Inhaled Iloprost Reverses Vascular Remodeling in Chronic Experimental Pulmonary Hypertension

Ralph Theo Schermuly, Hüseyin Yilmaz, Hossein Ardeschir Ghofrani, Kathrin Woyda, Soni Pullamsetti, Andreas Schulz, Tobias Gessler, Rio Dumitrascu, Norbert Weissmann, Friedrich Grimminger, and Werner Seeger

Department of Internal Medicine, Justus Liebig University Giessen, Giessen; and Schering Deutschland, Berlin, Germany

Rationale: **Inhaled iloprost is an effective therapy for pulmonary arterial hypertension (PAH). However, no study to date has addressed the effects of inhaled iloprost on changes to pulmonary vascular structure that occur in PAH.** *Objectives*: **The present study was designed to investigate chronic antiremodeling effects of inhaled iloprost in monocrotaline (MCT)-induced PAH in rats.** *Methods*: **Four weeks after a single injection of MCT, after full establishment of PAH, rats were nebulized with iloprost at a dose of 6 μg · kg^{-1} · day^{-1}, or underwent sham nebulization with saline.** *Results*: **After 2 weeks of inhalation therapy, right ventricular pressure and pulmonary vascular resistance were reversed in rats treated with iloprost, but not in sham-treated control animals. Systemic arterial pressure was unaffected. In addition, right heart hypertrophy, the degree of pulmonary artery muscularization, and the medial wall thickness of intraacinar pulmonary arteries regressed in response to iloprost. Furthermore, the MCT-induced increase in matrix metalloproteinase-2 and -9 activities and tenascin-C expression was suppressed.** *Conclusions*: **We conclude that the inhalation of iloprost reverses PAH and vascular structural remodeling in MCT-treated rats. This regimen suggests the possibility of an antiremodeling therapy in PAH.**

Keywords: iloprost; monocrotaline; pulmonary hypertension; remodeling

Pulmonary arterial hypertension (PAH) is a severe disease, characterized by elevated pulmonary artery pressure and pulmonary vascular resistance that ultimately leads to high mortality due to right heart failure (1). Infused epoprostenol has proven beneficial in the long-term treatment of patients with pulmonary hypertension (2). However, because of the nonselective route of application, systemic side effects accompany the pulmonary vasodilatory effect. Inhalation of aerosolized iloprost has been demonstrated to promote selective pulmonary vasodilation in severe pulmonary hypertension (3). Iloprost is a stable prostacyclin analog with strong vasodilatory (4) and antithrombotic (4) properties, and the long-term beneficial effects of daily repetitive iloprost inhalation have been documented (5–9). However, it remains controversial whether such beneficial long-term effects are due to the sustained pulmonary vasodilatory effects of the prostanoid, or whether they indicate reversal of structural changes that underlie the pathophysiology of PAH.

In the present study, we employed monocrotaline (MCT), a toxin derived from plants of the genus *Crotalaria* (10), to induce pulmonary artery smooth muscle hypertrophy and PAH in a rat model, as described previously (11). We then examined the acute pulmonary vasodilatory efficacy of inhaled iloprost in this model, and the antiremodeling effects of long-term inhalation of this agent.

(Received in original form February 23, 2005; accepted in final form May 2, 2005)

Supported by the Deutsche Forschungsgemeinschaft (SFB 547), Project C6.

Correspondence and requests for reprints should be addressed to Ralph Theo Schermuly, Ph.D., Zentrum für Innere Medizin, Justus Liebig Universität Giessen, Klinikstrasse 36, 35392 Giessen, Germany. E-mail: ralph.schermuly@innere.med.uni-giessen.de

Am J Respir Crit Care Med Vol 172. pp 358–363, 2005
Originally Published in Press as DOI: 10.1164/rccm.200502-296OC on May 5, 2005

METHODS

Study Protocols

Experiments were performed on Wistar rats (body weight, 300–350 g; Charles River, Sulzfeld, Germany). PAH was induced by a subcutaneous injection of MCT (60 mg · kg^{-1}; Sigma, Deisenhofen, Germany), dissolved in 0.1 M NaOH and adjusted to pH 7.40 with 0.1 M HCl, as described previously (12–16). The stable prostacyclin analog iloprost, or vehicle, was administered in nebulized form every day for 14 days, beginning 28 days after MCT injection. In a separate set of experiments, the acute hemodynamic effects of iloprost were investigated in animals 28 days after MCT injection. The experiments were performed according to institutional guidelines for the use and care of animals.

Study Groups

The animals were classified into the following four groups: (*1*) rats injected with saline (control, n = 10); (*2*) MCT-injected rats, 28 days postinjection (MCT_{28}, n = 14); (*3*) MCT-injected rats, 42 days postinjection and undergoing sham nebulization with saline from Days 28 to 42 (MCT_{42}, n = 14); and (*4*) MCT-injected rats, 42 days postinjection and undergoing inhalation of iloprost from Days 28 to 42 (MCT_{42}/Ilo, n = 10). For acute hemodynamic studies, 24 MCT_{28} rats (rats injected with MCT, 28 days postinjection) were used (inhalation of saline, n = 6; inhalation of iloprost at three different doses, n = 6 each).

Inhalation of Iloprost

Four weeks after a single MCT injection, rats were subjected to inhalation of iloprost in an unrestrained, whole body aerosol exposure system. The agent was aerosolized, via a jet nebulizer (Pari LC Star; Pari, Starnberg, Germany) connected to the cage, at a constant flow rate (6 L · minute^{-1}) for 15 minutes. This nebulizer was characterized by a mass median aerodynamic diameter of 2.8 μm and a geometric standard deviation of 2.5 (determined by laser difractometric measurements, as described previously [17]). Total lung deposition of nebulized material was 0.5%. This deposition fraction was determined in preceding experiments by nebulization of ^{99m}Tc-DTPA in the same nebulization system and subsequent quantification of ^{99m}Tc-DTPA in the lavage fluid of exposed rats. This value is in accordance with 0.3% lung deposition, which has been demonstrated previously in this model (16).

To assess the acute vasodilatory effects of inhaled iloprost, hemodynamic testing in response to the drug was performed 4 weeks after MCT injection. Hemodynamics were measured before and after a single inhalation of the agent (iloprost: 0.13, 0.65, and 1.3 μg · kg^{-1} · minute^{-1} for 15 minutes), using an ultrasonic nebulizer with a mass median aerodynamic diameter of 4.0 μm and a geometric standard deviation of 2.1 as described previously (18).

For assessment of chronic effects of inhaled saline or iloprost (total deposited dose, 6.0 μg · kg^{-1} · day^{-1}), 15-minute nebulizations were repeated 12 times per day for 2 weeks in rats that had already developed PAH due to MCT injection 4 weeks previously. Hemodynamic studies and assessment of structural changes were performed after completion of the 2-week period of inhalative therapy.

Surgical Preparation and Tissue Preparation

For monitoring hemodynamics, animals were anesthetized with ketamine–xylazine and tracheostomized, as described previously (13, 15). A polyethylene catheter was inserted into the left carotid artery to measure arterial pressure. A right heart catheter (PE-50 tubing) was inserted into the right ventricle through the right jugular vein for measurement of right ventricular systolic pressure with fluid-filled force transducers. Cardiac output was measured by a thermodilution technique. Briefly, a thermistor (1.5 F) was placed into the ascending thoracic aorta via the right carotid artery for measurement of transpulmonary thermodilution cardiac output (Cardiotherm 500-X; Hugo Sachs Electronik-Harvard Apparatus, March-Hugstetten, Germany). The cardiac output was averaged from three consecutive determinations and indexed to the weight of the animal to obtain cardiac index as described previously (13). After exsanguination, the left lung was fixed for histology in 10% neutral buffered formalin and the right lung was frozen in nitrogen.

Right Ventricular Hypertrophy

The right ventricle (RV) was dissected from the left ventricle (LV) and the septum (S) and weighed to determine the extent of RV hypertrophy from the ratio RV/(LV + S); this was done by an observer blinded to the treatment.

Histologic Examination of Lungs

The degree of muscularization of small peripheral pulmonary arteries was assessed by double-staining 3-μm sections with an anti-α-smooth muscle actin antibody (diluted 1:900; clone 1A4; Sigma, St. Louis, MO) and antihuman von Willebrand factor antibody (diluted 1:900; Dako-Cytomation, Hamburg, Germany). Sections were counterstained with hematoxylin and examined by light microscopy, using a computerized morphometric system (QWin; Leica Microsystems, Bensheim, Germany). In detail, at ×400 magnification 80 small pulmonary vessels of each animal ranging from 10 to 50 μm in external diameter were counted and noted as muscular, partially muscular, or nonmuscular by an observer blinded to treatment. To assess the degree of muscularization, the amount of α-smooth muscle actin–positive vessel wall area was determined in each vessel by the QWin image-processing system (Leica Imaging Systems, Bensheim, Germany). Nonmuscular arterioles were detected by endothelial anti–von Willebrand factor staining. Arteries that contained more than 70% α-actin–positive vessel wall area were defined as muscular; arteries with less than 4% α-actin–positive vessel area were defined as nonmuscular. Arteries that contained between 4 and 70% α-actin–positive vessel area were defined as partially muscularized. For all muscular arteries with an external diameter of 50 to 100 μm, the wall thickness of the media (i.e., the distance between external and internal elastic laminae) was measured along the shortest curvature and expressed as a percentage of medial wall thickness calculated as (2 × media thickness/external diameter) × 100. Media thickness was defined as the distance between the lamina elastica interna and lamina elastica externa.

Gelatin Zymography

Lung tissues were homogenized (20 mg of tissue weight per milliliter of buffer) at 4°C in 20 mM Tris-HCl, 150 mM NaCl, 1% (vol/vol) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 10 μM E-64, pH 7.5. The matrix metalloproteinase (MMP) activity was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis zymography, in which the enzymes hydrolyze the gelatin substrate present in the gel to form a clear band. After staining for protein in the gel, samples were separated on a 10% polyacrylamide gel copolymerized with gelatin (1 mg · ml^{-1}) at 4°C. The gel was subsequently incubated for 1 hour at 25°C in 2.5% (vol/vol) Triton X-100. The gel was washed with water (twice, 20 minutes each time) before incubating it overnight at 37°C in 50 mM Tris-HCl, pH 7.5. The gel was kept in fixative containing 40% (vol/vol) methanol and 7% (vol/vol) acetic acid for 1 hour, after which the gel was stained with 0.25% (vol/vol) Coomassie Brilliant Blue R250 dye for at least 1 hour and then destained in 10% (vol/vol) methanol and 7% (vol/vol) acetic acid. The gel was visualized with a BioDocAnalyze system (Whatman Biometra, Goettingen, Germany), in which activity of MMP-2 and MMP-9 was shown as a clear white band against a blue background.

Reverse Transcription–Polymerase Chain Reaction

To quantify tenascin-C expression changes in the various treatment groups, semiquantitative reverse transcription–polymerase chain reaction was employed. For this, total RNA was isolated from rat lungs with Trizol reagent (Life Technologies, Rockville, MD), and first-strand cDNA was synthesized with the ImProm-II reverse transcription system (Promega, Madison, WI), using oligo$(dT)_{12-18}$ primers according to the manufacturer's instructions. Afterward, 1 μg of cDNA product was used as a template in polymerase chain reaction amplifications together with appropriate primers as described. The final products were electrophoresed in a 2% agarose gel and detected by ethidium bromide staining. The expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were monitored as a loading control. Primers designed specifically for rat tenascin-C and GAPDH were as follows: for tenascin-C: forward primer, ATGTTGAATGGCGACAC; and reverse primer, CGGTCTCCAAACCCAG; and for GAPDH: forward primer, ACA GCCTGTTTCTGGT; and reverse primer, GATGCTGGATGCCTT TTAT.

Data Analysis

All data are given as means ± SEM. Differences between the groups were assessed by one-way analysis of variance and a Student–Newman–Keuls test for multiple comparisons, with a p value < 0.05 regarded as significant.

RESULTS

Acute Effects of Aerosolized Iloprost Administered to Rats 28 Days after MCT Treatment

Aerosolized iloprost dose dependently reduced right ventricular systolic pressure (Figure 1). As depicted, this pulmonary vasodilatation was accompanied by a slight decrease in systemic arterial pressure at the higher end of the dose range.

Chronic Effects of Aerosolized Iloprost: Hemodynamics

After injection of MCT, PAH developed in all rats (right ventricular systolic pressure on Day 28 = 68.9 ± 3.2 mm Hg [n = 11]

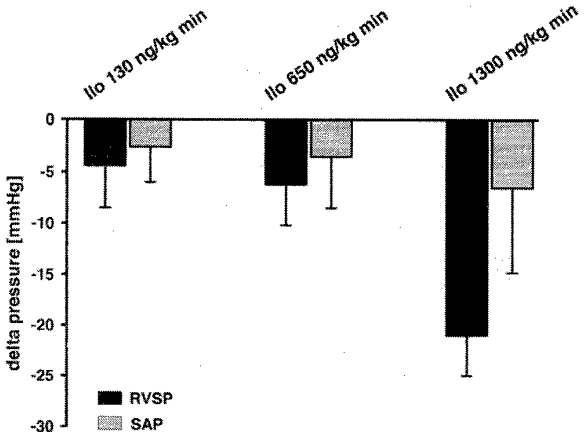

Figure 1. Acute pulmonary vasodilatory effect of inhaled iloprost in monocrotaline-induced pulmonary arterial hypertension. All animals had developed pulmonary arterial hypertension in response to monocrotaline treatment 28 days previously. Subsequent to catheterization, they received aerosolized iloprost during an inhalation period of 5 minutes (inhaled doses per kilogram bodyweight per minute are given). The decrease in right ventricular systolic pressure (RVSP, in mm Hg) and systemic arterial pressure (SAP, in mm Hg) in response to the inhalation maneuver is given (mean ± SEM of six independent experiments each).

and on Day 42 = 74.9 ± 5.1 mm Hg [n = 9], as compared with 27.3 ± 3.2 mm Hg in controls; Figure 2). No significant changes in systemic arterial pressure were observed. When compared with control animals (cardiac index, 36.5 ± 3.45 ml · minute^{-1} · 100 g bodyweight), the cardiac index was slightly decreased on Day 28 (27.4 ± 2.7 ml · minute^{-1} · 100 g bodyweight) and on Day 42 (31.8 ± 1.3 ml · minute^{-1} · 100 g bodyweight). The pulmonary vascular resistance index thus substantially increased, from 0.91 ± 0.05 to 2.41 ± 0.29 mm Hg · minute · ml^{-1} · 100 g bodyweight on Day 28 and to 2.36 ± 0.15 mm Hg · minute · ml^{-1} · 100 g bodyweight on Day 42. Inhaled iloprost (n = 8) decreased right ventricular systolic pressure to 52.9 ± 4.8 mm Hg ($p < 0.05$ vs. MCT_{42} and MCT_{28}), increased cardiac output, and decreased the pulmonary vascular resistance index to 1.48 ± 0.18 mm Hg · minute · ml^{-1} · 100 g bodyweight ($p < 0.05$ vs. MCT_{42} and MCT_{28}).

Chronic Effects of Aerosolized Iloprost: Right Ventricular Hypertrophy

Four weeks after injection of MCT, animals demonstrated significant right heart hypertrophy, indicated by an increase in the ratio of right ventricular weight to left ventricular plus septum weight (RV/[LV + S]) from 0.29 ± 0.02 (control animals) to 0.62 ± 0.02 (Figure 3). Rats that received inhaled vehicle for 2 weeks, from Days 28 to 42, showed further progression of right ventricular hypertrophy (RV/[LV + S] = 0.78 ± 0.07). Inhaled iloprost not only prevented this further progression, but also caused a significant regression of right ventricular hypertrophy as compared with Day 28 (MCT_{42}/Ilo = 0.51 ± 0.02).

Chronic Effects of Aerosolized Iloprost: Histopathology

Evaluation of the extent of muscularization of pulmonary arteries with external diameters of 15 to 50 μm demonstrated a significant reduction in normally nonmuscularized pulmonary arteries and a subsequent increase in fully muscularized vessels in response to MCT (Figures 4 and 5). Medial wall thickness was significantly increased in the MCT_{28} and MCT_{42} groups compared with the control group. We analyzed the medial wall thickness of arteries with external diameters of 50 to 100 μm (Figure 6). In comparison with control animals, the medial wall thickness (as a percentage of total wall thickness) increased significantly from 7.2 ± 1.8 to 28.0 ± 4.8% (MCT_{28}) and to 32.0 ± 5.7% (MCT_{42}). Both the percentage of fully muscularized pulmonary arteries and medial wall thickness (21.8 ± 2.1%) were significantly reduced by aerosolized iloprost ($p < 0.05$ vs. MCT_{42} and MCT_{28}).

Chronic Effects of Aerosolized Iloprost: MMPs and Tenascin-C

The activity of MMP-2 and MMP-9 was monitored by zymography. Significantly elevated gelatinolytic capacities were observed in response to MCT treatment, with MMP-2 having the major effect (Figure 7). The MMP-2 activity in lungs from MCT-treated rats was observed as a triplet, with molecular mass values of 66, 62, and 59 kD, with 62 kD being the predominant form, as previously described (19, 20). Activity of MMP-2 and MMP-9 decreased significantly in the iloprost treatment group, compared with both MCT_{42} and MCT_{28}. Similar results were obtained for

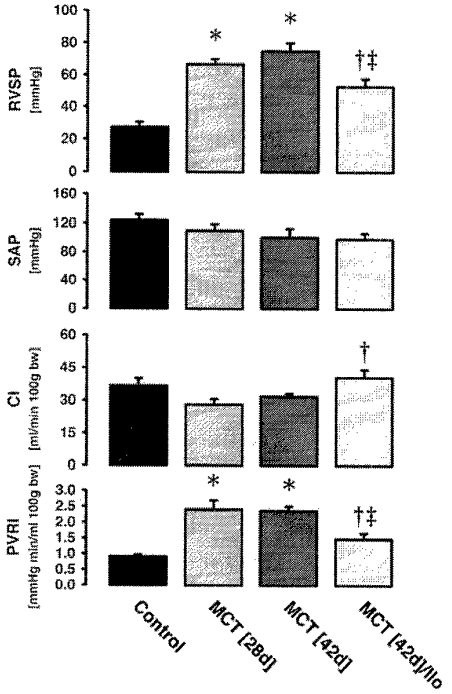

Figure 2. Influence of long-term treatment (Days 28–42) with inhaled iloprost (Ilo) on hemodynamics in monocrotaline (MCT)-induced pulmonary arterial hypertension. RVSP (in mm Hg), SAP (in mm Hg), cardiac index (CI, in ml · minute^{-1} · 100 g bodyweight), and pulmonary vascular resistance index (PVRI, in mm Hg · minute · ml^{-1} · 100 g bodyweight) are given. Iloprost was applied by repetitive inhalations from Days 28 to 42. *$p < 0.05$ versus control; †$p < 0.05$ versus MCT_{28}; ‡$p < 0.05$ versus MCT_{42}.

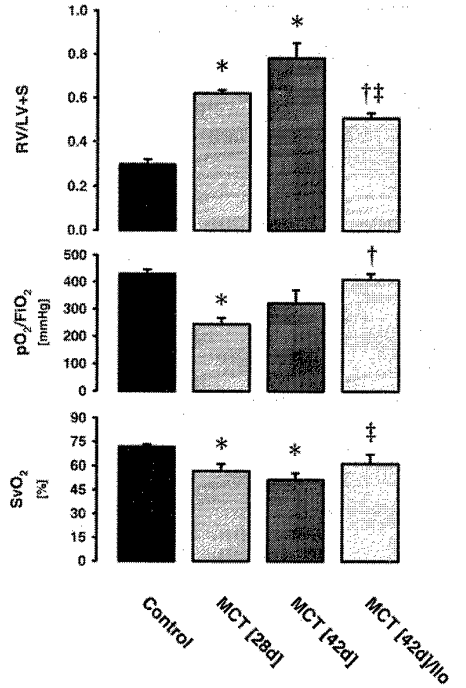

Figure 3. Influence of long-term treatment (Days 28–42) with inhaled iloprost (Ilo) on right heart hypertrophy and gas exchange in MCT-induced pulmonary arterial hypertension. The right-to-left ventricular ratio (RV/[LV + S]), arterial oxygenation (Po_2/Fi_{O_2}, in mm Hg), and central venous oxygen saturation (Sv_{O_2}, expressed as a percentage) are given. Iloprost was applied by repetitive inhalations from Days 28 to 42. *$p < 0.05$ versus control; †$p < 0.05$ versus MCT_{28}; ‡$p < 0.05$ versus MCT_{42}.

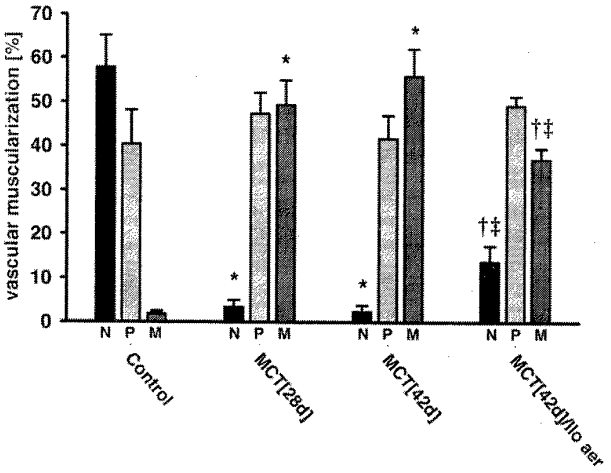

Figure 4. Influence of long-term treatment (Days 28–42) with inhaled iloprost (Ilo aer) on the degree of muscularization of peripheral pulmonary arteries. Percentages of nonmuscularized (N), partially muscularized (P), or fully muscularized (M) pulmonary arteries are given. Sixty to 80 intraacinar vessels were analyzed in each single lung. *p < 0.05 versus control; †p < 0.05 versus MCT_{28}; ‡p < 0.05 versus MCT_{42}.

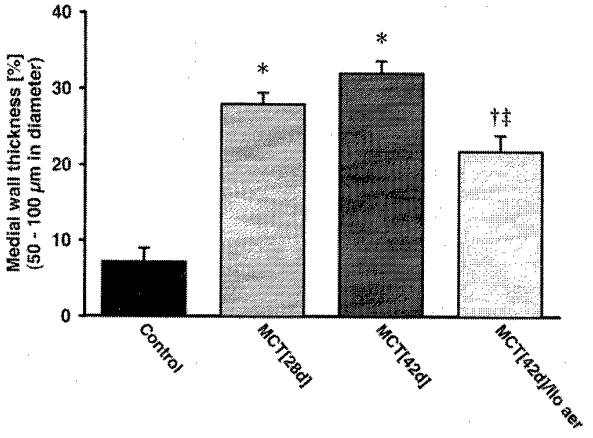

Figure 6. Effect of inhaled iloprost on medial wall thickness of pulmonary arteries (50–100 μm) in MCT-induced pulmonary arterial hypertension. *p < 0.05 versus control; †p < 0.05 versus MCT_{28}; ‡p < 0.05 versus MCT_{42}.

tenascin-C, which was fivefold upregulated in MCT-treated rats (p < 0.05) as compared with control animals (Figure 8) and downregulated by 25% in the iloprost-treated animals.

DISCUSSION

In the present study, we demonstrate that daily repetitive iloprost inhalation significantly improved pulmonary hemodynamics and reversed changes in vascular structure in MCT-induced PAH in rats. Similar to the abnormalities in human PAH, MCT treatment in rats is known to provoke endothelial injury, proliferation and hypercontraction of vascular smooth muscle cells, and inflammatory sequelae (21, 22). The animals die as a result of a progressive increase in precapillary lung vascular resistance with right heart failure. The inhalative therapy was commenced after establishment of severe PAH, 4 weeks after application of the toxin, which is in contrast to most previous investigations, in which the MCT and the agent of interest were coapplied (e.g., endothelin antagonist [23], prostaglandin E_1 [24], beraprost [14], or a phosphodiesterase type 5 inhibitor [14]). The limitation of such a preventive approach is that several pharmacologic interventions will result in a mitigation of PAH, because most of these compounds have some antiinflammatory potency (e.g., prostanoids, endothelin antagonists). However, as proven in many studies from our group and others, the inflammatory response levels off within the first week after MCT injection and is then replaced by a chronic vascular remodeling process, which in the later course of the disease appears to be no longer driven by inflammation. Therefore, more recent studies have applied therapeutic interventions once the disease was fully established (i.e., Rho kinase inhibitors [25], elastase inhibitors [12], statins [26], or phosphodiesterase inhibitors [13, 15]).

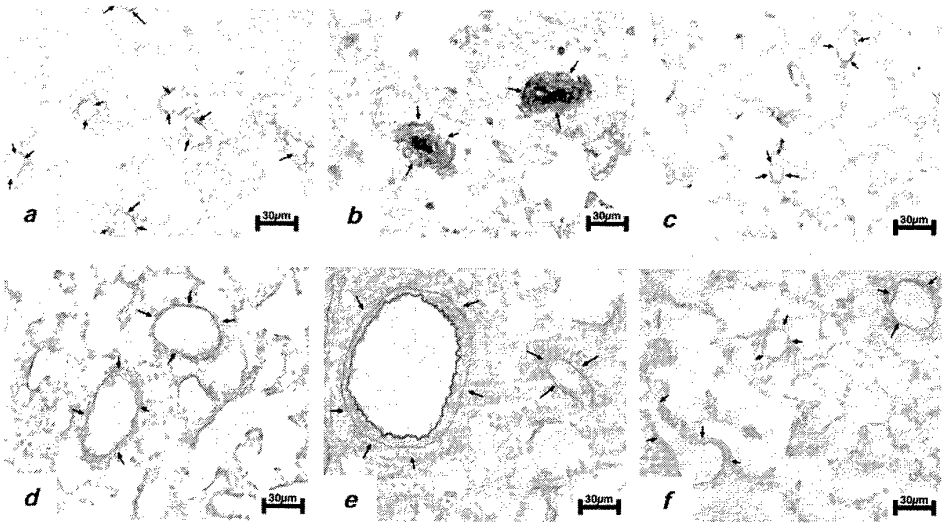

Figure 5. Effect of inhaled iloprost on the degree of muscularization (*A–C*) and medial wall thickness (*D–F*) of small pulmonary arteries. (*A* and *D*) Control lung; (*B* and *E*) MCT_{42} lung; (*C* and *F*) MCT_{42}/Ilo lung. The degree of muscularization is demonstrated by von Willebrand (*brown*) and α–smooth muscle actin (*purple*) staining for the identification of endothelium and vascular smooth muscle cells, respectively. The *arrows* indicate pulmonary arteries.

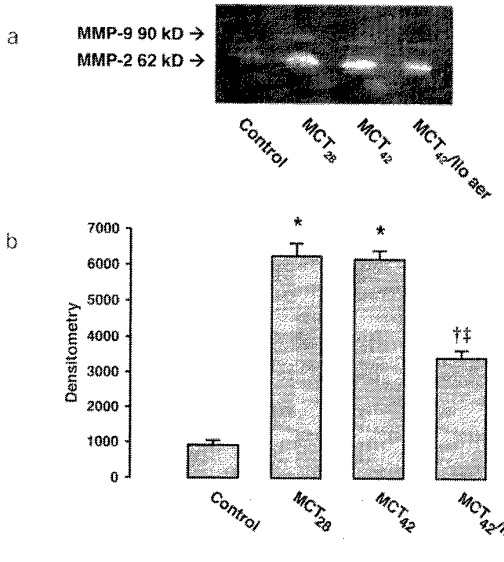

Figure 7. Influence of long-term treatment (Days 28–42) with inhaled iloprost (Ilo aer) on matrix metalloproteinase (MMP) activities. Homogenates of lung tissue from MCT-challenged animals receiving inhaled iloprost were examined and compared with control and sham-treated MCT animals. Zymography (*A*) and densitometric quantification of the bands (*B*) are indicated. Activity assays are representative of four gels for each group, which yielded identical results. *$p < 0.05$ versus control; †$p < 0.05$ versus MCT_{28}; ‡$p < 0.05$ versus MCT_{42}.

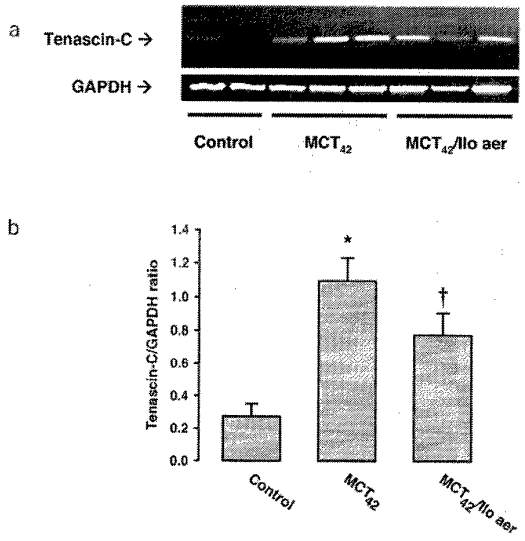

Figure 8. Influence of long-term treatment (Days 28–42) with inhaled iloprost (Ilo) on tenascin-C expression. Homogenates of lung tissue were examined from MCT-challenged animals receiving inhaled iloprost and compared with control and sham-treated MCT animals. Tenascin-C mRNA expression (*A*) and densitometric quantification of the bands (*B*) are indicated. All samples are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *$p < 0.05$ versus control; †$p < 0.05$ versus MCT_{42}.

Preceding *in vitro* studies have already suggested substantial antiproliferative potency of stable prostacyclin analogs in human pulmonary artery smooth muscle cells (27). The effects of the prostacyclin analogs are mediated by cell surface prostanoid receptors, in particular the prostaglandin I_2 (prostacyclin) receptor (28). This receptor is coupled to adenylyl cyclase via G proteins and increases cAMP in many cell types, including pulmonary artery smooth muscle cells (27, 29). Interestingly, distal human pulmonary artery smooth muscle cells, isolated from pulmonary arteries (less than 1 mm in external diameter), seem to be more susceptible to prostacyclin analog-induced inhibition of proliferation than are pulmonary artery smooth muscle cells from proximal pulmonary arteries (greater than 8 mm in external diameter) (29). These observations are well in line with our observation that MCT-induced structural changes in the intraacinar small pulmonary arteries (15–50 μm), representing the decisive segment for precapillary lung vascular resistance, were significantly improved by inhalation of iloprost.

The key finding of the study is the fact that regression of PAH was achieved by the inhalative prostanoid regimen. This is true for both hemodynamics and structural changes. As to hemodynamics, right ventricular systolic pressure, cardiac index, pulmonary vascular resistance, arterial oxygenation, and central venous oxygenation in the MCT_{42}/Ilo animals were significantly improved in comparison with both the sham-treated MCT_{42} animals and the MCT_{28} animals representing those before onset of therapy. Concerning structural changes, corresponding treatment effects were observed for right heart hypertrophy, the degree of muscularization (nonmuscular versus partially muscular versus fully muscular) of the small intraacinar pulmonary arteries, and the thickness of the medial wall of these vessels. These findings are supported by the observed reductions in MMP-2 and MMP-9 expression that we observed after iloprost application, because these two metalloproteinases are about four- to fivefold increased in MCT-treated rats. Both, MMP-2 and MMP-9 are regulated by intracellular cAMP levels (30, 31) and it has been shown that prostaglandin E_2 and dibutyryl-cAMP suppress membrane type 1 MMP, which activates MMP-2 (32). Furthermore, cAMP upregulates expression of tissue inhibitors of metalloproteinases (33, 34), which may further contribute to this reduced MMP-2 and MMP-9 activity observed in response to iloprost. It has been shown that pulmonary vascular remodeling is associated with induction of tenascin-C, which is a mitogenic cofactor produced through the action of MMPs (35). The reduction in tenascin expression that we found in response to iloprost may thus be directly linked to the downregulation of MMP-2 and MMP-9. In addition, it has been shown that inhibition of MMPs and vascular elastases suppresses tenascin-C expression (36, 37).

In conclusion, repeated inhalation of aerosolized iloprost reversed MCT-induced PAH, with respect to both hemodynamics and structural changes of the small intraacinar pulmonary arteries. The *in vivo* antiproliferative potency of inhaled iloprost, which even caused regression of structural remodeling of the lung vessels, has not been demonstrated previously, but is well in line with the beneficial effects reported for long-term treatment with inhaled iloprost in clinical studies (6–9). In addition to its potent lung vasodilatory effects, these findings suggest that inhaled iloprost also represents an effective antiremodeling therapy in the rat model of MCT-induced PAH.

Conflict of Interest Statement: R.T.S. received $25,000 in 2003 and 2004 as research grants from Bayer AG, $20,000 from Pfizer Ltd. as a research grant, and was funded by the Else Kroener Fresenius Foundation with $55,000 in 2004. H.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H.A.G. receives grant and contract support from Pfizer Ltd., and also serves on advisory boards for Pfizer Ltd., Schering, and Altana Pharma. K.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.P. does not have a financial relationship with a

commercial entity that has an interest in the subject of this manuscript. A.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. N.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. F.G. receives grant and contract support from Pfizer Ltd. and Altana Pharma, and also serves on the advisory board of Altana Pharma AG. W.S. receives grant and contract support from Schering AG, Pfizer Ltd., Altana Pharma AG, Lung Rx, and Myogen.

References

1. D'Alonzo GE, Barst RJ, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, Fishman AP, Goldring RM, Groves BM, Kernis JT. Survival in patients with primary pulmonary hypertension: results from a national prospective registry. *Ann Intern Med* 1991;115:343–349.
2. Rubin LJ, Mendoza J, Hood M, McGoon M, Barst R, Williams WB, Diehl JH, Crow J, Long W. Treatment of primary pulmonary hypertension with continuous intravenous prostacyclin (epoprostenol): results of a randomized trial. *Ann Intern Med* 1990;112:485–491.
3. Olschewski H, Walmrath D, Schermuly R, Ghofrani A, Grimminger F, Seeger W. Aerosolized prostacyclin and iloprost in severe pulmonary hypertension. *Ann Intern Med* 1996;124:820–824.
4. Witt W, Muller B. Antithrombotic profile of iloprost in experimental models of *in vivo* platelet aggregation and thrombosis. *Adv Prostaglandin Thromboxane Leukot Res* 1987;17A:279–284.
5. Hoeper MM, Olschewski H, Ghofrani HA, Wilkens H, Winkler J, Borst MM, Niedermeyer J, Fabel H, Seeger W, German PPH Study Group. A comparison of the acute hemodynamic effects of inhaled nitric oxide and aerosolized iloprost in primary pulmonary hypertension. *J Am Coll Cardiol* 2000;35:176–182.
6. Hoeper MM, Schwarze M, Ehlerding S, Adler-Schuermeyer A, Spiekerkoetter E, Niedermeyer J, Hamm M, Fabel H. Long-term treatment of primary pulmonary hypertension with aerosolized iloprost, a prostacyclin analogue. *N Engl J Med* 2000;342:1866–1870.
7. Olschewski H, Ghofrani HA, Schmehl T, Winkler J, Wilkens H, Hoper MM, Behr J, Kleber FX, Seeger W, German PPH Study Group. Inhaled iloprost to treat severe pulmonary hypertension: an uncontrolled trial. *Ann Intern Med* 2000;132:435–443.
8. Olschewski H, Simonneau G, Galie N, Higenbottam T, Naeije R, Rubin LJ, Nikkho S, Speich R, Hoeper MM, Behr J, *et al.* Inhaled iloprost for severe pulmonary hypertension. *N Engl J Med* 2002;347:322–329.
9. Olschewski H, Ghofrani HA, Walmrath D, Schermuly R, Temmesfeld-Wollbruck B, Grimminger F, Seeger W. Inhaled prostacyclin and iloprost in severe pulmonary hypertension secondary to lung fibrosis. *Am J Respir Crit Care Med* 1999;160:600–607.
10. Huxtable RJ. Activation and pulmonary toxicity of pyrrolizidine alkaloids. *Pharmacol Ther* 1990;47:371–389.
11. Rosenberg HC, Rabinovitch M. Endothelial injury and vascular reactivity in monocrotaline pulmonary hypertension. *Am J Physiol* 1988;255: H1484–H1491.
12. Cowan KN, Heilbut A, Humpl T, Lam C, Ito S, Rabinovitch M. Complete reversal of fatal pulmonary hypertension in rats by a serine elastase inhibitor. *Nat Med* 2000;6:698–702.
13. Schermuly RT, Kreisselmeier KP, Ghofrani HA, Samidurai A, Pullamsetti S, Weissmann N, Schudt C, Ermert L, Seeger W, Grimminger F. Antiremodeling effects of iloprost and the dual-selective phosphodiesterase 3/4 inhibitor tolafentrine in chronic experimental pulmonary hypertension. *Circ Res* 2004;94:1101–1108.
14. Itoh T, Nagaya N, Fujii T, Iwase T, Nakanishi N, Hamada K, Kangawa K, Kimura H. A combination of oral sildenafil and beraprost ameliorates pulmonary hypertension in rats. *Am J Respir Crit Care Med* 2004;169: 34–38.
15. Schermuly RT, Kreisselmeier KP, Ghofrani HA, Yilmaz H, Butrous G, Ermert L, Ermert M, Weissmann N, Rose F, Guenther A, *et al.* Chronic sildenafil treatment inhibits monocrotaline-induced pulmonary hypertension in rats. *Am J Respir Crit Care Med* 2004;169:39–45.
16. Nagaya N, Okumura H, Uematsu M, Shimizu W, Ono F, Shirai M, Mori H, Miyatake K, Kangawa K. Repeated inhalation of adrenomedullin ameliorates pulmonary hypertension and survival in monocrotaline rats. *Am J Physiol Heart Circ Physiol* 2003;285:H2125–H2131.
17. Schermuly R, Schmehl T, Gunther A, Grimminger F, Seeger W, Walmrath D. Ultrasonic nebulization for efficient delivery of surfactant in a model of acute lung injury: impact on gas exchange. *Am J Respir Crit Care Med* 1997;156:445–453.
18. Schermuly RT, Ghofrani HA, Enke B, Weissmann N, Grimminger F, Seeger W, Schudt C, Walmrath D. Low-dose systemic phosphodiesterase inhibitors amplify the pulmonary vasodilatory response to inhaled prostacyclin in experimental pulmonary hypertension. *Am J Respir Crit Care Med* 1999;160:1500–1506.
19. Frisdal E, Gest V, Vieillard-Baron A, Levame M, Lepetit H, Eddahibi S, Lafuma C, Harf A, Adnot S, Dortho MP. Gelatinase expression in pulmonary arteries during experimental pulmonary hypertension. *Eur Respir J* 2001;18:838–845.
20. Stanton H, Gavrilovic J, Atkinson SJ, d'Ortho MP, Yamada KM, Zardi L, Murphy G. The activation of proMMP-2 (gelatinase A) by HT1080 fibrosarcoma cells is promoted by culture on a fibronectin substrate and is concomitant with an increase in processing of MT1-MMP (MMP-14) to a 45 kDa form. *J Cell Sci* 1998;111:2789–2798.
21. Botney MD. Role of hemodynamics in pulmonary vascular remodeling: implications for primary pulmonary hypertension. *Am J Respir Crit Care Med* 1999;159:361–364.
22. Hoeper MM, Galie N, Simonneau G, Rubin LJ. New treatments for pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2002;165: 1209–1216.
23. Prie S, Stewart DJ, Dupuis J. Endothelin A receptor blockade improves nitric oxide-mediated vasodilation in monocrotaline-induced pulmonary hypertension. *Circulation* 1998;97:2169–2174.
24. Kato S, Sugimura H, Kishiro I, Machida M, Suzuki H, Kaneko N. Suppressive effect of pulmonary hypertension and leukocyte activation by inhaled prostaglandin E1 in rats with monocrotaline-induced pulmonary hypertension. *Exp Lung Res* 2002;28:265–273.
25. Abe K, Shimokawa H, Morikawa K, Uwatoku T, Oi K, Matsumoto Y, Hattori T, Nakashima Y, Kaibuchi K, Sueishi K, *et al.* Long-term treatment with a Rho-kinase inhibitor improves monocrotaline-induced fatal pulmonary hypertension in rats. *Circ Res* 2004;94:385–393.
26. Nishimura T, Faul JL, Berry GJ, Vaszar LT, Qiu D, Pearl RG, Kao PN. Simvastatin attenuates smooth muscle neointimal proliferation and pulmonary hypertension in rats. *Am J Respir Crit Care Med* 2002;166: 1403–1408.
27. Clapp LH, Finney P, Turcato S, Tran S, Rubin LJ, Tinker A. Differential effects of stable prostacyclin analogs on smooth muscle proliferation and cyclic AMP generation in human pulmonary artery. *Am J Respir Cell Mol Biol* 2002;26:194–201.
28. Coleman RA, Smith WL, Narumiya S. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* 1994;46: 205–229.
29. Wharton J, Davie N, Upton PD, Yacoub MH, Polak JM, Morrell NW. Prostacyclin analogues differentially inhibit growth of distal and proximal human pulmonary artery smooth muscle cells. *Circulation* 2000; 102:3130–3136.
30. McCawley LJ, Li S, Benavidez M, Halbleib J, Wattenberg EV, Hudson LG. Elevation of intracellular cAMP inhibits growth factor-mediated matrix metalloproteinase-9 induction and keratinocyte migration. *Mol Pharmacol* 2000;58:145–151.
31. Peracchia F, Tamburro A, Prontera C, Mariani B, Rotilio D. cAMP involvement in the expression of MMP-2 and MT-MMP1 metalloproteinases in human endothelial cells. *Arterioscler Thromb Vasc Biol* 1997;17:3185–3190.
32. Shankavaram UT, Lai WC, Netzel-Arnett S, Mangan PR, Ardans JA, Caterina N, Stetler-Stevenson WG, Birkedal-Hansen H, Wahl LM. Monocyte membrane type 1-matrix metalloproteinase: prostaglandin-dependent regulation and role in metalloproteinase-2 activation. *J Biol Chem* 2001;276:19027–19032.
33. Tanaka K, Iwamoto Y, Ito Y, Ishibashi T, Nakabeppu Y, Sekiguchi M, Sugioka Y. Cyclic AMP-regulated synthesis of the tissue inhibitors of metalloproteinases suppresses the invasive potential of the human fibrosarcoma cell line HT1080. *Cancer Res* 1995;55:2927–2935.
34. Zhong ZD, Hammani K, Bae WS, DeClerck YA. NF-Y and Sp1 cooperate for the transcriptional activation and cAMP response of human tissue inhibitor of metalloproteinases-2. *J Biol Chem* 2000;275:18602–18610.
35. Jones PL, Crack J, Rabinovitch M. Regulation of tenascin-C, a vascular smooth muscle cell survival factor that interacts with the $\alpha_v\beta_3$ integrin to promote epidermal growth factor receptor phosphorylation and growth. *J Cell Biol* 1997;139:279–293.
36. Cowan KN, Jones PL, Rabinovitch M. Regression of hypertrophied rat pulmonary arteries in organ culture is associated with suppression of proteolytic activity, inhibition of tenascin-C, and smooth muscle cell apoptosis. *Circ Res* 1999;84:1223–1233.
37. Cowan KN, Jones PL, Rabinovitch M. Elastase and matrix metalloproteinase inhibitors induce regression, and tenascin-C antisense prevents progression, of vascular disease. *J Clin Invest* 2000;105:21–34.