Sildenafil treatment in established right ventricular dysfunction improves diastolic function and attenuates interstitial fibrosis independent from afterload

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Borgdorff MA, Bartelds B, Dickinson MG, van Wiechen MP, Steendijk P, de Vroomen M, Berger RM. Sildenafil treatment in established right ventricular dysfunction improves diastolic function and attenuates interstitial fibrosis independent from afterload. Am J Physiol Heart Circ Physiol 307: H361–H369, 2014. First published May 30, 2014; doi:10.1152/ajpheart.00843.2013.—Right ventricular (RV) function is an important determinant of prognosis in congenital heart diseases, pulmonary hypertension, and heart failure. Preventive sildenafil treatment has been shown to enhance systolic RV function and improve exercise capacity in a model of fixed RV pressure load. However, it is unknown whether sildenafil has beneficial effects when treatment is started in established RV dysfunction, which is clinically more relevant. Our aim was to assess the effects of sildenafil treatment on RV function and fibrosis in a model of established RV dysfunction due to fixed afterload. Rats were subjected to pulmonary artery banding (PAB), which induced RV dysfunction after 4 wk, characterized by reduced exercise capacity, decreased tricuspid annular plane systolic excursion, and RV dilatation. From week 4 onward, 50% of rats were treated with sildenafil (100 mg·kg−1·day−1, n = 9; PAB-SIL group) or vehicle (n = 9; PAB-VEH group). At 8 wk, exercise capacity was assessed using cage wheels, and RV function was assessed using invasive RV pressure-volume measurements under anesthesia. Sildenafil treatment, compared with vehicle, improved RV ejection fraction (44 ± 2% vs. 34 ± 2%, P < 0.05; PAB-SIL vs. PAB-VEH groups), reduced RV end-diastolic pressure (2.3 ± 0.5 vs. 5.1 ± 0.9 mmHg, P < 0.05), and RV dilatation (end-systolic volume: 468 ± 45 vs. 643 ± 71 μL, P = 0.05). Sildenafil treatment also attenuated RV fibrosis (30 ± 6 vs. 17 ± 3%, P < 0.05) but did not affect end-systolic elastance, exercise capacity, or PKG or PKA activity. In conclusion, sildenafil improves RV diastolic function and attenuates interstitial fibrosis in rats with established RV dysfunction, independent from afterload. These results indicate that sildenafil treatment has therapeutic potential for established RV dysfunction.

RIGHT VENTRICULAR (RV) failure due to pressure overload is a major determinant of the outcome of congenital heart diseases (32) and in pulmonary arterial hypertension (17). RV function also determines outcomes in congestive heart failure (28, 45). Given the increasing incidence of heart failure as well as the quickly expanding population of adults with congenital heart disease, there is a growing need for therapies that specifically support RV function. Unfortunately, despite a growing interest in the mechanisms underlying RV failure (4, 24), so far no RV specific therapy is available. Treatments successful in left ventricular (LV) failure might be beneficial in RV failure, but application could be limited due to the fact that the RV is morphologically, functionally, and embryologically different from the LV (24, 40, 44).

However, recent studies have shown that, like LV failure, experimental RV failure is associated with ventricular dilatation, impaired systolic and diastolic function, and adverse myocardial remodeling, including hypertrophy and interstitial fibrosis (4, 10, 40). In LV failure due to pressure load, inhibition of phosphodiesterase (PDE)5A has been proven to successfully reduce hypertrophy and interstitial fibrosis and improve diastolic function (38). Therapeutic administration of PDE5A inhibitors (e.g., sildenafil) is now being tested in several clinical heart failure trials (16, 34).

PDE5A inhibitors have also been successfully used in patients with pulmonary arterial hypertension (22). In these patients, the beneficial effects of PDE5A inhibition on the RV might partly be explained by decreased RV afterload, resulting from sildenafil effects on the diseased pulmonary vasculature. However, there is emerging evidence that sildenafil also exerts direct beneficial effects on the pressure-loaded RV. PDE5A is activated in the RV of patients with a pressure-loaded RV (30). We have previously shown that sildenafil administered from the onset of pressure load (preventive treatment) enhanced systolic RV function, attenuated ventricular dilatation, and limited the decline in exercise tolerance in a rat model of fixed RV pressure overload but also modestly increased interstitial fibrosis (10). Since RV afterload was fixed in these experiments [pulmonary artery banding (PAB) is unaffected by the pulmonary vasodilatory effects of sildenafil], these findings indicated that sildenafil directly affected the RV myocardium.

In clinical practice, however, most patients already present with RV dysfunction, which disqualifies them for preventive treatment. Therefore, if sildenafil also has beneficial effects when started in established RV dysfunction, this would be very relevant for the clinical setting. The aim of the present study was to test whether sildenafil could improve systolic and diastolic function (measured with echocardiography and pressure-volume analysis) and attenuate fibrosis in a model of established, pressure load-induced RV dysfunction. In addition, we assessed whether changes in RV function and remodeling were associated with changes in exercise tolerance (measured as voluntarily run distance). To define the applicability of sildenafil treatment in different phases of RV dysfunction, we compared our results with those from the preventive strategy study.
MATERIALS AND METHODS

Animal model and study design. Animal care and experiments were conducted according to the Dutch Animal Experimental Act and conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). The Animal Experiments Committee of the University of Groningen approved the experimental protocol.

To induce fixed RV pressure overload, PAB was performed on Wistar rats (n = 20, male, 160–180 g, Charles River), which led to severe RV dysfunction in 4 wk (10). Animals were randomly assigned to the following two groups: 1) PAB with vehicle treatment (PAB-VEH group) and 2) PAB with sildenafil treatment (PAB-SIL group). The first 4 wk after PAB, both groups received regular drinking water. See Fig. 1A for the experimental setup. At 4 wk after PAB, RV dysfunction was confirmed by exercise testing and echocardiography. From 4 wk after PAB, the PAB-SIL group received drinking water to which sildenafil (Pfizer, New York, NY) was added (100 mg·kg−1·day−1). This dose is higher than that currently prescribed in humans (because of increased clearance rates in rodents (41)) but is frequently used in rodents (1, 36, 38, 42) and has been shown to lead to effective plasma levels that specifically inhibit PDE5A (10). In the same study (10), we have shown effective plasma levels resulting in the activation of PKG1. The PAB-VEH group continued to receive regular drinking water. At 8 wk after PAB, all rats were evaluated by exercise testing, echocardiography, and pressure-volume measurements.

Two animals (1 animal in the PAB-SIL group and 1 animal in the PAB-VEH group) died prematurely: one animal died from severe bleeding during surgery and the other died suddenly in the second week after surgery of unknown cause but without any sign of RV failure.

Exercise tolerance and clinical signs of failure. To measure voluntary exercise tolerance, running wheels were mounted in the rat cages, as previously described (10). Five days before surgery, 5 days before the 4-wk mark (halfway through the experiment), and 5 days before death at (8 wk), animals were allowed to run in the cage wheel. Running distance was thus recorded daily using a digital magnetic counter (Commodoor Cycle Odometer, Commodoor) (4, 10). Exercise tolerance was measured as the percent change in running distance at 4 or 8 wk compared with 0 or 4 wk, respectively, for each individual animal.

Throughout the experiment, rats were examined daily for clinical signs of RV failure according to a predefined ABCDE checklist, as previously described (10, 11). The ABCDE criteria were defined as follows: appearance and activity (A), body weight (B), cyanosis and circulation (C), dyspnea and tachypnea (D), and edema and effusion (E). appearance and activity symptoms were considered present when the animal had ruffled fur or red discoloration of the head and neck (due to decreased cleaning behavior) or was less active than previously despite stimulation. A body weight symptom was scored if there was a change in body weight of >15 g in <48 h. Cyanosis was checked at exposed skin on the head, paws, and tail. Hampered circulation was considered present if both front paws and hind legs/tail were pale and markedly colder than previously (regarded as a consequence of decreased perfusion). Dyspnea and tachypnea were qualitatively assessed and defined as markedly increased breathing effort and frequency, respectively. Edema and effusions were defined as fluid collection in the thorax and/or abdomen at euthanization.

Echocardiography. Echocardiography was performed at 4 wk to confirm RV dysfunction and at 8 wk as previously described (10) using a Vivid Dimension 7 system and 10S-transducer (GE Healthcare, Waukesha, WI). We used apical three- and four-chamber views and parasternal short- and long-axis views to measure RV and right atrial dimensions, tricuspid insufficiency, tricuspid annular plane systolic excursion, and continuous wave Doppler for the gradient across the PAB. Cardiac output was calculated as aorta diameter2 × 3.14 × velocity time integral × heart rate using systolic aorta diameter and pulsed wave Doppler measurements of aorta flow. Measurements from 6–12 consecutive beats were used to average out beat-to-beat variation.

RV hemodynamics. Hemodynamic characterization of the RV was performed by pressure-volume experiments obtained by RV catheterization using a combined pressure-conductance catheter (SPR-869, Millar Instruments, Houston, TX) at 8 wk after surgery according to a previously described protocol (10).

The volume signal of the conductance catheter was calibrated for parallel conductance and slope factor to obtain absolute volumetric values. The parallel conductance was estimated using the hypertonic saline method by infusing 10 μl hypertonic (10%) saline via the jugular vein cannula (2, 25). The slope factor was calculated as the uncalibrated conductance catheter cardiac output divided by LV cardiac output, as measured by echocardiography.

Load-independent parameters of contractility [end-systolic elastance (Ees) and preload recruitable stroke work (PRSW)] and diastolic function [end-diastolic elastance (Eed)] were measured during transient vena cava occlusion and could be obtained in 15 of 19 animals.
(n = 6 animals in the PAB-VEH group and 9 animals in the PAB-SIL). We calculated RV volume at a normalized end-systolic pressure of 70 mmHg as an additional measure of systolic function. Visual inspection of end-diastolic pressure-volume relationships (EDPVRs) revealed that relations were either highly linear or clearly exponential. Subjective scoring indicated that in the untreated group, four of six EDPVRs were clearly exponential, whereas in the treated group, only two of nine EDPVRs were clearly exponential. The presence of both linear and exponential EDPVRs complicated direct statistical comparison of both groups; slope factor (Ed) can be used for linear EDPVRs, the exponential coefficient can be used for exponential EDPVRs, but neither of them can be used in both. Therefore, to enable a single, objective analysis of all EDPVRs, we used the following approach.

Each EDPVR data set was divided into an upper volume range and a lower volume range, separated by the median end-diastolic volume. The upper and lower parts were each fitted with a linear curve, respectively: Ped = VEd-up + EEd-up × VEd and Ped = VEd-low + EEEd-low × VEd, where Ped is end-diastolic pressure, VEd is the upper volume range, EEd-up is EEd of the upper range, VEd is end-diastolic volume, VEd-low is the lower volume range, and EEEd-low is EEEd of the lower range. For EDPVRs that are essentially linear over the full volume range, EEd-up and EEd-low should be approximately equal, and EEd-up/EEd-low would be expected to be close to 1. For exponential EDPVRs, EEd-up will be substantially higher than EEd-low, and EEEd-low/EEd-up should be clearly <1. Thus, this ratio can be used as a simple, objective linearity index, with 1 indicating a linear relationship and values below 1 (increasingly steeper) indicating exponential curves.

Organ weights, hypertrophy, and fibrosis. After heart catheterization, rats were euthanized by removing the heart from the thorax. The heart, lungs, and liver were dissected. The RV, interventricular septum, LV, and both atria were separated and weighed. Tissue sections were fixed, transsectionally cut at 4-μm-thick sections, and stained with wheat germ agglutinin to assess cardiomyocyte size and with Masson trichrome to assess fibrosis. Cardiomyocyte size was measured as average surface area of cross-sectionally cut cardiomyocytes with a visible nucleus (Image-Pro, MediaCybernetics, Bethesda, MD) and photographed in a transsection of the entire RV. The extent of fibrosis was quantified as the blue-stained percentage of the total tissue area measured per whole ventricle (Image Scope 11, Aperio Technologies, Vista, CA), as previously described (10).

Quantitative RT-PCR. Expression of the fetal gene program (myosin heavy chain isoforms and natriuretic propeptides type A and B), markers of hypertrophy [sarco(endo)plasmic reticulum Ca2+ handling [sarco(endoplasmic reticulum Ca2+-ATPase (SERCA)2 and phospholamban], and genes of PDE pathways (PDE3 and PDE5) was measured to characterize the remodeling response and effects of sildenafil. Total RNA was extracted using the RNeasy fibrous tissue kit (Qiagen) following the manufacturer’s guidelines. Data were normalized to reference gene 36B4.

Protein kinase activity assays. PKG activity was assayed in RV tissue with the cyclex cGK-dependent protein kinase (cGK) assay kit (CycLex, Nagano, Japan). Samples were prepared in extraction buffer (50 mM potassium phosphate buffer, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, and 4 μl/ml phosphate inhibitor), potassium phosphate buffer, and DE buffer (20 mM Tris-HCl, 60 mM NaCl, 0.5 mM EDTA, 1 mM EGTA, and 4 μl/ml phosphatase inhibitors) according to the manufacturer’s protocol. Each prepared sample (100 μl) was then added to each well of the plate, which was precoated with recombinant G kinase substrate. After an incubation (30 min, 30°C), the wells were washed, and 100 μl of horseradish peroxidase-conjugated anti-phospho-specific antibody was added; this was incubated for 1 h at room temperature. The plate was then rinsed and substrate reagent was added for incubation of 15 min, after which stop solution terminated the reaction. Absorbance was read at dual wavelengths of 450/540 nm. Data are presented as quantities of cGK activity (expressed in units/μg protein) using the protein concentrations calculated with the Bio-Rad DC Protein Assay (Bio-Rad Laboratories).

PKA activity was measured using the MESACUP Protein Kinase Assay (MBL, Nagoya, Japan). Sample preparation was done as in the PKG sample preparation with extraction buffer (50 mM potassium phosphate buffer, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, and 4 μl/ml phosphate inhibitor), potassium phosphate buffer, and DE buffer (20 mM Tris-HCl, 60 mM NaCl, 0.5 mM EDTA, 1 mM EGTA, and 4 μl/ml phosphatase inhibitors). Each prepared sample (100 μl) was added to each well (incubated for 10 min, 25°C) followed by stop solution. After a wash, 100 μl biotinylated antibody 2B9 was added (incubated for 60 min, 25°C), and again the microplate was washed. The addition of POD-conjugated streptavidin (incubated for 60 min, 25°C), substrate solution (incubated for 3 min, 25°C), and stop solution was interspersed by washing steps. Finally, the microplate was read at a wavelength of 492 nm. Data are presented as relative PKA activity.

Statistical analysis. All quantitative data were tested for normality and are expressed as means ± SEM. PAB-VEH versus PAB-SIL differences were evaluated using Student’s t-tests or Mann-Whitney U-tests as appropriate. The group size was 8–9 animals/group except when otherwise specified. P values of <0.05 were considered significant (PASW Statistics 18 for Windows, SPSS, Chicago, IL).

RESULTS

PAB induced RV dysfunction. At week 4, before treatment was started, PAB had induced fixed RV pressure load (PAB gradient: 53 ± 4 mmHg), which led to RV dysfunction. RV dysfunction was characterized clinically by decreased exercise tolerance (running distance vs. baseline: −57 ± 8%). Echocardiography showed low tricuspid annular plane systolic excursion (2.1 ± 0.1 mm, normal value: ~3 mm), RV dilatation (end-diastolic diameter: 4.8 ± 0.2 mm, normal value: ~3.5 mm), and right atrial enlargement (maximal long-axis diameter: 4.3 ± 0.2 mm, normal value: ~3 mm) (12). After this clinical and echocardiographic evaluation, rats in the PAB-SIL group started with sildenafil treatment. Before treatment, there were no differences in characteristics, including RV function parameters, between PAB-SIL and PAB-VEH groups at 4 wk (Table 1).

The changes to 4 wk of PAB were similar to those described in previous studies (10, 12). In these studies, a similar magnitude of PAB led at 4 wk to increased contractility, as measured by Ees (+164%), RV dilatation, as measured by an increase in end-diastolic volume (+48%), as well as early phase deterioration of diastolic function, as measured by Ees.

Table 1. Echocardiographic characteristics at start of sildenafil therapy

<table>
<thead>
<tr>
<th>Echocardiographic parameters</th>
<th>PAB-VEH Group</th>
<th>PAB-SIL Group</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>PAB gradient, mmHg</td>
<td>49 ± 6</td>
<td>56 ± 4</td>
<td>0.33</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>315 ± 8</td>
<td>347 ± 15</td>
<td>0.10</td>
</tr>
<tr>
<td>CO, ml/min</td>
<td>56 ± 5</td>
<td>75 ± 9</td>
<td>0.12</td>
</tr>
<tr>
<td>TAPSE, mm</td>
<td>2.1 ± 0.2</td>
<td>2.1 ± 0.3</td>
<td>0.94</td>
</tr>
<tr>
<td>RVEDD, mm</td>
<td>5.0 ± 0.2</td>
<td>4.6 ± 0.4</td>
<td>0.48</td>
</tr>
<tr>
<td>RA diameter, mm</td>
<td>4.6 ± 0.2</td>
<td>4.1 ± 0.4</td>
<td>0.34</td>
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</tbody>
</table>

Values are means ± SE. PAB-VEH, vehicle-treated group with pulmonary artery banding (PAB); PAB-SIL, sildenafil-treated group with PAB; HR, heart rate; CO, cardiac output; TAPSE, tricuspid annular plane systolic excursion; RVEDD, right ventricular (RV) end-diastolic diameter; RA, right atrial.
Sildenafil beneficially affected RV dysfunction. After 8 wk, due to growth of the animals, PAB gradients had increased (74 ± 3 vs. 77 ± 5 mmHg, PAB-VEH vs. PAB-SIL groups, not significant). Sildenafil treatment improved ejection fraction and reduced end-systolic volume compared with untreated rats (Fig. 1, B and C) but did not significantly affect end-diastolic volume (Table 2). In untreated rats, RV pressure-volume loops showed ventricular dilatation (Fig. 2A). In the PAB-SIL group, pressure-volume loops showed a leftward shift on the volume axis, reflecting the (nonsignificant) reduced ventricular dilatation (Fig. 2A). Contractility, assessed by $E_{es}$ and PRSW, did not significantly differ between the two groups (Table 2). RV-pulmonary artery coupling, expressed as $E_{es}/E_{sa}$, tended to increase with sildenafil treatment, although the changes failed to reach statistical significance (0.68 ± 0.15 vs. 0.82 ± 0.15, PAB-VEH vs. PAB-SIL groups, $P = 0.054$). However, sildenafil treatment improved diastolic function: end-diastolic pressure was significantly lower in PAB-SIL group (Fig. 1D) and EDPVR differed markedly between the two groups (Fig. 2B). In the majority of untreated animals (4 of 6 animals, 66%), EDPVRs were shifted upward and displayed a clearly exponential behavior, indicating increased ventricular stiffness. In contrast, in sildenafil-treated animals, almost all RVs had relatively low end-diastolic pressures and a normal linear EDPVR (2 of 9 animals, 22%; Fig. 2B). To enable comparison of linear and nonlinear EDPVRs (neither $E_{ed}$ nor the exponential coefficient can be used in both), we divided the EDPVR data set of each animal into an upper volume range and lower volume range, separated by the median end-diastolic volume, and determined the slope ($E_{ed}$) for each part with a linear fit (see METHODS). $E_{ed-low}/E_{ed-up}$ was used as an index for linearity (with 1 indicating a linear EDPVR). In untreated rats, the linearity index was 0.35 ± 0.12; in sildenafil-treated rats, the linearity index was 0.90 ± 0.14 ($P = 0.01$; Fig. 1D), indicating significantly improved diastolic function. The improvement in RV function by sildenafil could not be correlated with improvements in exercise tolerance (Table 2), and symptoms of RV failure were seen in both groups (all animals in both groups were tachypneic and showed decreased cleansing behavior); none of the rats needed to be terminated prematurely because of severe RV failure symptoms. No unwanted side effects of sildenafil treatment were noted.

Sildenafil attenuated RV fibrosis but not RV hypertrophy. Sildenafil attenuated RV myocardial fibrosis (29 ± 3‰ vs. 17 ± 3‰, PAB-VEH vs. PAB-SIL groups, $P < 0.05$). This difference was not due to severity of loading, as the amount fibrosis per mmHg RV peak pressure was also decreased by sildenafil (Fig. 3, A–C). The degree of interstitial fibrosis correlated with end-diastolic pressure ($R^2 = 0.587, P = 0.001$; Fig. 3D), suggesting an interaction between fibrosis and diastolic function. TGF-$\beta$ mRNA expression was lower in sildenafil-treated animals, which is in line with the histological findings, but the difference failed to reach significance due to high variation (1.30 ± 0.14 vs. 0.97 ± 0.16, PAB-VEH vs. PAB-SIL groups, $P = 0.14$). mRNAs for galectin 3, TIMP, and collagen isoforms were equally expressed in both groups.

Pressure load led to hypertrophy, as shown by RV weight (1.2 ± 0.1 mg/g body wt; Table 2) and RV cardiomyocyte cross-sectional surface area (0.13 ± 0.01 $\mu m^2$) (10). The degree of RV hypertrophy in sildenafil-treated animals did not differ from that in untreated animals (Table 2).

No difference in the expression of RV remodeling-associated genes (natriuretic peptide precursor A, natriuretic peptide precursor B, skeletal muscle $\alpha$-actin 1, RCAN-1, myosin heavy chain 7/myosin heavy chain 6) could be demonstrated between treated and untreated groups (data not shown). Activities of PKG (3.2 ± 0.4 vs. 3.9 ± 0.8 units/$\mu g$ protein, PAB-VEH vs. PAB-SIL groups, $P = 0.43$) and PKA (1.6 ± 0.1 vs. 1.7 ± 0.1 units/$\mu g$ protein, PAB-VEH vs. PAB-SIL groups, $P = 0.68$) tended to be reduced in sildenafil-treated animals compared with untreated controls (3.2 ± 0.4 vs. 3.9 ± 0.8 units/$\mu g$ protein, PAB-VEH vs. PAB-SIL groups, $P = 0.07$) but did not significantly affect end-diastolic pressure-volume relations of PKG (3.2 ± 0.4 vs. 3.9 ± 0.8 units/$\mu g$ protein, PAB-VEH vs. PAB-SIL groups, $P = 0.07$). Activations of PKG (3.2 ± 0.4 vs. 3.9 ± 0.8 units/$\mu g$ protein, PAB-VEH vs. PAB-SIL groups, $P = 0.07$) and PKA (1.6 ± 0.1 vs. 1.7 ± 0.1 units/$\mu g$ protein, PAB-VEH vs. PAB-SIL groups, $P = 0.07$). Activations of PKG (3.2 ± 0.4 vs. 3.9 ± 0.8 units/$\mu g$ protein, PAB-VEH vs. PAB-SIL groups, $P = 0.07$) and PKA (1.6 ± 0.1 vs. 1.7 ± 0.1 units/$\mu g$ protein, PAB-VEH vs. PAB-SIL groups, $P = 0.07$).

Table 2. Pressure-volume parameters, exercise, and organ weights at 8 wk

<table>
<thead>
<tr>
<th></th>
<th>PAB-VEH Group</th>
<th>PAB-SIL Group</th>
<th>$P$ Value</th>
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<tbody>
<tr>
<td>pressure-volume parameters</td>
<td></td>
<td></td>
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<tr>
<td>HR, beats/min</td>
<td>283 ± 12</td>
<td>283 ± 11</td>
<td>1.00</td>
</tr>
<tr>
<td>CO, ml/min</td>
<td>89 ± 4</td>
<td>102 ± 5</td>
<td>0.07</td>
</tr>
<tr>
<td>$E_{es}$, mmHg/µl 0-1,000</td>
<td>96 ± 19</td>
<td>124 ± 25</td>
<td>0.38</td>
</tr>
<tr>
<td>PRSW, mmHg</td>
<td>39 ± 12</td>
<td>41 ± 7</td>
<td>0.89</td>
</tr>
<tr>
<td>voluntary exercise</td>
<td></td>
<td></td>
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<tr>
<td>Change at 8 wk vs. 4 wk, %</td>
<td>-46 ± 4</td>
<td>-45 ± 5</td>
<td>0.94</td>
</tr>
<tr>
<td>change at 8 wk vs. baseline, %</td>
<td>-79 ± 10</td>
<td>-81 ± 13</td>
<td>0.74</td>
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<tr>
<td>organ weights</td>
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<td></td>
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<tr>
<td>RV weight/body weight, mg/g</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>0.70</td>
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<tr>
<td>LV + IVS weight/body weight, mg/g</td>
<td>2.1 ± 0.03</td>
<td>2.1 ± 0.05</td>
<td>0.60</td>
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<tr>
<td>RA weight/body weight, mg/g</td>
<td>0.31 ± 0.08</td>
<td>0.26 ± 0.06</td>
<td>0.25</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>423 ± 15</td>
<td>442 ± 21</td>
<td>0.48</td>
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</table>

Values are means ± SE. $E_{es}$, end-systolic elastance; PRSW, preload recruitable stroke work; LV, left ventricle; IVS, interventricular septum.

Fig. 2. Pressure-volume analysis of sildenafil or vehicle in PAB. A: representative pressure-volume loops of untreated (PAB-VEH; top) and treated (PAB-SIL; bottom) rats with PAB during vena cava occlusion. End-systolic pressure-volume relations are marked by solid black lines; EDPVRs are marked by dashed black lines. B: overview of EDPVRs obtained during vena cava occlusion in PAB-VEH (black, $n = 6$) and PAB-SIL (gray, $n = 9$) groups. The pressure-volume loops in the top left corner are shown to indicate the area of the pressure-volume loops that is enlarged.
showing diastolic dysfunction while contractility (E_{stolic} dysfunction has been confirmed in experimental studies of pulmonary arterial hypertension (5). The importance of dia-
stolic RV function is associated with a poor outcome in increased right atrial pressure (as an indirect measure of dia-
tostic RV failure (23, 33). Clinical studies have shown that contractility and RV hypertrophy. Eventually, RV adaptation pro-
gresses into RV dysfunction and failure. Although the mech-
isms of RV dysfunction are incompletely understood, dia-
stolic dysfunction is thought to be important in the progression to RV failure (23, 33). Clinical studies have shown that
diastolic dysfunction is a prominent feature of RV failure, these beneficial effects of sildenafil on diastolic function may have therapeutic merit for patients with advanced RV dysfunction. Importantly, the present study,

In the present study, using pressure-volume measurements, we showed that sildenafil beneficially affects EDPVR and lowers end-diastolic pressure. The EDPVR reflects intrinsic diastolic stiffness (14). In humans, the RV EDPVR cannot be recorded accurately, but we and others have previously shown that within a physiological range, the EDPVR in a normal rat RV is linear (10, 18, 25).

The observed linear-to-exponential transformation of the EDPVR reflects increased stiffness of the diseased RV. In the low volume range, compliant elastin fibers and titin molecules are stretched, resulting in a shallow slope of the EDPVR. In the higher volume range, the slack length of titin and collagen fibers is exceeded, resulting in a much steeper slope of the EDPVR (14). Sildenafil treatment prevents the linear-to-exponential transformation of the EDPVR. This, in turn, results in lower end-diastolic pressures, highlighting the improved diastolic function. Since diastolic dysfunction is a prominent feature of RV failure, these beneficial effects of sildenafil on diastolic function may have therapeutic merit for patients with advanced RV dysfunction. Importantly, the present study, being a reversal study instead of a prevention study, is of great clinical relevance, because patients with a pressure-loaded RV will predominantly present with moderate to severe RV dys-
function, when preventive effects of sildenafil are not of much value to the patient.

In addition to the observed improvement in diastolic performance, parameters of systolic function and ventricular-arterial coupling tended to improve with sildenafil, although these changes failed to reach statistical significance. These slight improvements in systolic performance might have contributed to the increased cardiac output measured in this study. Enhanced contractility is a consistent finding in experimental models of RV pressure load (10, 12, 19, 23) as well as in patients (33). It is an adaptive mechanism that sustains stroke volume in increased afterload (12), a concept known as ventricular-arterial coupling. We speculate that in more progressed stages of RV pressure load, this compensatory mechanism reaches a maximum and cannot sustain stroke volume if...
pressure load persists. Hence, further therapy may be aimed at enhancing diastolic function in addition to efforts to reduce RV afterload.

 Potential mechanisms. The exponential behavior of the ED-PVR observed in untreated rats is not secondary to a shift toward a high volume range but also occurs at relatively normal volumes. These results suggest that sildenafil targets ventricular remodeling rather than just preventing ventricular dilatation. Sildenafil enhances PKG1 activity in early RV pressure load, as shown in our previous study (10), which may affect relaxation via phosphorylation of titin (27), but the activation of PKG1 failed to reach significance in this study. It could be that in this later stage of RV pressure load, PKG1 is already maximally activated by the pressure load. PKG1 activation thus does not mediate the beneficial effects of sildenafil on diastolic dysfunction. Additionally, we did not find differences in titin isoform expression (a switch from the stiffer N2B isoform to the less stiff N2Ba isoform), suggesting that the difference in ventricular stiffness is also not explained by regulation of titin stiffness at the transcriptional level. Alternatively, sildenafil has been suggested to activate PKA, which may affect Ca\(^{2+}\) handling, but, like in the preventive study (10), in this study, sildenafil did not change PKA activity. Another important component of RV dysfunction in experimental models is fibrosis (8, 11). Fibrosis plays a central role in the adaptation of the ventricle to stress in general and pressure load in particular (7, 43). In congenital heart disease and pulmonary hypertension, interstitial fibrosis has been shown to contribute to diastolic dysfunction (13, 15, 33). In the present study, the reduction of fibrosis in sildenafil-treated rats was strongly related to the reduction in end-diastolic pressures (Fig. 3D), suggesting an important component of the positive effects. In previous studies, beneficial effects of sildenafil on RV function have been observed, but these effects were all associated with reduced afterload due to improved pulmonary hemodynamics. Specifically, Xie et al. (42) showed that reducing afterload in a monocrotaline model of pulmonary hypertension also sustained T-tubule structure and improved RV Ca\(^{2+}\) handling, yielding insights into the mechanism by which afterload reduction improves RV function. It is possible that improved Ca\(^{2+}\) handling contributes to the effects observed in our study, although passive chamber properties appear to be more affected than active (Ca\(^{2+}\)-dependent) relaxation.

 Clinical implications. Sildenafil is increasingly being used in the treatment of various types of cardiovascular disease (37). In this study, we treated rats with moderate to severe RV dysfunction yet without signs of overt RV failure. That is, at the start of therapy, these rats had reduced exercise capacity, lower cardiac output, and increased right atrial diameter but no signs of ascites, pleural effusion, or inactivity. The clinical condition compares with New York Heart Association class 2–3, the class in which 80% of patients with pulmonary hypertension present (3, 6), and is seen frequently in the followup of patients with congenital heart defects (20, 32). In pulmonary hypertension, sildenafil therapy has been shown to be beneficial, but the place of PDE5A inhibitors in the treatment of RV dysfunction to fixed pressure overload is yet to be determined. A comparison with results of a preventive treatment strategy (10) indicated differences and similarities in the response (shown in Fig. 4) and highlights the importance of assessing the effects of treatment strategies in different stages of disease progression. Whereas the preventive strategy primarily further enhanced parameters of contractility (E\(_{\text{A}}\)), the reversal strategy affected diastolic function (EDPVR and end-diastolic pressure), although E\(_{\text{A}}\)/E\(_{\text{SV}}\) also appeared to subtly improve. One explanation of the less pronounced effect on contractility could be that contractility is almost maximally enhanced already. In contrast, a prominent feature of (more progressed) RV dysfunction is diastolic dysfunction. This study is the first to report a beneficial effect on diastolic dysfunction in a model of fixed afterload, which circumvents the potential effects on the pulmonary vasculature. Previous studies in rats have shown that the degree of RV dysfunction presented in this study (reduced cardiac output at rest, RV dilatation, and reduced exercise tolerance) can be clinically tolerated for an extended period of time (9). Similarly, in patients with congenital heart diseases, increased RV afterload may be clinically well tolerated (39). However, also similar to patients with a systemic RV, PAB rats exhibited a further decline in RV parameters and clinical function, i.e., RV dilatation and reduced exercise capacity (39).

These analyses also reveal that sildenafil may be associated with increased fibrosis in the early stage, whereas in the later stage of the disease, when fibrosis is a more prominent feature of RV remodeling, sildenafil attenuates fibrosis in the RV (Fig. 4). In both strategies, preventive and reversal, sildenafil limits ventricular dilatation (10) and has a positive effect on ejection fraction. The positive effects of sildenafil in our rats with pressure load appear to be in contrast with the recently reported negative results of the RELAX trial, which studied the long-term effects of sildenafil on patients with heart failure with preserved ejection fraction (34). However, in the RELAX trial, pulmonary artery pressure was only mildly elevated (41 mmHg); hence, RV dysfunction was probably also mild. Redfield et al. (34) suggested that RV dysfunction has to reach a certain limit for sildenafil to become effective in ventricular remodeling, a dose-effect relation that has been previously suggested from studies in mice with a LV load (29). Therefore, further trials are warranted to study the long-term effects of PDE5 inhibition in RV and LV failure [SIL-HF trial (16) and PITCH-HF trial (ID: NCT01910389)].

In this study, sildenafil did not improve exercise performance, as has been shown in a clinical study of pulmonary hypertension (21). Exercise tolerance, as measured with voluntary cage wheel exercise, can be compared with the clinically often used 6-min walking distance test (6MWT) rather than a maximal exercise test. The 6MWT is a valuable outcome parameter in many clinical trials in pulmonary hypertension or heart failure (22, 35). In all clinical trials in pulmonary hypertension, sildenafil treatment was associated with an improvement in 6MWT; however, in all these studies, pulmonary vascular resistance or pulmonary artery pressure also decreased (21, 22), suggesting that the effect might be due to reduced afterload and hence improved RV-pulmonary artery coupling. We studied a fixed afterload model using PAB, which may explain the lack of effect on exercise. Indeed, we have previously shown that in rats, there is a threshold of afterload above which exercise tolerance is decreased (12). Since we did not affect afterload in this study, we did not change this threshold. However, using this model, we were able to demonstrate beneficial effects, specifically on the RV. In patients with pulmonary arterial hypertension, the effects of sildenafil on the
pulmonary vasculature with the specific effects on RV function may add up, leaving sildenafil as one of the few drugs that is beneficial to both the heart and lungs (31).

Sildenafil has beneficial effects on the RV independent from its effects on the pulmonary vasculature. This is in contrast with recent reports of other targeted therapies (26, 31) that have shown adverse myocardial effects. This may induce a shift to use sildenafil not only as an add-on therapy but as a preferred therapy (21). Also, in the growing group of patients with RV dysfunction due to fixed afterload, direct beneficial effects on the RV of sildenafil are of high relevance.

**Limitations.** Our model of rats with RV dysfunction due to a fixed afterload comes with some limitations, which should be discussed. First, we did not address the effects of different dosages of sildenafil, which should be performed in future studies. Second, our relatively small animal study represents hypothesis-generating groundwork, based on which mechanistic studies and larger (clinical) trials can be designed. Third, no invasive pressure-volume measurements were performed at 4 wk, as the extent of this procedure precludes survival of the animals. However, data from our previous study (10) in this model clearly showed RV dysfunction at 4 wk, which was confirmed in the echocardiographic measurements we performed in the present study. Finally, survival analysis would have provided supplemental information on the therapeutic potential of sildenafil in this disease model. Even so, the strong effects of sildenafil on hemodynamics and fibrosis observed in the present and previous study (10) prompt further clinical study on sildenafil in the fixed pressure-loaded RV.

**Conclusions.** In this study in rats with fixed RV pressure overload, we show that sildenafil treatment, in established RV dysfunction, reduced end-diastolic pressure, altered EDPVRs, improved ejection fraction, and limited fibrosis. These changes were not mediated by activation of PKG or PKA. These data demonstrate that sildenafil has a direct beneficial effect on the pressure-loaded RV independent from effects on RV afterload. These results indicate that sildenafil treatment has therapeutic potential for the treatment of established RV dysfunction.
REFERENCES


