Sildenafil Preserves Lung Endothelial Function and Prevents Pulmonary Vascular Remodeling in a Rat Model of Diastolic Heart Failure

Running Title: Yin et al: Sildenafil in Cardiogenic Pulmonary Hypertension

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Abstract

**Background**—Pulmonary hypertension as frequent complication of left heart disease (PH-LHD) is characterized by lung endothelial dysfunction and vascular remodeling. While PH-LHD contributes to morbidity and mortality in heart failure, established therapies for PH-LHD are yet lacking. Here, we tested the effect of chronic sildenafil treatment in an experimental model of PH-LHD.

**Methods and Results**—In Sprague-Dawley rats, PH-LHD was induced by supracoronary aortic banding. Oral sildenafil treatment (60 mg/kg daily) was initiated after 7 days, and lung endothelial function (n=5), vascular remodeling, and right ventricular function (n=11 each) were analyzed 9 weeks post banding. As compared to sham-operated controls, aortic banding induced pulmonary hypertension and lung endothelial dysfunction evident as lack of endothelial NO production and endothelium-dependent vasodilation. These changes were associated with an increased pulmonary vascular resistance, medial thickening, and biventricular cardiac hypertrophy. Sildenafil treatment largely attenuated these pathologic changes, and was not associated with detectable adverse effects pertinent to lung vascular barrier function, edema formation, or systemic hemodynamics.

**Conclusions**—Our data identify sildenafil as a promising therapy for PH-LHD. In light of its documented protective effects at the myocardial level in heart failure, sildenafil presents a particularly attractive strategy in that it simultaneously targets cardiac remodeling and secondary PH-LHD.

**Key Words:** congestive heart failure, pulmonary hypertension, vascular remodeling, endothelial dysfunction
In 60%-80% of patients, congestive heart failure (CHF) is complicated by pulmonary hypertension (PH)\(^1,2\). Accordingly, PH owing to left heart disease (PH-LHD) constitutes one of the most common forms of PH. PH-LHD is not solely caused by a "passive" increase in pulmonary vascular pressures, but is frequently aggravated by a concomitant rise in pulmonary vascular resistance (PVR)\(^2\). This "reactive" increment in PVR further increases right ventricular (RV) afterload, promoting RV dysfunction and ultimately failure.

Although underlying causes differ, PH-LHD shares the common pathophysiological characteristics of pulmonary arterial hypertension (PAH): Lung vascular remodeling is manifest in experimental heart failure\(^3\) and in lungs from patients with CHF\(^4\). Lung endothelial dysfunction is evident in animal models of CHF as impaired endothelium-dependent vasodilation\(^5\), and was similarly observed in patients with CHF due to severe mitral stenosis\(^6\). The resulting imbalance between endothelial-derived vasoconstrictive and vasodilatory mediators is considered to constitute the major driving force for the increase in lung vascular tone and pulmonary vascular remodeling\(^7\). Yet, while the past decade has witnessed the introduction of a series of innovative strategies for the treatment of PAH, approved therapeutic options for non-PAH forms of PH including PH-LHD are still lacking.

Owing to its vasorelaxant effect and its relative selectivity for the pulmonary circulation, the phosphodiesterase inhibitor sildenafil has been established for the clinical treatment of PAH\(^8\). Based on the common pathologic features of PAH and PH-LHD, we speculated that sildenafil may be equally beneficial in PH-LHD, a notion that is supported by small clinical pilot trials. In CHF patients, acute administration of sildenafil selectively reduced pulmonary arterial pressure (PAP), increased peak VO\(_2\) during exercise\(^9\), and improved overall exercise capacity\(^10\). Chronic sildenafil treatment ameliorated exercise capacity and quality of life,
and improved aerobic efficiency in patients with PH-LHD\textsuperscript{11,12}. While these data have in part been attributed to the beneficial effects of sildenafil on cardiac remodeling and skeletal muscle perfusion, they may similarly attest to a therapeutic potential for sildenafil in PH-LHD. Yet, a comprehensive analysis of the effects of sildenafil on the pulmonary vasculature in CHF is so far lacking. Here, we analyzed the effects of chronic sildenafil on lung endothelial function, pulmonary vascular remodeling and right ventricular function in an experimental model of PH-LHD\textsuperscript{13,14}.

**Methods**

A detailed method section is provided in the supplementary material.

*Experimental design.* CHF was induced in male Sprague-Dawley rats (102±10 g body weight) by supracoronary aortic banding\textsuperscript{13,14}. A titanium clip with a residual open diameter of 0.8 mm was implanted across the ascending aorta distal to the origins of the coronary arteries. Sham-operated rats served as controls. Sildenafil treatment (60 mg/kg body weight daily p.o. in the drinking water) was initiated one week after aortic banding and continued for 8 weeks until PH and lung vascular adaptation were assessed. Comparable dosages were previously reported to be safe and effective in rats\textsuperscript{15}.

*Cardiac function and hypertrophy.* Transthoracic echocardiography was performed as described\textsuperscript{14}. Right ventricular (RV) end-diastolic diameter (RVEDD) and area, and left ventricular (LV) area and ejection fraction (LVEF) were measured. Tricuspid annular plane systolic excursion (TAPSE) and pulmonary artery acceleration time (PAAT) were quantified as...
indicators of RV systolic longitudinal function and PH. RV weight and the weight of the LV and septum (LV+S) were determined relative to body weight.

**Hemodynamics.** Arterial pressure (AP), central venous pressure, PAP, left atrial pressure (LAP), mixed venous oxygen saturation (SvO₂), and aortic flow were measured via implanted catheters and an ultrasonic flow probe, and systemic vascular resistance (SVR) and PVR were calculated. To assess lung vascular biomechanics in detail, we analyzed pressure-flow curves in the isolated perfused lung. Intrinsic lung vascular resistance R₀, i.e. the resistance that would exist if the vessels were at their respective diameter at zero vascular pressure, and the vascular distensibility factor α were calculated.

**Histostereology and biochemistry.** Lungs were fixed, paraffin-embedded, sectioned and stained with H&E. Blinded stereological analysis of mean wall thickness of lung arterial vessels (diameter 100-1000 μm) was performed. Lung phosphodiesterase 5 expression was analyzed by Western blot. In aortic blood samples, plasma concentrations of sildenafil and its main metabolite N-desmethyl-sildenafil were determined by high-performance liquid chromatography coupled to tandem mass spectrometry, and those of cyclic guanosine 3′,5′-monophosphate (cGMP) by enzyme-linked immunosorbent assay.

**Endothelial function.** Endothelial NO production, endothelium-dependent vasodilation and endothelial barrier function were assessed in isolated perfused rat lungs. Dose-dependent endothelial NO production in response to acetylcholine (ACh) was measured by fluorescence
imaging of the NO-sensitive dye DAF-FM. Endothelium-dependent vasodilation was assessed as dose-dependent reduction in PAP (ΔPAP) in response to ACh in lungs preconstricted by a continuous infusion of U46619 (50 pMol·min⁻¹). Lung vascular filtration coefficient (Kᵥ) was measured, and lung edema was evaluated by measurement of wet-to-dry lung weight ratios.

Statistical analysis. Data are presented as mean±SEM. Group sizes are n=11 for echocardiographic, histostereological and biochemical analyses. Western blots were replicated in triplicate. Hemodynamics and endothelial function were assessed in groups of n=5 each. Values of several groups were compared by Mann-Whitney U-tests and Kruskal-Wallis test for two or more independent groups. Spearman’s coefficient of correlation (rₛ) was calculated, and non-linear regression analyses were performed using SigmaPlot software. Statistical significance was assumed at P< 0.05.

Results

Plasma levels of sildenafil and N-desmethyl-sildenafil. In plasma samples from untreated rats, sildenafil and its metabolite N-desmethyl-sildenafil were always below the limit of quantification (<2 nMol/L). Of the sildenafil-treated rats, 22.7% and 50% had measurable plasma concentrations of sildenafil or N-desmethyl-sildenafil, respectively, with considerable variation between animals (Fig. 1A).

Ventricular hypertrophy and function. Within 9 weeks, rats with aortic banding showed significant increases in right and left ventricular weight (Figs. 1B,C), while body weights of CHF
and control animals did not differ (355±7 g in control, 352±6 g in CHF rats). Sildenafil treatment markedly attenuated both right and left ventricular hypertrophy (Fig. 1B,C). The notion that sildenafil may have protective effects on RV function was substantiated in echocardiographic measurements. As shown in representative B-mode images (Figs. 2A) and group data (Figs. 2B-G), aortic banding resulted in marked RV dilation, evident as increased end-diastolic area and diameter, that was associated with signs of PH and impaired RV systolic longitudinal function as indicated by reduced PAAT and TAPSE, respectively. Sildenafil reversed echocardiographic signs of RV dilation and PH at large, and increased TAPSE. LV end-diastolic area and LVEF did not differ between groups in line with the characteristics of diastolic heart failure with preserved ejection fraction.

Hemodynamics. As compared to sham-operated controls, mean PAP was approximately 7.5 mmHg higher in CHF rats (Fig. 3A). This increase was only in part attributable to a congestive "passive" elevation in lung vascular pressures, evident as ~2.4 mmHg increase in LAP (Fig. 3B), but similarly caused by a "reactive" increase in PVR. Sildenafil attenuated PH and reduced PVR (Fig. 3C) without causing a significant reduction in LAP. Mean arterial pressure (Fig. 3D) and aortic flow (Fig. 3E) did not differ between CHF and control animals and were not affected by sildenafil.

Lung vascular biomechanics. As compared to controls, isolated lungs from untreated CHF rats showed a marked reduction in vascular distensibility (Fig 4A) and a concomitant increase in intrinsic vascular resistance (Fig. 4B) consistent with the observed increase in PVR. Sildenafil largely reversed the effects of CHF on lung vascular biomechanics.
**Lung vascular remodeling.** Considerable lung vascular remodeling was evident in CHF rats as medial wall thickening in small (inner diameter < 150 μm), medium (diameter 150-250 μm), and large pulmonary arteries (diameter 250-1000 μm). Sildenafil prevented vascular remodeling in small and medium arteries, and mitigated medial thickening in large arteries (Fig. 5).

**PDE5 expression and cGMP plasma levels.** As demonstrated by representative immunoblots (Fig. 6A) and group data (Fig. 6B), PDE5 expression in lung homogenate was downregulated by ~50% in CHF as compared to control rats. To test whether sildenafil could still effectively increase cGMP concentrations, we measured cGMP levels in aortic plasma. Aortic cGMP levels in CHF rats were significantly lower as compared to controls (Fig. 6C). Sildenafil not only restored aortic cGMP levels, but almost doubled them as compared to untreated controls. Mean wall thickness over all vessel calibers correlated inversely with aortic cGMP levels and decreased exponentially with increasing concentrations of cGMP (Fig. 6D). Of note, mean wall thickness remained virtually unchanged over a wide concentration range of cGMP from 20-50 pMol/mL, but increased drastically when cGMP levels approached 10 pMol/mL or less.

**Lung endothelial function.** Fluorescence imaging of DAF-FM loaded endothelial cells identified a marked increase in NO production in response to ACh in pulmonary microvessels of sham-operated rats (Fig. 7A, top), that was absent in lungs of CHF rats (Fig. 7A, center). Sildenafil largely restored the endothelial NO response to ACh in CHF rats, as demonstrated by representative real-time images (Fig. 7A, bottom) and normalization of the characteristic dose-
response curve (Fig. 7B).

To verify the notion that sildenafil may preserve endothelial function in CHF, we measured the pulmonary pressure response to ACh in preconstricted lungs. In lungs of sham rats, ACh caused a dose-dependent decrease in PAP (Fig. 7C) that was completely abrogated in CHF lungs. In line with the reconstitution of endothelial NO synthesis in real-time imaging experiments, sildenafil largely restored the pulmonary vasodilatory response to ACh.

*Lung vascular barrier function.* Vascular permeability, quantified as filtration coefficient $K_f$, was reduced in lungs from CHF rats as compared to sham animals (Fig. 8A). This adaptive response partially compensated for the chronic elevation in lung hydrostatic pressure, which therefore resulted only in moderate lung edema as indicated by a slight increase in wet-to-dry lung weight ratio (Fig. 8B). Sildenafil further strengthened lung vascular barrier function as demonstrated by an even lower $K_f$, and this effect was associated with a reduction in lung edema (Figs. 8A,B). Changes in lung water content were inversely paralleled by respective changes in SvO₂, suggesting that CHF impaired and sildenafil restored tissue oxygenation (Fig. 8C).

**Discussion**

In the present study, we propose long term oral sildenafil as a novel strategy for the treatment of PH-LHD. In a rat model of CHF following aortic banding, sildenafil attenuated PH, RV hypertrophy and dysfunction. This effect was not solely attributable to an attenuation of LV diastolic failure, but likewise to the prevention of lung vascular remodeling and the restoration of endothelial function, resulting in a normalization of PVR.
Pulmonary hypertension with left heart disease. While PH-LHD develops in the majority of patients with CHF\textsuperscript{1,2}, it has received far less attention than PAH with respect to pathomechanistic, epidemiological or therapeutic studies\textsuperscript{20}. The past decades have witnessed a rapid advance in the treatment of PAH, with the clinical introduction of endothelin-1 receptor antagonists, prostacyclin analogs, and the phosphodiesterase inhibitor sildenafil\textsuperscript{21}. In consideration of the common pathological features of PAH and PH-LHD it seems conceivable that some of these interventions may also provide clinical benefit in patients with PH-LHD. Due to its capacity to attenuate cardiac hypertrophy and remodeling in severe heart failure\textsuperscript{22}, sildenafil presents a particularly attractive candidate for the treatment of PH-LHD.

Here, we analyzed the effects of chronic sildenafil treatment in a preclinical model of CHF that features the typical characteristics of PH-LHD, i.e. RV hypertrophy, elevated PVR, vascular remodeling, endothelial dysfunction and increased vessel tone\textsuperscript{13,14}. Notably, aortic flow, systemic arterial pressure and LVEF are normal in these animals. The model therefore reflects the clinical picture of heart failure with preserved ejection fraction, a category of heart failure that is attributed to abnormalities in diastolic rather than systolic ventricular function\textsuperscript{23} and associated with a high incidence of PH in patients\textsuperscript{2}. The observed beneficial effects of sildenafil on pulmonary hemodynamics, endothelial function and vascular remodeling suggest a direct effect of sildenafil on the pulmonary vasculature. In addition, improvement of LV diastolic function as suggested by the attenuation of LV hypertrophy can be expected to have contributed to the protective effect of sildenafil on pulmonary structure and function. Sildenafil has been shown to mitigate LV remodeling in severe cardiac failure by targeting specific maladaptive hypertrophy signaling cascades\textsuperscript{22,24}. Yet, sildenafil does not exert direct effects on functionally
compensated left ventricular hypertrophy, as these pathologic signaling cascades are little
activated. The observed reduction in LV mass in the treatment group suggests that sildenafil
may also attenuate marked LV hypertrophy in diastolic heart failure with preserved ejection
fraction, a notion that warrants further study given the current evaluation of sildenafil for the
clinical treatment of diastolic heart failure.

Lung vascular remodeling. Medial hypertrophy of muscular pulmonary arteries is a frequent
finding in severe CHF. This "remodeling" of the vascular wall consolidates PH and contributes
critically to the increased PVR in CHF patients. In the current study, considerable vascular
remodeling was evident in small, medium-sized and large pulmonary arteries and associated with
a concomitant increase in PVR. Chronic sildenafil treatment attenuated medial hypertrophy in
large arteries, and completely prevented vascular remodeling in medium-sized and small arteries.
This finding is in line with the well-documented anti-proliferative effect of sildenafil which
has been attributed to mechanisms including stimulation of the cGMP-dependent protein kinase
and regulator of G protein signaling 2 pathway, to upregulation of mitogen-activated protein
kinase phosphatase-1 and inhibition of extracellular signal-regulated kinase 1/2
phosphorylation, as well as to inhibition of reactive oxygen species generation and RhoA/Rho
kinase activation. While the prevention of lung vascular remodeling by sildenafil was
paralleled by a complete normalization of PVR, CHF-induced pulmonary hypertension was only
partially reversed, and PAP remained elevated by ~2 mmHg (without reaching p<0.05) reflecting
the persistent increase in LAP as a consequence of the sustained banding.

Sildenafil is most prominently known for its inhibitory effect on PDE5. PDE5 is
markedly upregulated in lungs and right ventricles of patients with PAH or congenital heart
disease\textsuperscript{28,29}, but its expression was reduced in lung endothelial cells of rats with aortic banding.

Our finding is in line with the reported decrease in myocardial PDE5 expression in pacing-induced heart failure\textsuperscript{30} and may indicate a differential regulation of PDE5 expression in PH and ventricular remodeling of different etiologies. In addition to blocking PDE5, sildenafil also blocks PDE1, and like PDE5 blockers, specific PDE1 inhibition can reverse lung vascular remodeling and RV hypertrophy in rodent models of PAH\textsuperscript{31}. Yet in contrast to the cGMP-specific activity of PDE5, PDE1 hydrolyzes both cAMP and cGMP\textsuperscript{22}. The reported $IC_{50}$ of sildenafil for PDE5 in rats is 3.4 nMol/L\textsuperscript{32} while $IC_{50}$ for PDE1 can be estimated as 250-300 nMol/L based on available data in humans\textsuperscript{33}. In the present study, plasma concentrations of sildenafil and its active metabolite $N$-desmethyl-sildenafil were well below the $IC_{50}$ for PDE1 in sildenafil-treated rats.

Yet, due to the approx. 10x faster elimination of sildenafil in rats as compared to humans\textsuperscript{34}, considerably higher sildenafil concentrations may have been achieved immediately after drug uptake with the drinking water which may have sufficed to inhibit PDE1 at least temporarily. The extremely short plasma half-life of sildenafil in rats in combination with individual differences in the time lag between the last oral sildenafil uptake and the actual experiment is also likely to account for the large variability in plasma concentrations.

As compared to controls, plasma cGMP levels in aortic blood of CHF rats were markedly reduced. Given the short half-life of cGMP in plasma, this finding likely signifies endothelial dysfunction in the pulmonary rather than the systemic circulation. Aortic cGMP levels correlated inversely with vessel wall thickness suggesting that inhibition of cGMP hydrolysis may be critically involved in the detected anti-remodeling effect. Remarkably, this inverse relationship seems to be pathognomonic for PH-LHD: On the one hand, patients or animal models with PAH consistently show supranormal urinary or plasma cGMP levels\textsuperscript{35,36}. LHD without pulmonary
involvement, on the other hand, likewise results in elevated cGMP plasma levels in peripheral blood due to increased circulating levels of natriuretic peptides. In contrast, severe heart failure with pulmonary hypertension causes natriuretic peptide resistance, in that pulmonary cGMP release no longer matches the transpulmonary extraction of natriuretic peptides, thus resulting in subnormal cGMP levels in aortic blood. In line with this notion, pulmonary cGMP release was recently shown to correlate inversely with pulmonary vascular resistance in patients with heart failure.

The fact that wall thickness remains unchanged over a large range of cGMP concentrations but increases steeply at very low cGMP levels implies that aortic cGMP concentrations may present a potential biomarker for lung vascular remodeling in CHF. In the present study, a cut-off value of 24 pMol/mL for aortic cGMP levels would identify banded rats with a sensitivity of 100% and a specificity of 36.4%. The area under the respective receiver operating characteristic (ROC) curve (supplementary Fig. 1) is 0.756. Provided that sensitivity and specificity would be similar in patients, cGMP levels in aortic blood obtained e.g. during left heart catheterization may help to differentiate PH-LHD from PAH patients on the one, and patients with LHD without pulmonary involvement on the other side. PH-LHD could be effectively excluded in patients with normal or supranormal aortic cGMP levels, while those with lower values would require further diagnostic evaluation.

**Lung endothelial dysfunction.** While restoration of endothelial-derived NO synthesis may present a particularly promising strategy in PH-LHD based on the demonstrated inverse relationship between cGMP and vascular remodeling, therapeutic strategies targeting lung endothelial function in CHF are still lacking. In line with previous data, marked lung endothelial
dysfunction was evident in CHF rats. Lung endothelial function was at large preserved by sildenafil and may contribute relevantly to its beneficial properties in PH-LHD by acting in addition to or in conjunction with its anti-proliferative effects. Notably, preservation of endothelial function may potentiate the direct effect of sildenafil in that inhibition of cGMP hydrolysis will coincide with enhanced NO-triggered cGMP synthesis. To our knowledge the present study is the first to directly demonstrate a protective effect of sildenafil on lung endothelial function, a finding that is in line with previous reports that sildenafil may improve endothelial function in the systemic circulation. Several mechanisms may account for the suppression of endothelial dysfunction by sildenafil, in that sildenafil can restore the expression of endothelial NO synthase (eNOS), promote its posttranslational activation by stimulating Akt-dependent eNOS phosphorylation and endothelial Ca\textsuperscript{2+} influx, and inhibit Nox2 expression and superoxide production. As lung endothelial dysfunction in the aortic banding model results primarily from increased \textbeta-actin expression and subsequent F-actin polymerization, cGMP-dependent activation of vasodilator-stimulated phosphoprotein (VASP) and subsequent inhibition of actin filament formation may present a particularly intriguing mechanism by which sildenafil may restore endothelial function in this specific setting.

**Lung vascular barrier function.** In CHF, lung microvessels are constantly exposed to elevated hydrostatic pressures and adapt by thickening of the capillary wall and a concomitant silencing of pro-edematous signaling pathways. While attenuation of medial proliferation and preservation of endothelial NO synthesis are declared therapeutic targets in PH-LHD, prevention of structural remodeling and endothelial adaptation at the level of alveolar capillaries may promote barrier failure and alveolar flooding. The use of vasodilators in PH-LHD has also been...
viewed critically in that opening of precapillary sphincters may increase capillary pressure and thus, escalate edema formation. Here we show that sildenafil decreases $K_f$ in CHF lungs, thus strengthens the microvascular barrier, attenuates lung edema and improves systemic oxygenation. This barrier-protective effect of sildenafil is consistent with our previous observation that cGMP signaling negatively regulates microvascular barrier failure and that sildenafil attenuates hydrostatic edema formation.

Clinical implications. Our results identify sildenafil as a promising strategy to attenuate lung vascular remodeling and RV hypertrophy in patients with left heart disease and at risk for PH-LHD. The lack of systemic hemodynamic effects of sildenafil is particularly noteworthy in consideration of the catastrophic results from a series of clinical trials that addressed the effectiveness of systemic vasodilators in CHF and at large resulted in increased patient morbidity and/or mortality. Based on its FDA approval for the treatment of PAH, sildenafil may be rapidly exploitable as novel therapy in PH-LHD. Importantly, sildenafil presents a particularly attractive strategy in this context due to its concomitant beneficial effects on the myocardium in severe CHF and its current clinical evaluation in diastolic heart failure in the NIH-sponsored "RELAX" trial (NCT00763867). Taken together, sildenafil may provide a dual protective effect in CHF, in that it reverses cardiac hypertrophy and remodeling, while simultaneously attenuating PH-LHD.
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Disclosures

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Figure Legends

**Figure 1. Sildenafil concentration and ventricular hypertrophy.** (A) Box plots show plasma concentrations of sildenafil and its main active metabolite N-desmethyl-sildenafil in sildenafil-treated and untreated rats (Data are from n=11 rats each). Limits of quantification are shown as dotted line. Group data show right ventricular weight (RV) over body weight (BW) (B) and the combined weight of the left ventricle and the interventricular septum (LV+S) over body weight (C), respectively, in sham-operated rats and in rats with aortic banding (CHF) with or without sildenafil (sil) treatment. Data from n=5 rats each, *p<0.05 vs. sham, #p<0.05 vs. CHF.

**Figure 2. Echocardiographic analyses.** Representative echocardiographic B-mode images (A) show left (LV) and right (RV) ventricle in end-diastole in sham operated (left), banded (CHF, center), and sildenafil-treated banded rats (CHF+sil, right). Note marked dilation of the right ventricle in banded rats, which is attenuated by sildenafil. Replicated in n=11 rats each. Bar graphs show end-diastolic RV area (B) and diameter (RVEDD; C), left ventricular ejection fraction (LVEF; D), LV area (E), pulmonary artery acceleration time (PAAT; F); and tricuspid annular plane systolic excursion (TAPSE; G) in sham operated, banded, and sildenafil-treated banded rats. Data from n=11 rats each, *p<0.05 vs. sham, #p<0.05 vs. CHF.

**Figure 3. Pulmonary and systemic hemodynamics.** Group data show (A) pulmonary arterial pressure (PAP), (B) left atrial pressure (LAP), (C) pulmonary vascular resistance (PVR), (D) arterial pressure (AP), and (E) aortic flow in sham operated, banded (CHF), and sildenafil-treated banded rats (CHF+sil). Data from n=5 rats each, *p<0.05 vs. sham, #p<0.05 vs. CHF.
Figure 4. Lung vascular biomechanics. Group data show (A) lung vascular distensibility (α) and (B) intrinsic lung vascular resistance (R₀) in sham operated, banded (CHF), and sildenafil-treated banded rats (CHF+sil). Data from n=5 rats each, *p<0.05 vs. sham, #p<0.05 vs. CHF.

Figure 5. Lung vascular remodeling. (A) Representative images of HE-stained lung sections from sham operated (top) and banded (bottom) rats. Note different scale bars and considerable vascular remodeling after aortic banding. Replicated in n=11 rats each. Group data show mean vascular wall thickness of pulmonary arterioles with (B) an inner diameter (Ø) < 150 μm, (C) Ø of 151–250 μm, and (D) Ø of 250-1000 μm in lungs from sham operated, banded (CHF), and sildenafil-treated banded rats (CHF+sil). Data from n=11 rats each, *p<0.05 vs. sham, #p<0.05 vs. CHF.

Figure 6. PDE5 expression and cGMP plasma levels. Representative Western blots from lung homogenate (A) and quantitative bar graph (B) show decreased PDE5 expression and α-tubulin as loading control in lungs of banded (CHF) as compared to sham operated rats. Data from n=3 rats each, *p<0.05 vs. sham. (C) Group data show plasma concentration of cyclic guanosine 3′,5′-monophosphate (cGMP) in sham operated, banded (CHF), and sildenafil-treated banded rats (CHF+sil). Data from n=11 rats each, *p<0.05 vs. sham, #p<0.05 vs. CHF. (D) Mean vascular wall thickness of pulmonary arterioles was plotted against plasma cGMP concentration of the respective animal in sham operated, banded, and sildenafil-treated banded rats (n=11 each). Data were fit to a three-parameteric exponential decay by non-linear regression analysis.
Inverse correlation was tested by Spearman’s coefficient of correlation ($r_s = -0.687$, $p<0.001$).

Figure 7. Lung endothelial function. (A) Representative images of DAF-FM loaded capillaries show endothelial cells color-coded for NO production in response to acetylcholine (ACh) in lungs from sham operated (top), banded (CHF; center), and sildenafil-treated banded rats (CHF+sil; bottom). Images were obtained at baseline (left) and 10 min after administration of $5 \times 10^{-6}$ Mol/L ACh (right). Vessel margins are marked as white lines. (B) Group data show dose-dependent changes in capillary DAF-FM fluorescence in response to stimulation with ACh in lungs from sham operated, banded (CHF), and sildenafil-treated banded rats (CHF+sil). Dose-response curves were constructed from four-parametric logistic curve fits. Data from $n=5$ rats each, *$p<0.05$ vs. sham, #$p<0.05$ vs. CHF. (C) Group data show endothelium-dependent vasorelaxation response to ACh in isolated perfused rat lungs preconstricted by U46619 (50 pMol/min). Vasorelaxation was determined as dose-dependent pressor response and is expressed as reduction in pulmonary arterial pressure ($\Delta$PAP) relative to the respective vehicle control. Dose-responses were constructed from four-parametric logistic curve fits. Data from $n=5$ rats each, *$p<0.05$ vs. sham, #$p<0.05$ vs. CHF.

Figure 8. Lung vascular barrier function and venous oxygenation. Group data show (A) lung vascular filtration coefficient ($K_i$), (B) wet-to-dry lung weight ratio, and (C) mixed venous oxygen saturation (SvO$_2$) in sham operated, banded (CHF), and sildenafil-treated banded rats (CHF+sil). Data from $n=5$ rats each, *$p<0.05$ vs. sham, #$p<0.05$ vs. CHF.
sham aortic banding

B

Ø <150 μm

mean vascular wall thickness (μm)

0 5 10 15 20 25

* #

Ø 150-250 μm

mean vascular wall thickness (μm)

0 5 10 15 20 25 30

* #

Ø 250-1000 μm

mean vascular wall thickness (μm)

0 20 40 60 80 100

sham CHF CHF+sil

D

mean vascular wall thickness (μm)

* #

mean vascular wall thickness (μm)
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Sildenafil preserves lung endothelial function and prevents pulmonary vascular remodeling in a rat model of diastolic heart failure

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Supplemental Material
Materials and Methods

Animals. Male juvenile (92±10 g, bw) Sprague-Dawley rats were obtained from Charles River Laboratories (Sulzfeld, Germany). All animals received care in accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academy Press, Washington, DC 1996). The study was approved by the animal care and use committee of the local government authorities (LAGeSo Berlin, Germany).

Experimental groups. 108 rats were randomly assigned to one of three experimental groups: In group 1 (n=36), congestive heart failure (CHF) was induced by supracoronary aortic banding without further treatment. In group 2 (n=36), supracoronary aortic banding was performed and treatment with sildenafil (60 mg/kg bw daily per os via the drinking water; kindly provided by Pfizer Inc., Groton, UK) was started on postoperative day eight. Individual sildenafil uptake was monitored and adjusted on a daily basis. In group 3 (n=36), sham-operated animals served as controls.

Supracoronary aortic banding. CHF was induced by supracoronary aortic banding in juvenile rats as described previously\(^1\text{-}^3\). Following anesthesia by intraperitoneal injection of ketamin (87 mg/kg bw; Pharmacia, Erlangen, Germany) and xylazine (13 mg/kg bw; Bayer-Schering, Leverkusen, Germany), rats were placed in supine position, the chest wall was shaved and sterilized, and a left thoracotomy was performed in the third intercostal space while rats were ventilated with 100% O\(_2\). The ascending aorta was
isolated from the connective tissue and partially occluded by a titanium clip (Hemoclip™, Weck closure system, Research Triangle Park, NC) with a defined internal diameter of 0.8 mm that was implanted across the ascending aorta immediately distal to the origins of the coronary arteries. Sham-operated rats of similar body weight, in which the clip was positioned in the surrounding mediastinal tissue, served as controls. After supracoronary aortic banding or sham operation, respectively, the thoracic cavity was resealed by sterile surgical suture, and rats were allowed to recover from anesthesia. For postoperative analgesia, subcutaneous injections of caprofen (4 mg/kg bw; Rimadyl™, Pfizer GmbH, Karslruhe, Germany) were given for 3 days post-operatively. After recovery, the rats were separated and placed in cages with free access to water and standard laboratory diet for 9 weeks.

**Evaluation of pulmonary hypertension and lung vascular adaptation.** Nine weeks after aortic banding or sham operation, rats entered the experimental protocol. By this time, rats had attained body weights of 354±5 g with no differences between experimental groups. From each group, 11 animals underwent transthoracic echocardiography and subsequent histostereological analysis, while 5 animals each were used for i) *in vivo* hemodynamic evaluation, ii) endothelial NO imaging, or evaluation of iii) lung vascular biomechanics, iv) endothelium-dependent vasorelaxation, or v) endothelial barrier function in isolated perfused lung preparations, respectively.

**Echocardiography.** Rats were anesthetized by intraperitoneal injection of ketamin (87
mg/kg bw) and xylazine (13 mg/kg bw) and allowed to breathe spontaneously in a left recumbent position. Transthoracic 2-dimensional (2D), M-mode and Doppler imaging were performed with a 10-MHz transducer using VIVID 7 echocardiography equipment (General Electric, Horten, Norway) as described before\(^1\), and images were digitally stored for subsequent off-line analysis. Right (RV) and left (LV) ventricular geometry were assessed in the parasternal midpapillary short axis as LV and RV end-systolic and end-diastolic areas, and LV ejection fraction (LVEF) was calculated as described\(^4\). RV end-diastolic diameter (RVEDD) was measured in the apical four chamber view. To assess RV systolic longitudinal function, the base-to-apex movement during systole, measured as the tricuspid annular plane systolic excursion (TAPSE) of the lateral portion of the tricuspid annular plane, was recorded in the M-mode under 2D echocardiographic guidance from the apical 4-chamber view. Pulsed wave Doppler of the pulmonary outflow was recorded in the parasternal view at the level of the aortic valve. For this purpose, the sample volume was positioned proximal to the pulmonary leaflets and aligned to maximize laminar flow. The pulmonary artery acceleration time (PAAT) was used as an echocardiographic indicator of pulmonary hypertension and measured from the onset of systolic flow to peak pulmonary outflow velocity. For each parameter, three complete cardiac cycles were analyzed and averaged. After completion of echocardiographic measurements, blood samples were obtained from the aorta for subsequent measurements of sildenafil, \(N\)-desmethyl-sildenafil, and 3’-5’-cyclic guanosine monophosphate (cGMP) concentrations in plasma and lungs were harvested.
for histostereological analyses as outlined below.

*Sildenafil and N-desmethyl-sildenafil plasma concentration.* Due to the shorter half-life of sildenafil in rodents (0.4–1.3 h) as compared to humans (3.7 h)\(^5\), concentrations of both sildenafil and its main metabolite *N*-desmethyl-sildenafil in rat plasma were determined by reversed phase high-performance liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS, API 365; AB Sciex Germany GmbH, Darmstadt, Germany) after liquid/liquid extraction using sildenafil, *N*-desmethyl-sildenafil and d\(_3\)-sildenafil (internal standard) as reference compounds (Toronto Research Chemicals TRC, Toronto, ON). In brief, rat plasma (100 µL) was mixed with internal standard (80 ng), alkalinized with borate buffer (pH 9.0, 100 µL), and extracted with tertiary butyl methyl ether (2 mL, 10 min over-head shaking). After centrifugation (10 min at 3000g), the extract (1.5 mL) was evaporated to dryness (nitrogen at 40°C) and reconstituted by liquid chromatography (LC) eluent (100 µL). An aliquot (20 µL) was injected into the LC system (Shimadzu LC-10, Duisburg, Germany) and chromatographed (Phenomenex Synergy Polar RP 4 µm, 150 x 2.0 mm, 40°C) under isocratic conditions (eluent A: 5 mMol/L aqueous ammonium acetate incl. 0.1% acetic acid; eluent B: acetonitrile; 48%/52%). The LC eluent flow (0.35 mL/min) was directly introduced into the electrospray ion source. In the selected reaction monitoring mode positive ion transitions of sildenafil (m/z 475.2→m/z 100.2), *N*-desmethyl-sildenafil (m/z 461.2→283.1), and d\(_3\)-desmethyl-sildenafil (m/z 478.2→m/z 103.1) were monitored. The calibrated range for
both compounds was 2-5267 nMol/L with a limit of quantification at 2 nMol/L. Linear regression of area ratio (analyte/internal standard) was performed by 1/x weighing and resulted in correlation coefficients ($r^2$) of $>0.999$. Quality control in the lower, medium, and upper calibration range was performed according to the FDA requirements. The day-to-day coefficient of variation of sildenafil (N-desmethy-sildenafil) quality control samples was 1.3%, 6.9%, and 3.0% (1.6%, 1.4%, and 4.8% respectively) at concentrations of 12, 1511 and 3205 nMol/L (14, 1696 and 396 nMol/L, respectively). The corresponding accuracy was 105.3%, 103.7%, and 104.2% (98.9%, 101.7%, and 99.2%). The analytical method validation fulfilled all requirements of the FDA Guideline concerning selectivity, accuracy, precision, recovery, and stability.

cGMP plasma concentration. Aortic blood samples (1.5 ml) were collected in EDTA tubes with IBMX (33 mg/ml; Sigma-Aldrich Chemie GmbH), centrifuged at 4°C (5000g, 10 min) and stored at -80°C. cGMP concentrations were determined by enzyme-linked immunosorbent assay microplate immunoassay kits according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

Histostereology. Lungs were fixed by intratracheal instillation of 4% paraformaldehyde for 30 min; tissue sections of 1 cm$^3$ were excised and fixed for another 16 h. Dehydrated tissues were paraffin-embedded, and 5 µm sections were cut and mounted on slides coated with 10% poly-L-lysine (Sigma-Aldrich Chemie GmbH, Munich, Germany). Lung sections were stained with hematoxylin and eosin and analyzed in a blinded fashion. Each
section was completely scanned through with a light microscope (BX50, Olympus, Ballerup, Denmark) and an adapted magnification of predominantly 100x to identify arterial blood vessels. The microscope was equipped with a mechanical stage linked to a computer running a stereological software (Cast version 2.1.6.1., Olympus). The measurement of mean vascular wall thickness was performed according to a stereological approach described by Nyengaard and colleagues\(^7\). The diameter was defined by determining the longest diameter with a measurement line perpendicular to the longest axis. The start and end points were the outermost layer of the arterial wall. Next, the opposing vessel wall thicknesses were measured in the region of the diameter. Every evaluated arterial structure was marked to avoid over- and underestimation. The mean of both wall thickness measurements gave the final result for the wall thickness related to the diameter. In each group, 800 to 1000 pulmonary arterioles were analyzed and vessels were categorized according to their external diameters as small (<150 µm in diameter), medium sized (diameter 151-250 µm), and large (diameter 251-1000 µm) pulmonary arterioles.

*Western Blot Analysis.* Aliquots of frozen rat lungs were homogenized in phosphate buffered saline (PBS) containing protease inhibitor mixture (Complete Mini; Roche Diagnostics GmbH, Mannheim, Germany), 1 mmol/L phenylmethansulfonyl fluoride, and 1% Triton-100. Total protein concentration was determined by Bradford Protein Assay (Bio-Rad, Munich, Germany). Sample proteins (50 µg/slot) and a pre-stained
protein-weight marker (Bio-Rad) were size-fractionated by SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell, Dassel, Germany). Membranes were blocked, washed, and incubated with matching primary antibodies for PDE5 (Cell Signaling Technology, Danvers, MA) and appropriate secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized by enhanced chemiluminescence (ECL; Perkin Elmer GmbH, Freiburg, Germany), and intensity of protein bands was quantified using Image J (NIH, Bethesda, MD).

**Hemodynamics.** For *in vivo* measurement of pulmonary and systemic hemodynamics, rats were anesthetized by intraperitoneal injection of medetomidine (0.5 mg/kg bw; Domitor™, Dr. E. Graeub AG, Basel, Switzerland), fentanyl (0.05 mg/kg bw; JanssenCilag, Neuss, Germany), and midazolam (5 mg/kg bw; Dormicum™, Roche, Basel, Switzerland) as previously described². The neck and chest wall were shaved, and rats were placed in a supine position on a thermostatically controlled electronic heating blanket (Homeothermic Blanket Control Unit; Harvard Apparatus, March-Hugstetten, Germany) to maintain the body temperature at 38°C throughout the experiment. After tracheal intubation, rats were mechanically ventilated (Animal respirator advanced 4601-1; TSE system GmbH, Bad Homburg, Germany) with a tidal volume of 6 mL/kg at 100 breaths/min and a peak inspiratory pressure of 10.2±1.1 cmH₂O. Polyvinyl catheters (internal diameter 0.58 mm) were advanced into the aorta and the vena cava *via* the left
carotid artery and the right jugular vein, respectively. A median sternotomy was performed, and catheters were introduced into the left atrium and the pulmonary artery via the left auricle and the right ventricle, respectively. An ultrasonic flowprobe (Transonic™; Transonic Systems Inc., Ithaca, NY) was positioned around the ascending aorta distal to the branching of the coronary arteries and proximal to the truncus brachiocephalicus. Arterial pressure (AP), central venous pressure (CVP), pulmonary arterial pressure (PAP), left atrial pressure (LAP), and aortic flow were continuously monitored (HSE Type 705/1; Hugo Sachs Elektronik, March-Hugstetten, Germany) and recorded by use of the software package DasyLab™32 (DasyLab, Moenchengladbach, Germany). Systemic vascular resistance (SVR) and pulmonary vascular resistance (PVR) were calculated as arteriovenous pressure differences over flow.

Following hemodynamic characterization, animals were killed by exsanguinations. Heart and lungs were removed, and RV weight and the combined weight of the LV and the interventricular septum (LV+S) relative to body weight were determined. The excised lungs were weighed, dried by a microwave desiccation technique⁸, re-weighed, and the wet-to-dry lung weight ratio was calculated as a measure of lung edema.

*Isolated perfused rat lung.* For imaging of endothelial NO production, and evaluation of lung vascular biomechanics, endothelium-dependent vasorelaxation, and endothelial barrier function, isolated perfused rat lungs were prepared from sham operated, banded, or sildenafil-treated banded rats, respectively, as previously described⁹,¹⁰. In brief,
isolated lungs were constantly inflated with a gas mixture of 21% O₂, 5% CO₂, and balance N₂ at a positive airway pressure of 5 cmH₂O. Lungs were perfused with Krebs-Henseleit buffer containing 3% bovine serum albumin (Sigma-Aldrich Chemie GmbH) at 38°C in a circulatory manner. At baseline, LAP was adjusted to 5 cmH₂O, yielding a PAP of 10±1 cmH₂O at a perfusion rate of 14 mL/min. PAP and LAP were continuously monitored and digitally recorded (DASYlab™ 32).

**Lung vascular biomechanics.** To characterize changes in lung vascular biomechanics independent of the actual hemodynamic situation *in vivo*, we determined lung vascular distensibility (α) and intrinsic lung vascular resistance (R₀) by recording of lung pressure-flow curves and nonlinear regression analysis to a one-compartment distensible vessel model as previously described¹¹. In this model, R₀ describes the pulmonary vascular resistance that would exist if the vessels were at their respective diameter at zero vascular pressure; α reflects the corresponding vascular distensibility factor.

Briefly, lungs were perfused at a constant flow (Q) of 50 ml·kg⁻¹·min⁻¹ as described above. Perfusate flow via the roller pump (Stoeckert Instrumente; Sorin Group Deutschland GmbH, Munich, Germany) was then adjusted to 25, 50, 75, and 100 ml·kg⁻¹·min⁻¹ in a randomized manner, and LAP was adjusted to maintain constant at 2 mmHg by adapting the height of the venous outflow reservoir. The resulting changes in PAP were recorded (DASYlab™ 32), and α and R₀ were calculated according to the equation
as previously reported\textsuperscript{12}.

\textit{Real-time fluorescence microscopy}. \textit{In situ} fluorescence microscopy of isolated perfused rat lungs was performed as previously described\textsuperscript{9,10}. In brief, lungs were positioned on a vibration-free table and superfused with saline at 37\textdegree C to prevent drying. For fluorescence measurement of NO production \textit{in situ}, we loaded the cell-permeant, NO sensitive dye 4-amino-5-methylamino-2\textsuperscript{\prime},7\textsuperscript{\prime}-difluorofluorescein diacetate (DAF-FM DA; 5 \textmu mol/L; Invitrogen GmbH, Darmstadt, Germany) to lung microvascular endothelial cells via a microcatheter that was wedged into a pulmonary vein draining a capillary area on the lung surface\textsuperscript{9,10}. DAF-FM DA de-esterifies intracellularly to DAF-FM which is converted via an NO dependent mechanism to an intensely fluorescent benzotriazole derivative with fluorescence intensity linearly reflecting NO concentration\textsuperscript{9,10}.

Endothelial DAF-FM fluorescence was excited at 480 nm by a near monochromatic beam from a digitally controled galvanometric scanner (Polychrome IV; T.I.L.L. Photonics, Martinsried, Germany). Fluroescence emission was collected through an upright intravital microscope (Axiotech\textsuperscript{Vario} 100 HD; Zeiss, Jena, Germany) equipped with an apochromat objective (UAPO 40x W2/340; Olympus, Hamburg, Germany) and appropriate dichroic and emission filters (FT 510 and LP 520; Zeiss, Jena, Germany) by a CCD camera (Sensicam; PCO, Kelheim, Germany) and subjected to digital image analysis.
Single venular capillaries were viewed at a focal plane corresponding to maximum vessel diameter (14-28 µm). All fluorescence images were background corrected, with background determined in images captured before fluorophore loading. Fluorescence was quantified in 4 µm² areas along the microvascular wall, representing single lung endothelial cells.

Endothelial DAF-FM fluorescence was recorded in 10 s intervals at baseline, and following stimulation with increasing concentrations of acetylcholine (ACh; 10⁻⁹–10⁻⁴ mol/L; Sigma-Aldrich Chemie GmbH). DAF-FM fluorescence intensity (F) was expressed relative to its individual baseline (F₀). Because the NO-dependent conversion of DAF-FM into a fluorescent benzotriazole derivative is irreversible, the ratio F/F₀ reflects cumulative NO production over time, while its first derivative ΔF/F₀ determined in 5 s intervals reflects actual NO production.

Endothelium-dependent vasodilation. In isolated perfused rat lungs, endothelium-dependent vasodilation was determined as previously described¹³ as change in lung perfusion pressure (ΔPAP) in response to ACh (10⁻⁹–10⁻⁵ mol/L) in lungs preconstricted by the thromboxane A₂ analog U46619 (Calbiochem, Darmstadt, Germany). U46619 was continuously infused at a final concentration of 50 pMol/min resulting in a sustained increase in PAP. Ten min after the start of U46619, increasing concentrations of ACh were infused via the pulmonary arterial line as 50 µL boluses in 3-min intervals. PAP was continuously recorded and expressed as ΔPAP relative to the respective vehicle control.
(0.9% NaCl).

**Lung vascular barrier function.** Lung vascular filtration coefficient \( (K_f) \) was measured in isolated perfused rat lungs by a gravimetric technique as recently reported\(^7\). In brief, isolated perfused lungs were positioned on a microscale (PRS 320-3; Kern, East Sussex, United Kingdom), and lung weight, PAP and LAP were continuously monitored (HSE Type 705/1) and digitally recorded (DASYlab 32). After we had ensured that lungs were under isogravimetric conditions for > 5 min, LAP was increased by 4 cmH\(_2\)O and \( K_f \) was determined from the resulting weight gain \( (\Delta W) \) by dividing the rate of weight gain \( \Delta W/\Delta t \) measured between 18 and 20 min after the LAP increment by the resultant elevation in capillary pressure\(^14\). Results were normalized to 100 grams initial lung weight, assuming the density of the filtered fluid to be 1 g/mL. Pulmonary capillary pressure (Pc) was calculated from PAP and LAP as described before\(^14\). Next, LAP was reset to baseline and deviations from the isogravimetric state were corrected for by subtracting the linear regression of \( \Delta W/\Delta t \) at baseline LAP from the actual \( \Delta W/\Delta t \) measured between 18 and 20 min\(^15\).

**Statistical analysis.** Statistical analyses were performed by use of SigmaStat software (SigmaStat 3.0, Systat Software Inc., Erkrath, Germany). Data are presented as mean ± SEM. Values of several groups were compared by Mann-Whitney U-tests and Kruskal-Wallis test for two or more independent groups, respectively. Spearman’s coefficient of correlation (\( r_s \)) was calculated to test correlation between parameters, and
non-linear regression analyses were performed using SigmaPlot software (Version 9.0, Systat Software Inc.). Receiver operating characteristic (ROC) curve was generated and area under the curve calculated to determine the overall discriminatory power of plasma cGMP levels for prediction of supracoronary aortic banding and subsequent pulmonary hypertension owing to left heart disease in rats. Statistical significance was assumed at P<0.05.
Receiver operating characteristic (ROC) curve for plasma cGMP concentration as a "predictor" of aortic banding in rats. Sensitivity represents the fraction of true positives detected by cGMP and is plotted against the fraction of false positives (1-specificity). The total area under the ROC curve reflecting the overall discrimination performance of the test was calculated as 0.756.
References


