor treatment compared with saline treatment for 2 weeks significantly lowered RV systolic pressure to 43.4 ± 2.1 mm Hg but with no significant effects on systemic arterial pressure, cardiac index, or body weight (Figure 6A through 6C). Furthermore, the partial arterial oxygenation and central venous oxygen saturation that were decreased in MCT-PH rats treated with saline became normalized (≈70%) (Figure 5).

Figure 5. Dimethylarginine dimethylaminohydrolase (DDAH) isoform expression in endothelial cells treated with cytokines. A and B, mRNA expression of DDAH2 by real-time polymerase chain reaction in human umbilical vein endothelial cells (HUVECs) treated with different concentrations of growth factors or cytokines (platelet-derived growth factor [PDGF], 30 ng/mL; transforming growth factor-β1 [TGF-β1], 2 and 5 ng/mL; tumor necrosis factor-α [TNF-α], 1 or 10 ng/mL; interferon-γ [IFN-γ], 10 ng/mL; interleukin [IL]-1β, 10 ng/mL; IL-4, 10 ng/mL; IL-6, 10 or 100 U; IL-8, 10 or 100 ng/mL). All values were normalized to porphobilinogen deaminase, and relative changes were expressed as ΔCT. Western blots (C) and subsequent densitometric quantification (D) of DDAH2 in HUVECs treated with cytokines TNF-α, IFN-γ, and IL-1β for 24 hours. All values are mean ± SEM (n = 4). *P < 0.05 vs control.

Figure 6. Influence of inhaled phosphodiesterase (PDE) 3/4 inhibitor on hemodynamics, gas exchange, right ventricular (RV) hypertrophy and pulmonary vascular remodeling in monocrotaline (MCT)-induced pulmonary hypertensive rats. Physiological measurements were taken 28 days after MCT injection (MCT [28 days]). PDE3/4 inhibitor or saline was applied by repetitive inhalations from day 14 to 28 (MCT[28 days]/PDE3/4i or MCT[28 days]/saline). Control animals received a sham injection of saline. A, RV systolic pressure (RVSP); B, systemic arterial pressure (SAP); C, body weight (g); D, ratio of arterial oxygen to the fraction of inspired oxygen (PaO₂/FiO₂); E, central venous oxygen saturation (SvO₂); and F, measurement of RV hypertrophy [RV/(LV+S)] are given (n = 8 to 14). Medial wall thickness (G) and muscularization (H) of pulmonary resistance arteries, as percentage of total pulmonary artery cross section (size 20 to 50 μm), are given (n = 10). All values are mean ± SEM. *P < 0.05 vs control; †P < 0.05 vs MCT[28 days]/saline.
in PDE3/4 inhibitor–treated rats (Figure 6D and 6E). Importantly, inhaled PDE3/4 inhibitor reduced established RV hypertrophy to 0.37 ± 0.03 (Figure 6F).

**Effect of PDE3/4 Inhibitor on Pulmonary Vascular Remodeling**

To assess PDE3/4 inhibitor effects on pulmonary vascular remodeling, we quantitatively assessed the degree of muscularization of pulmonary resistance arteries. In MCT-PH rats treated with saline, a dramatic increase in muscularization and medial wall thickness of pulmonary arteries occurred compared with control rats (Figure 6G and 6H). Aerosolized PDE3/4 inhibitor treatment compared with saline treatment for 2 weeks significantly lowered fully muscularized pulmonary resistance arteries (24.1 ± 4.6% versus 49.6 ± 7.5%) and medial wall thickness (25.0 ± 0.4% versus 31.4 ± 0.5%; Figure 6G and 6H).

**Effects of Aerosolized PDE3/4 Inhibitor on Total cAMP-Specific PDE Activity in Different Tissues**

To study the widespread/tissue-specific effects of aerosol containing PDE3/4 inhibitor in different tissues, total cAMP-specific PDE activity was measured in the lung, kidney, and liver. Interestingly, aerosolized PDE3/4 inhibitor treatment significantly reduced total cAMP-specific PDE activity only in lung tissues compared with kidney and liver tissues of MCT-PH rats (Figure VA through VC in the online-only Data Supplement).

**Effect of PDE3/4 Inhibitor on Methylarginine Production**

Two weeks of treatment of MCT-PH rats with PDE3/4 inhibitor caused a substantial and significant decrease in the plasma ADMA levels. Plasma ADMA but not L-arginine levels were decreased in PDE3/4 inhibitor–treated MCT-PH rats compared with saline-treated MCT-PH rats from 1.65 ± 0.15 to 0.31 ± 0.04 (Figure 7A and 7B).

**Effect of PDE3/4 Inhibitor on DDAH Expression and Activity**

Two weeks of treatment of MCT-PH rats with PDE3/4 inhibitor increased DDAH2 mRNA levels compared with saline-treated MCT-PH rats (Figure 7C). Western blot analysis confirmed that DDAH2 expression also increased with PDE3/4 inhibitor treatment in MCT-PH rat lungs (Figure 7D and 7E). Furthermore, DDAH activity decreased by 4-fold in saline-treated MCT-PH rat lungs compared with control lungs and in organs other than lung, ie, in primary organs responsible for ADMA metabolism (kidney and liver; Figure 7F and Figure VIA and VIB in the online-only Data Supplement). Importantly, 2 weeks of PDE3/4 inhibitor administration in MCT-PH rat lungs restored DDAH expression and activity to a nearly normal level.

**Effect of PDE3/4 Inhibitor on cGMP and NO Production**

Parallel to alteration in DDAH activity, PDE3/4 inhibitor also increased NO synthesis. Four weeks after MCT injection, rats...
showed a remarkable decrease in NOx levels. Compared with saline treatment, treatment of MCT-PH rats with PDE3/4 inhibitor significantly elevated plasma NOx levels and plasma cGMP levels (Figure 8A and 8B).

Effects of PDE3/4 Inhibitor on Endothelial Regeneration

Quantitative analysis of von Willebrand factor–positive blood vessels in lung sections indicated a lower vascular density in MCT-injected rat lungs compared with control rat lungs. Interestingly, the vascular density was increased in PDE3/4 inhibitor–treated MCT-PH rats compared with saline-treated MCT-PH rats (Figure 8C and 8D). The number of proliferating (proliferating cell nuclear antigen–positive) ECs increased significantly in the PDE3/4 inhibitor–treated group compared with the saline-treated group (Figure 8E). In contrast, the number of apoptotic ECs increased significantly 2 weeks after MCT injection. PDE3/4 inhibitor inhalation significantly decreased the number of apoptotic pulmonary ECs (Figure 8F).

Discussion

This study has 4 salient findings. First, the combined PDE3/4 inhibitor tolafentrine increases DDAH2 promoter activity, upregulates DDAH2 expression, and inhibits ADMA-induced apoptosis in ECs via a cAMP-PKA–dependent pathway. Second, inhalation therapy of PDE3/4 inhibitor improved pulmonary hemodynamics and reversed structural changes in

Figure 8. Effect of phosphodiesterase (PDE) 3/4 inhibitor on plasma concentrations of nitrate and nitrate (NOx), cGMP, lung vascularization, proliferation, and apoptosis in monocrotaline (MCT)-induced pulmonary hypertensive rats. Plasma concentrations of NOx, cGMP, lung vascularization, proliferation, and apoptosis were assessed in control, MCT-injected rats that were either treated by repetitive inhalations from day 14 to 28 with saline (MCT[28 days]/saline) or PDE3/4 inhibitor (MCT[28 days]/PDE3/4 inhibitor). Plasma NOx (A) and cGMP (B) levels (n=10). *P<0.05 vs control; †P<0.05 vs MCT[28 days]/Saline. C, Quantitative analysis of vessel density. All values are mean±SEM (n=10). *P<0.05 vs control; †P<0.05 vs MCT[28 days]/Saline. D, Representative immunohistochemical microphotographs of von Willebrand factor–positive endothelial cells in vessels of lungs. Scale bars=40 µm. E, Cell proliferation was assessed by staining for proliferating cell nuclear antigen (PCNA; red nuclei). F, Apoptosis was assessed by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay (green cells are TUNEL-positive cells). White arrows indicate PCNA- and TUNEL-positive cells in E and F, respectively. Scale bars=20 µm. G, Schematic depiction of molecular mechanisms responsible for augmented nitric oxide (NO) production by PDE3/4 inhibitor. By increasing intracellular cAMP levels, PDE3/4 inhibitor activates protein kinase A (PKA) and enhances NO production by modulating the asymmetrical dimethylarginine–dimethylarginine dimethylaminohydrolase (ADMA–DDAH) axis, which was altered during progressive pulmonary hypertension. ADMA is endogenously derived from the proteolysis of methylated arginine residues by protein–arginine methyl transferases (PRMT). 5′AMP indicates adenosine 5′-monophosphate; iPKA and aPKA, inhibited and activated PKA; CAT, cationic amino acid transporter; CREB, cAMP response element binding; DMA, dimethyl amine; eNOS, endothelial NO synthase; NP, nuclear protein; and SAH and SAM, S-adenosyl homocysteine and methionine.
MCT-PH rats. Third, PDE3/4 inhibitor restores DDAH expression and activity in MCT-PH rats, reduces ADMA levels, and elevates NO levels. Fourth, treatment alters pulmonary EC apoptosis, proliferation, and vascularization, which contribute to the regeneration of pulmonary ECs (Figure 8G).

Impairment of pulmonary vascular and endothelial homeostasis plays a significant role in the pathobiology of PAH. Despite the uncertainty of the exact mechanisms causing the pulmonary arteriolar obstruction, EC apoptosis could initiate microvascular degeneration.4 Thus, regeneration of the pulmonary vascular bed could be a novel therapeutic approach for the treatment of PAH. In this scenario, although it is generally accepted that cAMP signaling is most likely involved in various EC events,14 there is currently a paucity of information regarding the role of cAMP elevation in regulating the function of these cells. We found here that the PDE3 and PDE4 isoforms are expressed in ECs. Inhibition of these isoforms by combined PDE3/4 inhibitor inhibited ADMA-induced apoptosis and augmented VEGF-stimulated proliferation.

ADMA, an analog of l-arginine present in blood of both humans and animals, can inhibit NOS18 and is the overmarker of endothelial dysfunction–associated diseases.8,19 In addition, endogenous ADMA and exogenous ADMA inhibit acetylcholine-induced vascular endothelium–dependent relaxation.20 ADMA also increases EC motility, oxidative stress, and upregulated genes encoding endothelial adhesion molecules that are redox sensitive.21 The present study shows that ADMA induces apoptosis in ECs, in agreement with a study from Jiang et al.9 Furthermore, treatment with the PDE3/4 inhibitor forskolin and 8-Br-cAMP reduced ADMA-induced apoptosis in ECs by accumulating cAMP. This event was inhibited by inhibition of PKA activity, indicating that PDE3/4 inhibitor inhibits ADMA-mediated endothelial apoptosis in a cAMP/PKA-dependent manner. The effective concentrations of ADMA used in in vitro studies are higher than the plasma level of ADMA achieved in patients and experimental models of PH.8,22 However, the intracellular ADMA level in endothelium has been reported to be much more concentrated, even 10-fold higher than the reported range for plasma values. The intracellular levels of ADMA in patients with PAH and its apoptotic effects on ECs in vivo remain to be determined.

A central finding of the present study is that PDE3/4 inhibitor increases DDAH2 expression, and in silico analysis demonstrates 6 putative CREB-binding elements in DDAH2 promoter. In addition, increased DDAH2 promoter/reporter activity in the presence of PDE3/4 inhibitor confirmed the function role of these CREB responsive elements. This supposition is strengthened by the additional finding that treatment with 8-Br-cAMP increased DDAH2 promoter/reporter activity and inhibited it by cotreatment with PKA inhibitor. In addition, because eNOS appears to be the central source of NO in endothelium, we evaluated the expression and activation of eNOS followed by direct measurement of intracellular NO.23 Interestingly, the PDE3/4 inhibitor increased NO production/NOS activity with no changes in the expression/phosphorylation of eNOS, indicating that PDE3/4 inhibitor increases NO production as a result of changed DDAH expression and activity.

Because it has been well demonstrated that PDE3 and/or PDE4 inhibitors block synthesis of proinflammatory cytokines,24 we assessed the influence of PDE3/4 inhibitor on the synthesis/release of inflammatory cytokines by ECs in vitro. We observed that tolfenetrine caused a decrease in tumor necrosis factor-α–stimulated IL-8 secretion. Vice versa, proinflammatory cytokines such as tumor necrosis factor-α and interferon-γ mediated DDAH2 downregulation. These observations in vitro may have important implications for the pathogenesis and treatment of PH. First, they show the involvement of ADMA and DDAH in MCT-induced endothelial dysfunction. From our results and previous reports,8,25 we postulate that MCT induces oxidative stress and inflammatory milieu by downregulating DDAH, which then induces ADMA production, thus causing endothelial apoptosis, a process that precedes progressive PH and vascular remodeling. Second, the fact that tolfenetrine modulated DDAH, which is dysregulated in both experimental and human PAH,8,22 suggests a therapeutic potential of PDE3/4 inhibitors.

Interestingly, an increased serum- and VEGF-stimulated proliferation was observed in ECs after treatment with PDE3/4 inhibitor. This observation can be explained by augmented VEGF signaling in these settings either by adenylyl cyclase/PKA-dependent VEGF induction26 or by induction of DDAH2, which in turn can induce VEGF, resulting in increased proliferation and migration of ECs.11

To address the therapeutic potential of PDE3/4 inhibition, MCT-PH rats were treated with tolfenetrine from day 14 to 28 by repetitive inhalations. We aimed to achieve a selective pulmonary vasodilation by using a 15-fold lower dose of tolfenetrine compared with the intravenous dose used in our previous studies.27 In accordance, analysis of total cAMP-specific PDE activity in different tissues demonstrated significant PDE inhibition in lungs but not in kidney and liver after tolfenetrine treatment, suggesting a less widespread inhibition of PDE activity after aerosolized tolfenetrine treatment. Hemodynamics and RV hypertrophy were significantly improved, as were the structural changes in the lung vasculature evoked by MCT. These most impressive beneficial effects of PDE3/4 inhibitor on structural remodeling may be explained in part by cAMP-mediated inhibition of proliferation, cell cycle progression, and migration of pulmonary arterial smooth muscle cells.28

In line with these hemodynamic data, a strong impact of PDE3/4 inhibitor on plasma ADMA, an endogenous NOS inhibitor of MCT-challenged animals, was noted. PDE3/4 inhibitor treatment of MCT-PH rats for 2 weeks reduced ADMA levels, which was paralleled by increased DDAH expression and activity in lungs and, to a lesser extent, in kidneys that become dysregulated during development of MCT-induced PH.9 Because DDAH is key regulator of endogenous ADMA levels, increased DDAH expression and activity may accelerate the degradation of endogenous ADMA, enhancing the activity of eNOS and eventually augmenting the synthesis of NO. In line with this hypothesis, NOx levels returned to close to control values in PDE3/4 inhibitor–treated PH rats. To the best of our knowledge, this is the first study to demonstrate cAMP-PDE regulation of NO/cGMP signaling by reducing the endogenous NOS inhibitor ADMA. Interestingly, our results with tolfen-
trine identify cAMP-elevating agents in general and combined PDE3/4 inhibitor specifically as transcriptional modulators of DDAH2, in addition to the previously recognized all-transretinoic acid.\(^9\) In addition to transcriptional regulation, combined PDE3/4 inhibitor may directly influence DDAH activity by its potent antiinflammatory properties. Thus, these 2 pathways together enhance the bioavailability of NO, thereby reducing the impairment of endothelial-dependent relaxation/endothelial degeneration induced by MCT.

MCT and environmental stress induce pulmonary EC injury and decrease the number of pulmonary capillaries,\(^30\) contributing to the development of PH, suggesting that regeneration of lung microvasculature may be a novel and effective therapeutic strategy for restoring pulmonary hemodynamics in experimental and clinical PH. In fact, recent studies have demonstrated that transplantation of somatic cells or endothelial-like progenitor cells transduced with eNOS rescues MCT-induced PH,\(^3\) suggesting that NO-elevating agents such as PDE3/4 inhibitors may be involved in endothelial regeneration. In line with this reasoning, inhalation of PDE3/4 inhibitor enhanced expression of proliferating cell nuclear antigen, a marker for cell proliferation, in pulmonary ECs. Interestingly, PDE3/4 inhibitor also increased the number of pulmonary capillaries in MCT-injected rats, suggesting attenuation of MCT-induced PH by PDE3/4 inhibitor, possibly by protecting against EC apoptosis or by inducing microvascular angiogenesis.

Conclusions

Therapeutic inhalation of combined PDE3/4 inhibitor tolfen-trine reduced MCT-induced PH, ie, improved hemodynamic values, and reduced vascular remodeling and endothelial degeneration by modulating ADMA-DDAH axis. Thus, regeneration of the pulmonary vascular bed could be a novel therapeutic approach; hence, PDE3/4 inhibitors may be useful for the treatment of pulmonary vascular disease.

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Disclosures

None.

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### CLINICAL PERSPECTIVE

Endothelial dysfunction is a key feature of chronic systemic and pulmonary vascular disorders. The nitric oxide pathway plays a central role in maintaining physiological organ function. Alterations of this pathway have been attributed to be centrally involved in the course of diseases like chronic heart failure, systemic and pulmonary arterial hypertension, and arteriosclerosis. The activity of nitric oxide synthases, particularly nitric oxide synthase-3, was found to be suppressed by its endogenous inhibitor asymmetrical dimethylated arginine (ADMA). In the present study, we show that dimethylarginine dimethylaminohydrolase (DDAH), the key regulator of ADMA levels, is downregulated in experimental pulmonary hypertension. In untreated animals, endothelial dysfunction, pulmonary vascular pruning, and right heart dysfunction were associated with reduced nitric oxide. We show a mechanistic link between cAMP-increasing agents and the restoration of endothelial function in progressive pulmonary hypertension. Administration of the phosphodiesterase 3/4 inhibitor tolafertrine led to an increase in the expression of DDAH2 in endothelial cells via a protein kinase A–dependent activation of the DDAH2 promoter. This resulted in decreased ADMA levels and subsequent increased nitric oxide production. In addition, this cAMP-elevating agent prevented vascular pruning and decreased right heart hypertrophy. Prostanoids are one mainstay of the treatment of pulmonary hypertension that operate mainly via elevation of cAMP and subsequent downstream signaling. However, their clinical utility is hampered in part by their immanent side effect profile and/or the route of administration (eg, inhaled, subcutaneous, intravenous). Thus, phosphodiesterase 3/4 inhibitors could represent an independent new class of drugs that warrant further investigation in pulmonary vascular disorders.
cAMP Phosphodiesterase Inhibitors Increases Nitric Oxide Production by Modulating Dimethylarginine Dimethylaminohydrolases


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cAMP phosphodiesterase inhibitors increases nitric oxide production by modulating dimethylarginine dimethylaminohydrolases

Pullamsetti, Modulators of ADMA-DDAH
MATERIALS AND METHODS

Animal experiments

Adult male Sprague Dawley rats (300-350 g body weight) were obtained from Charles River Laboratories (Sulzfeld, Germany). The experiments were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals. Both the University Animal Care Committee and the Federal Authorities for Animal Research of the Regierungspräsidium Giessen (Hessen, Germany) approved the study protocol.

MCT treatment

MCT (Sigma, Deishofen, Germany) was dissolved in 0.5 N of HCl, and the pH was adjusted to 7.4 with 0.5 N of NaOH. The solution was given as a single subcutaneous injection (60 mg/kg) to male Sprague Dawley rats. MCT is a pyrrolizidine alkaloid of plant origin. Monocrotaline is metabolized in the liver to a pyrrole derivative with short half-life that induces endothelial injury in the pulmonary circulation. Vascular reaction in response to MCTP includes initial inflammatory mononuclear infiltration, pulmonary artery endothelial cell (PAEC) damage and subsequent endothelial cell apoptosis. The resolution of MCT-induced damage involves proliferation of PASMCs and subsequent vascular remodeling, which result in pulmonary hypertension, and finally, in right ventricular failure1-3.

Experimental groups

The animals were classified into the following groups: (1) rats injected with saline (Control, n=8); (2) rats injected with MCT who underwent sham nebulization with saline from days 14 to 28 post-injection (MCT[28d]/Saline, n=14); and (3) rats injected with MCT who underwent inhalation of PDE3/4-inhibitor (tolafentrine; see below) from days 14 to 28 post-injection (MCT[28d]/PDE3/4i, n = 10).
Nebulization of tolfentrine

Two weeks after a single MCT injection, rats were subjected to inhalation of tolfentrine or sham nebulization in an unrestrained, whole-body aerosol exposure system as described\(^4\). Inhaled saline or tolfentrine (dose deposited in the lungs \(\sim\)120\(\mu\)g/kg/day) was delivered as 15min nebulization maneuvers using a jet nebulizer with a constant flow rate of 6 l/min (Pari LC Star, Pari, Starnberg, Germany) repeated 12 times per day for 2-weeks (days 14–28).

Surgical preparation, measurement of hemodynamics, and tissue preparation

To monitor hemodynamics, the animals were initially anesthetized intraperitoneal with ketamine and xylazine. The left carotid artery was cannulated, and a right heart catheter was inserted through the right jugular vein for measurement of RV pressure with fluid filled force transducers. Cardiac output in rats was measured by a thermodilution technique (Cardiotherm 500-X; Hugo-Sachs Electronic—Harvard Apparatus GmbH, March, Germany) as described\(^5\). After exsanguination, the left lung was fixed for histology in 10% neutral buffered formalin, and the right lung was snap-frozen in liquid nitrogen for molecular biology experiments.

Assessment of RV hypertrophy

The RV wall was separated from the left ventricular (LV) wall and ventricular septum. Dry weight of the RV wall, free LV wall, and ventricular septum was determined. RV hypertrophy was expressed as the ratio of weight of the RV wall to that of the free LV wall and ventricular septum (S) [RV/(LV+S)].

Assessment of pulmonary vascular remodeling
Paraffin lung sections (3 μm) were double-stained with 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). Sections were counterstained with hematoxylin and examined by light microscopy using a computerized morphometric system (Qwin, Leica, and Wetzlar, Germany) for assessing the degree of muscularization of small peripheral pulmonary arteries. In addition, lung sections were stained for Elastin-Nuclear Fast Red to assess the medial wall thickness. Percentage of fully muscularized pulmonary arteries, related to the total number of pulmonary arteries and measurement of medial wall thickness (given in percentage of total wall thickness) of pulmonary arteries sized from 20 to 50 μm were performed as previously described (7).

**Assessment of microvessel density, apoptosis and proliferation in vivo**

Detection of blood vessels was performed by immunohistochemistry for von Willebrand factor (vWF) (rabbit anti-human vWF, Dako, Glostrup, Denmark). Frozen 3-μm thick lung sections equilibrated to room temperature were fixed in acetone for 10 min and endogenous peroxidases were blocked with 0.3% (v/v) hydrogen peroxide in methanol and then blocked with 5% (v/v) goat serum in phosphate-buffered saline (PBS), followed by incubation with anti-vWF antibody (rabbit anti-human vWF, Dako). Sections were washed in PBS, and antibody binding was determined using a Vector ABC kit (Vector Laboratories, Burlingame, CA). After extensive washing, sections were stained with 3,3′-diaminobenzidine and visualized under the light microscope. The vWF-staining was quantified in lung sections as described. Tissue sections were also stained for proliferating cell nuclear antigen (PCNA) using polyclonal anti-PCNA rabbit (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and visualized with Alexa 555–conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). All sections were counterstained with nuclear 4′-6-diamidino-2-phenylindole (DAPI) and
mounted with fluorescent mounting media (all from DAKO). To assess apoptosis, sections were visualized using terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end-labeling (TUNEL) with an in situ cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany) as specified by the manufacturer. At the end of the procedure, the slides were examined by fluorescence microscopy after DAPI staining. Positive control of TUNEL labeling was prepared using DNase I (Sigma, St. Louis, MO) treatment. After pretreatment, histological sections were incubated with DNase I (5µg/ml) in a 37°C humidified chamber for 10min to induce DNA strand breaks. Negative control was obtained by omitting terminal transferase from the labeling procedure.

**Endothelial cell isolation and culture**

Primary human umbilical vein endothelial cells (HUVECs) were obtained from human umbilical veins as described. HUVECs (passage 1–2) were cultured in M199 medium supplemented with 2.5µg/ml endothelial cell growth supplement and 5% fetal calf serum. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. Human Pulmonary Artery Endothelial Cells (HPAEC) (Lonza, Walkersville, MD) and cultured in endothelial cell basal medium-2 and supplemented with hydrocortisone, 0.2 ml; hFGF-B, 2 ml; VEGF, 0.5 ml; IGF-1, 0.5 ml; ascorbic Acid, 0.5 ml; Heparin, 0.5 ml; FBS, 10 ml; hEGF, 0.5 ml; GA-1000, 0.5 ml.

ECV304 (American Type Culture Collection, Manassas, VA) was cultured in M199 medium supplemented with 10% fetal calf serum, 100µg/ml penicillin, and 100µg/ml streptomycin in a humidified incubator with 5% CO₂ at 37°C. For each experimental set up, several preparations of HUVECs were made during the course of the study (three separate independent experiments). 3-petris/ 8-wells were used per each independent experiment. All the petries/wells for a particular experiment were from pooled HUVECs.
**Immunofluorescence staining of endothelial cells**

Immunofluorescence labeling of HUVECs was performed as described. Antibodies directed against PDE3A, PDE3B, PDE4A, PDE4B, PDE4C, PDE4D (1:100; all from FabGennix Inc., Frisco, TX) were used and visualized with Alexa 488- and Alexa 555– conjugated goat anti-rabbit IgG (Molecular Probes). At the end of the procedure, the slides were examined by fluorescence microscopy after DAPI staining.

**Cytokine stimulation of endothelial cells**

HUVECs were made quiescent in serum-free medium and then incubated with different concentrations of growth factors or cytokines [PDGF (30ng/ml), TGF-β (2ng/ml and 5ng/ml), TNF-α (1ng/ml and 10ng/ml), IFN-γ (10ng/ml), IL-1β (10ng/ml), IL4 (10ng/ml), IL6 (10 U and 100U), IL8 (10ng/ml and 100ng/ml)]. After 6h, RNA was isolated followed by cDNA synthesis and real-time reverse-transcription PCR for DDAH2 expression changes. Similarly, HUVECs were incubated with cytokines [TNF-α (10ng/ml), IFN-γ (10ng/ml), IL-1β (10ng/ml)]. After 24h, protein was isolated followed by western blot analysis.

**PDE activity assays**

PDE activity was determined using 1μM cyclic nucleotide (cAMP or cGMP) as substrate via a two-step radioimmunoassay procedure adapted from Thompson and Appleman. Briefly, substrate and protein sample were incubated over a period in which PDE activity is linear, after which they were boiled for 2min to terminate the reaction. Results are expressed relative to the protein concentration. To identify the contribution of activity of specific PDEs, we performed assays in the presence of specific PDE-inhibitors [30 μM vinpocetine, 30 μM 8-
methoxy-methyl-3-isobutyl-1-methylxanthine (8-MM-IBMX, for PDE1), 10µM erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) in the presence of excess cGMP (for PDE2), 10µM trequisin (for PDE3), and 10µM rolipram (for PDE4) and excess calcium in the presence of EGTA (for PDE1)].

**Apoptosis assessment in endothelial cells**

The cell death TUNEL assay was used to detect cytoplasmic histone-associated DNA fragments to assess apoptosis as described\(^6\). Briefly, HUVECs were made quiescent in serum-free medium for 24h and then incubated with 0.1% FCS or ADMA (10µM, 20µM), with or without PDE3/4-inhibitor (0.05µM, 0.1µM, 0.5µM, 1µM) for 24h. Further, to check the influence of cAMP analogues, PKA-inhibitors and PPAR\(_\gamma\) antagonists on ADMA induced apoptosis, HUVECs were treated with 20µM ADMA followed by treatment (i) with PDE3/4-inhibitor (0.5µM, 1µM), Forskolin (10µM, 50µM) or 8-Br-cAMP (0.01mM, 0.1mM, 1mM), (ii) with PKA-inhibitors, H89 (10µM, 25µM) or KT5720 (5µM) in the absence/presence of PDE3/4-inhibitor (0.5µM, 1µM) or (iii) with PPAR\(_\gamma\) antagonist, GW9662 (10µM, 20µM) in the absence/presence of PDE3/4-inhibitor (0.5µM, 1µM). After 24h, HUVECs were incubated for 30min with lysis buffer at room temperature and then centrifuged at 200g for 10min at 4°C. Aliquots of the supernatant (20µl) were placed into microtiter plate wells coated with streptavidin and the TUNEL assay was performed according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). At the end of assay procedure, absorbance of samples was read spectrophotometrically at 405nm and at reference wavelength 490 using a Tecan ELISA reader (Tecan, Crailsheim, Germany).

**Assessment of proliferation in endothelial cells**
The bromodeoxyuridine (BrdU) incorporation assay was used as a measure of DNA synthesis as described. HUVECs were made quiescent in serum-free medium for 24h and then incubated with 10% FCS or VEGF (30 ng/ml), with or without PDE3/4-inhibitor (0.05µM, 0.1µM, 0.5µM, 1µM) for 24h. BrdU was added during the last 4 h before BrdU incorporation was determined by a colorimetric ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The ELISA plate was read at 370nm and at reference wavelength 492 using a Tecan ELISA reader (Tecan, Crailsheim, Germany). All experiments were done with 6–8 wells per experiment and repeated at least three times.

**Cell viability assessment in endothelial cells**

The MTT assay was performed to measure cell viability as described previously. HUVECs were made quiescent in serum-free medium for 24h and then incubated with 0.1% FCS, 10% FCS or VEGF (30ng/ml), with or without PDE3/4-inhibitor (0.05µM, 0.1µM, 0.5µM, 1µM) for 24h. After treatment, medium containing 1mg/ml MTT (Promega, Mannheim, Germany) was added to cells for a final concentration of 0.2mg/ml and incubated at 37°C for 1-2h. At the end of this period, the extent of MTT reduction to formazan within cells was quantified spectrophotometrically (TEKAN, Crailsheim, Germany) at 490nm and taken as an indicator of cell viability.

**DDAH promoter activity assay**

A restriction fragment spanning nucleotides -1755 to -216 of the human DDAH2 gene was isolated from a human DDAH2 genomic clone and cloned into the multiple cloning site of the pGL3basic luciferase vector (Promega, Mannheim, Germany) to produce a DDAH2 promoter/reporter gene construct (pGL3sal). Transient transfections of ECV304 cells with promoter/reporter constructs were achieved using Lipofectamin-2000 according to the
manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA) in a 48-well plate. Twenty-four hours after transfection, PDE3/4-inhibitor (1µM), 8-Br-cAMP (0.1mM) or KT5720 (5µM) was added to the culture medium and cells maintained in culture for a further 24h. The cells were lysed in 4°C luciferase lysis buffer (Promega, Mannheim, Germany), and firefly and renilla luciferase activities were measured by a bioluminometer (TEKAN, Crailsheim, Germany).

**Cytokine measurements from endothelial cells**

To study the influence of PDE3/4-inhibitor on cytokine release from HUVECs, cells were incubated with or without PDE3/4-inhibitor (0.1µM, 1µM) for 24h. In addition, HUVEC cells were stimulated with TNF-α (10ng/ml) in the absence/presence of PDE3/4-inhibitor (0.1µM, 1µM) for 24h, to measure the influence of PDE3/4-inhibitor on TNF-α increased cytokine secretion. IFN-γ, IL-1β, IL-4, IL-6, IL-8, TGF-β1 was measured in HUVEC supernatants using a DuoSet enzyme-linked immunosorbent assay (ELISA) development kit (R & D Systems, Minneapolis, MN, USA) as described11.

**Western blotting**

Lung tissues and HUVECs were homogenized separately in lysis buffer containing 50mM Tris-HCl pH 7.6, 10mM CaCl₂, 150mM NaCl, 60mM NaN₃ and 0.1% (w/v) Triton X-100 using a tissue homogenizer. Samples were centrifuged at 13000rpm for 20min at 4°C, and the supernatant protein content was measured using Dye Reagent Concentrate (Bio-Rad, Muenchen, Germany). Extracts containing equal amounts of protein were denatured and subjected to electrophoresis on a 10% SDS polyacrylamide gel and blotted on to polyvinylidene fluoride membrane with a semidry transfer unit (Biometra, Goettingen,
Germany). The membrane was then incubated with Anti–phospho-eNOS (Ser1177, Cell Signaling Technology, Beverley, MA), Anti–phospho-eNOS (Thr495, Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-eNOS (Santa Cruz Biotechnology Inc., Santa Cruz, CA) anti-DDAH2 (Abcam, Cambridge, MA) and then with the appropriate HRP-conjugated secondary antibody as described. Equal protein loading was confirmed by blotting membranes with an antibody against GAPDH. The bands were visualized using an enhanced ECL detection kit (Amersham Bioscience, Freiburg, Germany) and quantified by densitometry.

**DDAH activity assay**

DDAH activity was assayed by determining L-citrulline formation in tissue homogenates by a colorimetric method in 96-well microtiter plates as described. As a negative control, tissue homogenates were boiled for 10 min to inactivate the enzyme. Background values obtained were subtracted from the experimental data to provide corrected DDAH activity. One unit enzyme was defined as the amount that catalyzed the formation of 1 μmol L-citrulline from ADMA per minute at 37°C.

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

Lung tissue and HUVECs were homogenized, and RNA was extracted using Tri Reagent according to the manufacturer’s protocol to obtain total cellular RNA (Biozol, Eching, Germany). For cDNA synthesis, reagents and incubation steps were applied as described previously. Briefly, total RNA was reverse transcribed using the ImProm-II™ Reverse Transcription System (Promega, Mannheim, Germany). Aliquots were used for real-time [reverse transcription (RT)] polymerase chain reactions (PCRs) using the Stratagene Mx3000p Real-Time PCR machine (Stratagene, La Jolla, CA) and SYBR Green as the
fluorescence signal. The expression of PDE3A, PDE3B, PDE4A, PDE4B, PDE4C, PDE4D was normalized to the housekeeping gene porphobilinogen deaminase (PBGD) and expressed as $\Delta CT$. In addition, expression of DDAH1 and 2 was analyzed by real-time RT-PCR using the $\Delta\Delta CT$ method for the calculation of relative changes. The primers used in this study are shown in Table 1.

**Intracellular NO/NOS activity measurements**

The production of NO/NOS activity in HUVECs was measured using the DAF-2 DA detection system (Cell Technology Inc, Mountain View, CA) according to the manufacturer's instructions. Briefly, HUVECs were made quiescent in serum-free medium for 24h and then incubated with or without PDE3/4-inhibitor (0.1$\mu$M, 0.5$\mu$M, 1$\mu$M) for 24h. The cells were incubated in DAF-2 DA (10$\mu$M) for 60 min at 37°C. The cells were then washed in HBSS buffer, followed by counter staining with DAPI. Fluorescence images were captured with a digital camera (DC 300 FX; Leica Microsystems) attached to the microscope. Quantification was carried out by computer-aided image analysis using the image processing and analysis system Leica DMLA and QWin 500IW software (Leica Instruments). For each specimen, the immunostained area was analyzed in six randomly selected measurement areas (500 x 370 $\mu$m) stained with DAPI, and fluorescence intensity of DAF-2 DA of the whole measurement area.

In addition, HUVECs were plated in 96 well plates and were treated with the PDE3/4-inhibitor as described above. The fluorescent signal was then measured in a on a fluorescence microplate reader (Tecan Infinite® M1000 Microplate Reader) at 485 nm excitation and 520 nm emission. Data are presented as percentage of control values. To confirm the effects of nitrite with those of another NO-releasing compound, we also incubated the HUVECs with Diethylamine NONOate sodium salt hydrate (Sigma), which is considered a rather pure NO
donor (0.5, or 5mM), a NO synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME) (Calbiochem) was used (data not shown).

**Measurement of ADMA and L-arginine**

ADMA and L-arginine were measured by a modified method based on high-performance liquid chromatography with fluorescence detection as described. Briefly, plasma was mixed with an internal standard and PBS and applied to Oasis MCX solid-phase extraction cartridges. After washing with HCl and methanol, amino acids were eluted with 1.0ml of concentrated ammonia/water/methanol (10/40/50). The solvent was evaporated under a stream of nitrogen, and the amino acids were derivatized with o-phthaldialdehyde reagent containing 3-mercaptopropionic acid. The derivatized amino acids were separated by isocratic reversed-phase chromatography on a C_{18} column at a column temperature of 30°C using a mobile phase consisting of potassium phosphate buffer (50mmol/l, pH 6.5), containing 8.7% acetonitrile at a flow rate of 0.3ml/min. Fluorescence detection was performed at excitation and emission wavelengths of 340 and 455nm, respectively.

**Measurement of NOx and cGMP**

NO is rapidly converted to nitrite and nitrate (NOx) in human plasma. Plasma NOx levels were measured in triplicate after conversion of nitrate to nitrite by nitrate reductase, and nitrite was measured using a Nitrate/Nitrite Colorimetric Assay Kit based on the Griess reaction (Cayman, Ann Arbor, MI). Similarly, plasma cGMP was measured in duplicate using a cGMP radioimmunoassay (Coulter-Immunotech, Hamburg, Germany). The results are expressed as pmol/ml.

**Statistical analysis**
Data are presented as mean±SEM. Unless otherwise stated, statistical comparisons of samples were performed by ANOVA followed by Dunnett’s post-hoc test. Residuals were checked for normal distribution by normal quantile-quantile plots and Shapiro-Wilk tests, homogeneity of variances was checked using Bartlett’s test. Dose-dependency was checked by Spearman’s correlation. Statistical comparisons for real-time PCR experiments are based on Δct values, samples were compared using Student’s t-tests with pooled variances, p values were corrected for multiple testing using Holm’s procedure.
RESULTS

PDE isoform activity in endothelial cells

Using cAMP PDE inhibitors [PDE1, 30µM vinpocetine and 30µM 8-MM-IBMX; PDE2, 10µM EHNA in the presence of excess cGMP; PDE3, 10µM milrinone; PDE4, 10µM rolipram; and PDE1, the addition of excess calcium in the presence of EGTA], we calculated the relative contribution of each isoform to total cAMP PDE activity in HUVECs and found that PDE3 and PDE4 were the major contributors (Supplemental Figure 1).

PDE isoform expression in HPAECs

Using real-time [RT-]PCR, we found that PDE isoforms PDE3A, 3B, 4A, 4C and 4D, but not PDE4B, are expressed in HPAECs (Supplemental Figure 2A). These results were confirmed by immunostainings (Supplemental Figure 2B, C).

In silico analysis of DDAH2 promoter for the CREB response elements

We searched for the location of the CREB response element (CREB-RE) within the human DDAH2 promoter using Genomatix/MatInspector software. This *in silico* analysis demonstrated the presence of 6 putative CREB-RE sequences between -1755 to -216 bp of the gene (Supplemental Figure 3).

Effect of PDE3/4 inhibitor on cytokine release from endothelial cells

To study the influence of the PDE3/4 inhibitor on cytokine release from endothelial cells, ECs were stimulated with TNF-α (10ng/ml) followed by treatment with different concentrations of the PDE3/4 inhibitor (0.1µM, 1µM). After 24 h, supernatant was collected and determined for the changes in the secretion of IFN-γ, IL-1β, IL-4, IL-6, IL-8 and TGF-β1 by ELISAs.
Detectable levels of IL-1β, IL-4, IL-6 and IL-8 were secreted from HUVECs and IL6 and IL8 secretion was elevated after TNF-α stimulation (Supplemental Figure 4A-D). The PDE3/4 inhibitor caused a decrease in TNF-α stimulated IL-1β and IL8 secretion from HUVECs.

**Effects of aerosolized PDE3/4 inhibitor on total cAMP-specific PDE activity in different tissues**

To study the wide spread/ tissue specific effects of aerosol containing the PDE 3/4 inhibitor in different tissues, total cAMP-specific PDE activity was measured in the lung, kidney and liver. A 40% increase in total cAMP-specific PDE activity was found in lung tissue from MCT-PH rats treated with aerosolized saline as compared to lung tissue from control rats (Supplemental Figure 5A). In contrary, a 35% decrease in total cAMP-specific PDE activity was found in kidneys from MCT-PH rats treated with aerosolized saline as compared to kidneys from control rats (Supplemental Figure 5C). Interestingly, aerosolized PDE3/4 inhibitor treated rat lung tissues had significantly reduced total cAMP-specific PDE activity (Supplemental Figure 5A). Although non-significant, slight decrease in total cAMP-specific PDE activity was observed between aerosolized PDE3/4 inhibitor treated- and aerosolized saline- treated in MCT-PH rat kidneys (Supplemental Figure 5C). No significant decrease in total cAMP-specific PDE activity in kidney and liver was observed between inhaled PDE3/4 inhibitor and saline treated MCT-PH rats (Supplemental Figure 5B, C).

**Effect of a PDE3/4 inhibitor on DDAH activity in kidney and liver tissues**

Further, assessment of DDAH activity in organs other than lung (kidney and liver) i.e. in the primary organs responsible for ADMA metabolism showed a reduction in DDAH activity in saline-treated MCT-PH rat kidneys as compared to control kidneys. However, slight change
in kidneys and no changes in liver tissue were observed in aerosolized PDE3/4 inhibitor-
treated MCT-PH rats as compared to saline-treated MCT-PH rats (Supplemental Figure 6A, B)
REFERENCES


FIGURE LEGENDS

Supplemental Figure 1: PDE3 and PDE4 activities in endothelial cells.

HUVECs cells were analyzed for cAMP PDE activity assay using PDE inhibitors (30µM 8-MM-IBMX, 30µM EHNA, 1µM Trequisin, 10µM Rolipram). The activity inhibition by each inhibitor to the percentage of total cAMP PDE activity was calculated. All values are means ± SEM (n=3). *,p<0.05 versus total activity.

Supplemental Figure 2: PDE3 and PDE4 isoform expression in HPAECs

(A) mRNA expression as analyzed by real-time [RT-]PCR and (B, C) protein expression and localization by immunofluorescence staining for PDE3 and PDE4 isoforms in HPAECs. For (A) all values were normalized to porphobilinogen deaminase (PBGD), and relative changes were expressed as ΔCT (n=3). Scale bar=10µm; 40x magnification.

Supplemental Figure 3: In silico analysis of DDAH2 promoter for the CREB response elements

Schematic representation of the cAMP response element binding protein (CREB) binding sites within the DDAH2 promoter region. Putative consensus binding sites for selected transcription factor family matrices were identified using the Genomatix (MatInspector) export function. Sequences for putative binding sites are underlined and their position was indicated.

Supplemental Figure 4: Influence of PDE3/4 inhibitor on cytokine release from endothelial cells.

(A-D) The influence of PDE3/4 inhibitor (0.1µM, 1 µM) on cytokine release (IL6, IL8, TNF-α, IFN-γ) in HUVECs in the absence or presence of TNF-α stimulation was determined by
ELISA after 24 h. All values are means ± SEM (n=9-12). *,p<0.05 versus non-stimulated; †,p<0.05 versus TNF-α stimulated cells.

Supplemental Figure 5: Influence of inhaled PDE3/4 inhibitor on cAMP-specific PDE activity in MCT-induced pulmonary hypertensive rats.

cAMP-specific PDE activity was measured 28 days after MCT injection (MCT[28d]). PDE3/4 inhibitor or saline was applied by repetitive inhalations from day 14 to day 28 (MCT[28d]/PDE3/4i) or (MCT[28d]/Saline). Control animals received a sham injection of saline. (A-C) Total cAMP-specific PDE activity in the lung, liver and kidney of the above treated rats was measured. All values are means ± SEM (n=8). *,p<0.05 versus control; †,p<0.05 versus MCT[28d]/Saline.

Supplemental Figure 6: Effect of PDE3/4 inhibitor on DDAH activity in MCT-induced pulmonary hypertensive rats.

DDAH activity was measured rats treated with saline (Control), MCT injection plus saline (MCT[28d]/Saline), or MCT injection plus PDE3/4 inhibitor for 2 weeks (MCT[28d]/PDE3/4i). (A, B) DDAH enzyme activity was determined by in vitro assay from kidney and liver homogenates in above mentioned treatment groups. All values are means ± SEM (n=4). *,p<0.05 versus control; †,p<0.05 versus MCT[28d]/Saline.
Supplemental Figure 1
Supplemental Figure 2

A

\( \Delta CT \)

PDE3A  PDE3B  PDE4A  PDE4B  PDE4C  PDE4D

B

-DAPI  +DAPI

PDE3A

PDE3B

C

-DAPI  +DAPI

PDE4A

PDE4B

PDE4C

PDE4D

100 \( \mu \)m  100 \( \mu \)m  100 \( \mu \)m  100 \( \mu \)m
Supplemental Figure 3

EMBOSS_001  1 AACAGACTAGCAAAGGAAATACATCCCGAATTTTGGGAGAATAATGCT  50
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EMBOSS_001  151 CTTCCTGTCCTCGGTATCACTGCCAAATTCCCAGTATTACTTTTGAAGAATGCT  200
EMBOSS_001  201 AACAGACTAGCAAAGGAAATACATCCCGAATTTTGGGAGAATAATGCT  250
EMBOSS_001  251 CAACACTGAGACTAATCGTCTAAGCAGATTGAGAAGATACAGGCT  300
EMBOSS_001  301 AACACTGAGACTAATCGTCTAAGCAGATTGAGAAGATACAGGCT  350
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-360 AG
Supplemental Figure 4
Supplemental Figure 5
Supplemental Figure 6

A

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<tr>
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<tr>
<td>PDE3/4i</td>
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B

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### Table 1

**Forward and reverse primer sequences**

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