Chronic inhibition of phosphodiesterase 5 does not prevent pressure-overload-induced right-ventricular remodelling

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Aims Inhibition of phosphodiesterase 5 (PDE5) decreases pulmonary pressure and improves symptoms in patients with pulmonary arterial hypertension. It is unclear however, whether inhibition of PDE5 can prevent myocardial remodelling during right-ventricular pressure overload.

Methods and results Right-ventricular pressure overload was produced in male rats in a pulmonary hypertension model (monocrotaline 60 mg/kg s.c.) or by surgical pulmonary artery banding. PDE5 inhibition using oral sildenafil (50 mg/kg/day in drinking water) or placebo was initiated 14 days after monocrotaline treatment and continued for 14 days until final examination. In the pulmonary artery banding groups, rats were treated with sildenafil (50 mg/kg/day) or placebo for 21 days following surgical pulmonary artery banding. At the final experiments, right-ventricular haemodynamics were measured and remodelling was analysed using histological, biochemical, and gene expression markers. Both monocrotaline and pulmonary artery banding increased right-ventricular systolic pressure to \[ P \approx 80 \text{ mmHg} \]. In parallel, both interventions induced markers of hypertrophy (upregulation of natriuretic peptides, increase in myocyte diameter) and fibrosis (upregulation of collagen types 1A2 and 3A1) as well as mRNA expression of the tissue inhibitor of matrix metalloproteases 1 and osteopontin in the right ventricle. In monocrotaline model, sildenafil decreased pulmonary pressure, reduced right-ventricular hypertrophy, and prevented fibrosis marker gene upregulation. After pulmonary artery banding, in contrast, sildenafil increased markers of myocardial remodelling and right-ventricular myocyte diameter.

Conclusion Sildenafil prevents myocardial remodelling in pulmonary hypertension through an indirect action via right-ventricular unloading.

KEYWORDS
Pulmonary hypertension; Remodelling; Sildenafil

1. Introduction
Pulmonary arterial hypertension is a severe disease characterized by an elevation of pulmonary arterial pressure and secondary right-ventricular (RV) remodelling. A key feature of the disease is the dysregulation of important vasodilatory mechanisms in the pulmonary circulation, including increased expression of phosphodiesterase 5 (PDE5). The resulting vasoconstriction leads to pressure overload of the right ventricle, pathological remodelling of the myocardium, cardiac failure, and, ultimately, death. In line with this pathophysiological concept, the PDE5 inhibitor sildenafil, originally developed for the treatment of erectile dysfunction, reduced pulmonary pressure in acute and chronic experimental models of pulmonary hypertension. Subsequently, after confirmation of clinical efficacy of sildenafil to reduce pulmonary pressure and improve exercise capacity in randomized trials, sildenafil was approved for the treatment of patients with pulmonary arterial hypertension. While the pulmonary dilatory effects of sildenafil have been clearly demonstrated in animal models as well as in patients, the effect of PDE5 inhibition on RV remodelling has not been thoroughly investigated so far. In rats with monocrotaline-induced pulmonary hypertension, RV weight was reduced in parallel with a decrease in pulmonary arterial pressure, but in those experiments a distinction between...
secondary effects due to RV unloading and a potential direct myocardial effect is not possible. In addition, the direct effects of PDE5 inhibition on the underlying components leading to cardiac remodelling, such as myocardial hypertrophy, interstitial fibrosis, and the regulators of these processes, have not been addressed. Regarding the left ventricle, sildenafil has been reported to attenuate hypertrophy and fibrosis after aortic banding, and in isoprenaline- or doxorubicin-induced cardiac damage in rodent models; however, the effect of PDE5 inhibition on RV remodelling is unknown. Because of the major differences between left and right heart haemodynamics and potentially differing responses to pressure overload, the data from the left-ventricular myocardium cannot simply be extrapolated to the right ventricle.

In the present study, therefore, we sought to investigate the effect of PDE5 inhibition on RV remodelling. To that end, we analysed markers of myocardial hypertrophy, fibrosis, and the regulators of these processes in two rat models of RV pressure overload. In order to differentiate indirect from direct myocardial effects, we used both the rat model of monocrotaline-induced pulmonary hypertension (where sildenafil reduces pulmonary pressure and thus RV overload) and surgical banding of the pulmonary artery (where RV load is kept constant throughout the experiment). Our results indicate that while sildenafil prevents myocardial remodelling in pulmonary hypertension via RV unloading, there is no direct anti-remodelling effect when pressure overload is constant.

2. Methods

All animal experiments were performed in accordance with the European Community guidelines for the use of experimental animals and with the German law for the protection of animals. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1 Pharmacokinetics

In a satellite experiment, a single dose of sildenafil (25 mg/kg) was administered to four Sprague-Dawley rats (body weight ~350 g) via the drinking water over 1 h. Blood samples were drawn with heparinized syringes for subsequent determination of plasma sildenafil levels at 1 and 4 h after exposure. Plasma was obtained by centrifugation and stored below –15 °C until analysis. Quantification of the compound was conducted after addition of an internal standard, protein precipitation with acetonitrile, and separation by high-pressure liquid chromatography coupled to a tandem mass spectrometer (Applied Biosystems MD Sciex API 3000). The lower limit of quantitation was 2 nmol/L. At 4 hours after exposure, the sildenafil treated animals and four control animals were sacrificed for determination of plasma and RV cGMP content.

2.2 Monocrotaline and treatment with sildenafil

Adult male Sprague-Dawley rats weighing 250--280 g were purchased from Charles River Laboratories (Sulzfeld, Germany). Rats were given a single subcutaneous injection of 60 mg/kg monocrotaline (MCT, Sigma-Aldrich Chemie GmbH, München, Germany) or vehicle under isoflurane anaesthesia (2% vol/vol) under conditions of artificial ventilation (RUS-1321-RA, FMI Führ Medical Instruments GmbH, Seeheim/Ober-Beerbach, Germany). FiO2 was set at 0.5, respiration volume was 10 mL/kg at 60 strokes/min; inspiration to expiration ratio was 1:1 and the positive end-expiratory pressure was 1.0 cm H2O. Core body temperature was maintained at 37 °C using a controlled heating pad. A Millar microtip catheter (SPR-671, FMI Führ Medical Instruments GmbH, Seeheim/Ober-Beerbach, Germany) was inserted into the left carotid artery to measure heart rate and systemic arterial pressure. A fluid filled polyethylene catheter (PE 50) was inserted through the right jugular vein into the right ventricle for measurement of RV pressure. Cardiac output was measured by transpulmonary thermodilution technique, averaged from three consecutive measurements. All haemodynamic measurements were performed with a PowerLab System using the Chart 5.0 Software with the cardiac output module (ADInstruments GmbH, Spechbach, Germany). After exsanguination, the right ventricles were snap-frozen on dry ice for RNA extraction and determination analysis of hydroxyproline content. For histological analyses (separate experiments), the right ventricle was fixed in formaline.

2.5 Assessment of RV hypertrophy

The animals were exsanguinated and the heart was excised. The RV wall was separated from the left-ventricular wall and the
ventricular septum. The ratio of the right ventricle to left ventricle plus septum weight [RV/(LV+S)] was calculated as an index of RV hypertrophy.

For determination of RV cardiomyocyte transverse diameter, the RV mid-cavity was sliced in 3 μm sections. After haematoxylin-eosin staining, sections were analysed using the Nikon Eclipse TE 2000-U system with Lucia General 5.03 Nikon DS-U1 software (Tokyo, Japan). To assess the circumferential diameters of the cardiomyocytes, selected cells (which had to be cut transversely and a visible nucleus) from fields were chosen randomly and their equivalent diameter determined by the software. More than 100 myocytes were measured for each animal (n = 3/group).

2.6 Determination of hydroxyproline in right ventricular myocardium

The content of 4-hydroxyproline in the RV was determined as a marker for myocardial collagen content using the chloramin-T method. Two independent tissue samples per animal (n = 6 per group) were weighted and hydrolysed in 6 M HCl. Analysis was done in duplicate for each sample.

2.7 Measurement of plasma biomarkers

Blood was collected in heparinized vials. Samples were mixed thoroughly and centrifuged at 1000g at a temperature of 4°C for 10 min. Plasma was separated and frozen immediately at −80°C. Plasma CGMP levels were measured using a commercially available ELA kit (cGMP Enzymeimmunoassay Biotrak System, Amersham # RPN 226). Plasma concentrations of NT-pro-BNP were measured with a commercially available radiolmmunoassay kit (PhoenixPep- tide, Belmont, CA, USA) according to the manufacturer’s instructions. There was no cross-reactivity with rat ANP.

2.8 RNA extraction and quantitative real-time polymerase chain reaction

Samples of the right ventricle were immediately snap-frozen on dry ice and total RNA was extracted using the RNeasy fibrous tissue kit (Qiaegen), following the manufacturer’s guidelines. Integrity of obtained RNA was checked on a Bioanalyzer (Agilent). For reverse transcription, 1 μg of total RNA was first digested with RNase-free DNase I (Gibco) for 15 min at room temperature and then reverse-transcribed using Promiscript (Promega) in a total reaction volume of 40 μL according to the standard protocol of the kit supplier. Real-time polymerase chain reaction was carried out as described previously. The resulting expression is given in arbitrary units.

In RV samples, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were used as markers of hypertrophy and type 3 collagen α-1 chain (COL3A1) and type 1 collagen α-2 chain (COL1A2) were used as markers of cardiac fibrosis. Tissue inhibitor of metalloproteinase 1 (TIMP-1) was used as a molecular marker for turnover of extracellular matrix and osteopontin (OPN) as a marker for myofibroblast activation. Primer/probe sequences are given in Table 1.

2.9 Statistical analysis

Data are expressed as means ± SEM. Differences between groups were evaluated by the use of one-way ANOVA, followed by Student-Newman-Keuls post hoc analysis for multiple comparisons (GraphPad Prism 4.0 Software GraphPad Software Inc., San Diego, CA, USA). A P-value <0.05 was regarded significant.

3. Results

3.1 Pharmacokinetics

After oral exposure to 25 mg/kg sildenafil, plasma levels of sildenafil were 170 ± 50 nmol/L at 1 h, and 22 ± 8 nmol/L.
at 4 h. These values exceed the IC50 values of sildenafil at the rat PDE5, which has been determined to be in the single digit nanomolar range.16 At 4 h, plasma cGMP levels were 8.9 ± 2.4 nmol/L, compared with 1.1 ± 0.1 nmol/L in placebo treated controls (P < 0.05). In the RV, cGMP concentrations tended to be increased after sildenafil (227 ± 46 fmol/mg), compared with placebo-treated controls (147 ± 28 fmol/mg, P > 0.05).

3.2 Monocrotaline model

Right-ventricular systolic pressure was increased to 82.9 ± 6.0 mmHg at 28 days post-MCT treatment, compared with 27.0 ± 0.5 mmHg in saline-injected control animals (Figure 1A). In parallel, RV end-diastolic pressure was increased (7.7 ± 1.3 vs. 2.0 ± 0.2 mmHg) and cardiac index (20.8 ± 2.1 vs. 28.0 ± 1.2 mL/100 g) and stroke volume decreased (0.18 ± 0.02 vs. 0.32 ± 0.02 mL; Figure 1B-D). There were no significant differences in systolic arterial pressure in the systemic circulation and heart rate between the groups (Table 2). The haemodynamic effects of MCT were paralleled by extensive RV hypertrophy: the RV weights were 120 ± 8 mg/100 g body weight, compared with controls (47 ± 2 mg/100 g body weight), and the RV/(LV+S) ratio was 0.51 ± 0.02, compared with 0.24 ± 0.01 in saline-treated controls (Figure 2A, Table 2), and plasma NT-pro-BNP levels rose to 41.1 ± 4.9 pg/100 μL, when compared with 14.0 ± 0.8 pg/100 μL in the control group (Figure 2B). RV equivalent cardiomyocyte diameter was increased to 31.2 ± 0.4 μm, compared with control (24.8 ± 0.4 μm, Figure 2D), whereas hydroxyproline content was decreased (Figure 2C). RV mRNA expression of the hypertrophy markers ANP and BNP was significantly elevated in MCT rats, compared with non-treated controls (Figure 3A and B). Likewise, expression of type I collagen alpha-2 chain (COL1A2) and type 3 collagen alpha-1 chain (COL3A1) as indicators of interstitial fibrosis was also increased in RV myocardium. The expression levels of TIMP-1, an important regulator of extracellular matrix turnover and OPN, a matricellular protein involved in myofibroblast activation, were strongly increased after MCT.

Sildenafil substantially improved haemodynamic and morphological parameters in MCT treated rats. Compared with vehicle, sildenafil reduced RV systolic pressure to 55.8 ± 5.6 mmHg, and RV end-diastolic pressure to 3.9 ± 0.5, respectively (Figure 1A and B). Sildenafil normalized cardiac index and stroke volume in animals with MCT-induced pulmonary arterial hypertension (Figure 1C and D), whereas systemic arterial pressure and heart rate were not statistically different across groups (Table 2). The RV unloading effect of sildenafil was paralleled by a reduction of RV hypertrophy (Figure 2A, Table 2). Plasma NT-pro-BNP levels were decreased to 27.5 ± 3.7 pg/100 μL (Figure 2B). In the sildenafil group, myocardial hydroxyproline content was not changed, but cardiomyocyte diameter was decreased to normal. Treatment with sildenafil also strongly suppressed the expression of the hypertrophy marker genes, reduced collagen mRNA expression to control levels, and reduced TIMP-1 and OPN levels to near normal (Figure 3).

3.3 Pulmonary artery banding

RV afterload was drastically elevated by pulmonary artery banding. The RV systolic pressure was 83.8 ± 8.2 mmHg (compared with 31.6 ± 1.5 mmHg in sham-operated rats,
Figure 4, which was virtually identical to the RV pressure in the placebo-treated monocrotaline animals. In parallel, RV end-diastolic pressure was increased (9.1 ± 1.4 vs. 3.0 ± 0.3 mmHg), cardiac index (28.7 ± 1.3 vs. 38.6 ± 1.3 mL/100 g), and stroke volume decreased (0.31 ± 0.01 vs. 0.43 ± 0.01 mL; Figure 4B–D). Systemic blood pressure and heart rate were not different among the groups (Table 2).

This RV pressure overload of the pulmonary artery banding animals was reflected by extensive RV hypertrophy: RV weight was increased to 112 ± 7 mg/100 g body weight, compared with 50 ± 2 mg/100 g body weight in sham-operated animals, and the RV/(LV + S) ratio was 0.57 ± 0.03, compared with 0.25 ± 0.01 in sham-operated animals (Figure 5A, Table 2). Plasma NT-pro-BNP levels rose to 31.4 ± 2.5 pg/100 µL, when compared with 13.3 ± 1.3 pg/100 µL in the sham-operated rats (Figure 5B). Similarly, RV hydroxyproline content and RV cardiomyocyte diameter were increased, compared with sham (Figure 5).

Pulmonary artery banding also induced a strong increase in the mRNA expression of myocardial hypertrophy marker genes ANP and BNP as well as an induction of the fibrosis marker genes type I collagen alpha-2 chain (COL1A2) and type 3 collagen alpha-1 chain (COL3A1). Furthermore, the mRNA expression of OPN and tissue inhibitor of metalloproteinase 1 (TIMP-1) was also strongly increased (Figure 6).

In contrast to its effects in the monocrotaline model, chronic sildenafil treatment had no beneficial effects in the pulmonary artery banding model. At constant RV systolic pressure (86.7 ± 13.3 mmHg), sildenafil affected neither the RV functional parameters (Figure 4B–D), nor RV hypertrophy or hydroxyproline content (Figure 5A–C). Cardiomyocyte diameter was even increased in the sildenafil compared with the placebo group (Figure 5D). On the mRNA level, the expression of hypertrophy marker genes ANP and BNP

<table>
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<th>Table 2 Summarized data of haemodynamics and heart weights in rats with monocrotaline-induced pulmonary arterial hypertension and in rats with pulmonary artery banding</th>
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<td><strong>Haemodynamics</strong></td>
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<td>Heart rate</td>
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<td><strong>MCT</strong>, monocrotaline; <strong>BPsys</strong>, systolic blood pressure; <strong>BPdia</strong>, diastolic blood pressure; <strong>RVw</strong>, right-ventricular wet weight; <strong>LV+S</strong>, left-ventricular + septum wet weight.</td>
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<td>*P &lt; 0.05 vs. MCT/placebo or banding/placebo, respectively.</td>
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tended to be increased in sildenafil group (but not statistically significant), while the increase in COL1A2, COL3A1, and OPN reached statistical significance (Figure 6).

3.4 Levels of cGMP in plasma

MCT treatment or pulmonary artery banding alone had no significant effect on plasma cGMP levels, compared with the respective sham groups. However, sildenafil increased plasma cGMP levels from 0.9 ± 0.4 to 1.8 ± 0.2 nmol/L in MCT-treated rats (compared with MCT placebo) and from 3.1 ± 0.3 to 7.0 ± 0.4 nmol/L in pulmonary artery banding (compared with sham).

4. Discussion

In this study, we demonstrate that the PDE5 inhibitor sildenafil attenuates RV remodelling in the monocrotaline model of pulmonary hypertension, but not after surgical banding of the pulmonary artery. If anything, chronic sildenafil aggravated rather than attenuated RV remodelling under conditions of constant pressure overload. It seems therefore that the anti-remodelling effects of sildenafil depend on the RV loading conditions.

Progressive RV remodelling, including increased wall thickness and myocardial hypertrophy as well as interstitial fibrosis, is a hallmark of pulmonary arterial hypertension. Also known as cor pulmonale, this process frequently leads to cardiac failure and death. Contemporary therapy of pulmonary arterial hypertension is based on pulmonary vasodilation through prostanoids, endothelin receptor antagonists, and PDE5 inhibitors. While these principles have convincingly demonstrated a reduction in pulmonary pressure and an improvement of clinical function parameters, their effects on the processes underlying the myocardial remodelling in pulmonary hypertension have not been characterized so far.

In the monocrotaline model, RV remodelling is characterized by myocardial hypertrophy, which includes an increased

Figure 3 Effects of sildenafil on monocrotaline-induced mRNA expression of markers of hypertrophy and remodelling in the right ventricle. (A) Atrial natriuretic peptide (ANP); (B) Brain natriuretic peptide (BNP); (C) Type 1 collagen alpha-2 chain (COL1A2); (D) Type 3 collagen alpha-1 chain (COL3A1); (E) Tissue inhibitor of metalloproteinase 1 (TIMP-1); (F) Osteopontin. Data are mean ± SEM of n = 10 animals per group; *P < 0.05 vs. monocrotaline/placebo.
Figure 4  Effect of treatment with sildenafil on right-ventricular and systemic haemodynamics in pulmonary artery banding. (A) Right-ventricular systolic pressure (RVPsys); (B) Right-ventricular end-diastolic pressure (RVEDP); (C) Cardiac index (CI); (D) Stroke volume (SV). Data are mean ± SEM (n = 8–10/group); *P < 0.05 vs. banding/placebo.

Figure 5  Effects of sildenafil on right-ventricular hypertrophy and fibrosis markers in pulmonary artery banding. (A) Right to left-ventricular weight ratio [RV/(LV+S)]; (B) plasma NT-pro-BNP; (C) Right-ventricular 4-hydroxyproline content (4OH-P); (D) Right-ventricular cardiomyocyte diameter. Data are mean ± SEM (n = 8–10/group); *P < 0.05 vs. banding/placebo.
mass of the right ventricle and upregulation of hypertrophy marker gene expression, such as ANP\textsuperscript{18,19} and BNP\textsuperscript{20}. In addition, there is excessive interstitial myocardial fibrosis in pulmonary hypertension, evidenced by an increased collagen deposition and thus upregulation of collagen expression\textsuperscript{21} which contributes to RV failure in the process of the disease. Here, we demonstrate that mRNA expression of the two major myocardial collagen isoforms Col1A2 and Col3A1 as well as the tissue inhibitor of matrix metalloproteinase 1 (TIMP-1); (F) Osteopontin. Data are mean ± SEM of \( n = 8–10 \) animals per group; *\( P < 0.05 \) vs. banding/placebo.

A dysbalance between synthesis and degradation of extracellular matrix due to an altered expression of important regulators has previously been identified in left-ventricular remodelling. These regulators include TIMP-1,\textsuperscript{23,24} and OPN, a matricellular protein that has only recently been identified to orchestrate quantitative and qualitative control of extracellular matrix proteins\textsuperscript{25} as well as myofibroblast activation.\textsuperscript{26} At least regarding these key regulators, the processes underlying pressure-induced myocardial remodelling seem to be similar in the right and left ventricle.

Our data from the monocrotaline model demonstrate that the PDE5 inhibitor sildenafil, an approved therapy for the treatment of pulmonary hypertension, not only reduces pulmonary pressure, but also attenuates key features of RV remodelling (Figures 1–3). The observed down-regulation of collagen1A2, collagen 3A1, TIMP-1, and OPN, indicate a potential benefit on extracellular matrix composition, too. Collectively, these findings are important because RV remodelling, cor pulmonale, and cardiac decompensation are the key drivers of morbidity and mortality in pulmonary arterial hypertension.\textsuperscript{1,3}

Although our observations will have to be confirmed in clinical trials, we speculate that the (indirect) RV

![Figure 6](https://example.com/figure6.png)

**Figure 6** Effects of sildenafil on pulmonary artery banding-induced mRNA expression of biomarkers of hypertrophy and remodelling in the right ventricle. (A) Atrial natriuretic peptide (ANP); (B) Brain natriuretic peptides (BNP); (C) Type I collagen alpha-2 chain (COL1A2); (D) Type 3 collagen- alpha-1 chain (COL3A1); (E) Tissue inhibitor of metalloproteinase 1 (TIMP-1); (F) Osteopontin. Data are mean ± SEM of \( n = 8–10 \) animals per group; *\( P < 0.05 \) vs. banding/placebo.
anti-remodelling effects contribute to the clinical improvement and, potentially, better survival mediated by PDE5 inhibition in pulmonary hypertension.

In contrast to the results from the monocrotaline model, sildenafil did not favourably influence RV remodelling in the presence of a fixed pulmonary stenosis. Despite an almost identical increase in RV systolic pressure load in the monocrotaline and pulmonary artery banding models, respectively (to ~80 mmHg, Figures 1A and 4A), and despite a greater increase in plasma cGMP levels, sildenafil increased rather than decreased myocardial hypertrophy and markers of myocardial remodelling in the banding model (Figures 5 and 6). The major difference between the monocrotaline and the pulmonary artery banding model is the influence of PDE5 inhibition on loading conditions. While in the monocrotaline model, sildenafil produced a pronounced reduction in pulmonary pressure and, hence, RV afterload, loading conditions remain constant in the fixed stenosis model (Figure 4A). We therefore conclude that the beneficial effects of PDE5 inhibition on RV remodelling are dependent on the simultaneous effect on pulmonary haemodynamics.

In a mouse model of aortic banding and, consequently, left-ventricular overload, sildenafil was reported to reduce myocardial hypertrophy and fibrosis.9,27 Also, sildenafil was left-ventricular overload, sildenafil was reported to reduce haemodynamics. 


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