Right Ventricular Diastolic Impairment in Patients With Pulmonary Arterial Hypertension

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Background—The role of right ventricular (RV) diastolic stiffness in pulmonary arterial hypertension (PAH) is not well established. Therefore, we investigated the presence and possible underlying mechanisms of RV diastolic stiffness in PAH patients.

Methods and Results—Single-beat RV pressure-volume analyses were performed in 21 PAH patients and 7 control subjects to study RV diastolic stiffness. Data are presented as mean±SEM. RV diastolic stiffness (β) was significantly increased in PAH patients (PAH, 0.050±0.005 versus control, 0.029±0.003; P<0.05) and was closely associated with disease severity. Subsequently, we searched for possible underlying mechanisms using RV tissue of PAH patients undergoing heart/lung transplantation and nonfailing donors. Histological analyses revealed increased cardiomycocyte cross-sectional areas (PAH, 453±31 μm² versus control, 218±21 μm²; P<0.001), indicating RV hypertrophy. In addition, the amount of RV fibrosis was enhanced in PAH tissue (PAH, 9.6±0.7% versus control, 7.2±0.6%; P<0.01). To investigate the contribution of stiffening of the sarcomere (the contractile apparatus of RV cardiomycocytes) to RV diastolic stiffness, we isolated and membrane-permeabilized single RV cardiomycocytes. Passive tension at different sarcomere lengths was significantly higher in PAH patients compared with control subjects (>200%; Pinteraction<0.001), indicating stiffening of RV sarcomeres. An important regulator of sarcomeric stiffening is the sarcomeric protein titin. Therefore, we investigated titin isoform composition and phosphorylation. No alterations were observed in titin isoform composition (N2BA/N2B ratio: PAH, 0.78±0.07 versus control, 0.91±0.08), but titin phosphorylation in RV tissue of PAH patients was significantly reduced (PAH, 0.16±0.01 arbitrary units versus control, 0.20±0.01 arbitrary units; P<0.05).

Conclusions—RV diastolic stiffness is significantly increased in PAH patients, with important contributions from increased collagen and intrinsic stiffening of the RV cardiomycocyte sarcomeres. (Circulation. 2013;128:2016-2025.)

Key Words: diastole ■ heart failure ■ hypertension, pulmonary ■ sarcomeres

Idiopathic pulmonary arterial hypertension (PAH) is a rare but fatal disease with a survival rate of 58% at 3 years.¹ Present therapy is unable to normalize pulmonary arterial pressures, and PAH patients ultimately develop right heart failure.²

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Previous studies have demonstrated that PAH patients have reduced systolic function as measured by right ventricular...
(RV) ejection fraction. However, knowledge of the role of RV diastolic stiffness in PAH is limited. Measuring RV diastolic stiffness has been hindered until now because noninvasive techniques (echocardiography, magnetic resonance imaging [MRI]) provide information only on relaxation velocities, not on diastolic stiffness per se. In addition, these measures are highly sensitive to the confounding effects of increased preload and afterload and are therefore not reliable in the setting of PAH. On the other hand, the gold standard of measuring load-independent diastolic stiffness by pressure-volume (PV) analysis is not without risk in PAH patients because it requires temporal preload reduction. In left heart failure, this was circumvented by the development of single-beat analyses of diastolic PV relationships. However, it is unclear whether this analysis could also be used for the RV in PAH.

There are several possible contributing factors explaining RV diastolic stiffness in PAH. Hypertrophy and fibrosis are known to increase ventricular stiffness. However, RV diastolic stiffness could also be caused by changes in the contractile apparatus of RV cardiomyocytes: the sarcomeres. Sarcomeric stiffness is tightly regulated by the giant sarcomeric protein titin. Titin consists of 2 isoforms: the stiff N2B isoform and the compliant N2BA isoform. Besides changes in isoform composition, titin compliance is regulated by phosphorylation. Whether these factors are altered in human PAH pathophysiology is unknown.

Therefore, the aims of this study are to determine the presence of RV diastolic stiffness in PAH patients and to explore the contribution of collagen formation, sarcomeric stiffening, and posttranslational modifications of titin in RV tissue of PAH patients.

Methods

Assessment of RV Diastolic Stiffness

Hemodynamic data were obtained from digitally stored routine clinical patient records. Patients eligible for this study were referred to the VU University Medical Center for evaluation of pulmonary hypertension and patients with PAH undergoing follow-up analysis between September 2001 and November 2011. Standard clinical care included right heart catheterization (balloon-tipped flow-directed 7F Swan-Ganz catheter, 131HF7, Baxter Healthcare Corp, Irvine, CA) and cardiac MRI (1.5-T whole-body system, Siemens Sonata, Siemens Medical Solutions, Erlangen, Germany). During right heart catheterization, radial or femoral blood samples were collected, and standard laboratory tests, including N-terminal probrain natriuretic peptide level (NT-proBNP), were performed. New York Heart Association class and 6-minute walking distance were registered during the same clinical evaluation. All patients were evaluated in stable hemodynamic condition while in the supine and breathing at normal frequencies.

Patients were selected on the basis of the following criteria: good-quality recordings of right heart catheterization pressure curves with cardiac MRI performed within the same hospital admission and under the same hemodynamic condition (n=28). PAH was diagnosed according to the PAH diagnostic guidelines (n=21). Control subjects were selected from referred patients suspected to have PAH but in whom the condition was ruled out after normal pulmonary pressures were recorded during right heart catheterization (n=7).

Right Heart Catheterization

The following invasive variables were recorded: right atrial pressure (RAP), RV pressure, mean pulmonary artery pressure (mPAP), and pulmonary capillary wedge pressure (PCWP). Cardiac output (CO) was determined by Fick method, and pulmonary vascular resistance (PVR) was calculated from the following formula:

\[
PVR=(mPAP-PCWP)/CO.\]

Diastolic filling pressures were measured at the minimum pressure point (recorded after tricuspid valve opening) and noted as begin-diastolic pressure (BDP). End-diastolic pressure (EDP) was recorded at the maximal diastolic filling pressure point before the onset of isovolumic contraction (Figure 1A).

Cardiac MRI

RV volumes were calculated with Mass software (MEDIS, Medical Imaging Systems, Leiden, the Netherlands) from multiple short axial slice MRI analysis. End-systolic volume was considered to correspond to BDP and is further referred to as begin-diastolic volume (BDV), whereas end-diastolic volume (EDV) corresponded to EDP (Figure 1A). Stroke volume (SV) was calculated from MRI-derived pulmonary artery flow and used to accurately determine RV BDV. RV volumetric filling curves were obtained from the stack of short-axis cine images for the quantification of RV early (E) and atrial (A) induced peak filling rate (E/A ratio), as previously described.

Single-Beat PV Analysis

PV relations were constructed by fitting a nonlinear exponential curve through the diastolic PV points using the following formula:

\[
P(t)=t^a(\beta^t-1),\]

where \(P\) is pressure, \(t\) is a curve-fitting constant, \(\beta\) is a diastolic stiffness constant, and \(V\) is volume.

The slope of the curve was characterized by the exponential term \(\beta\) and the curve constant \(t\), which were further used to quantify RV diastolic stiffness. The first 2 points used to construct the PV relation were the BDP-BDV point and the EDP-EDV point. The third PV point used to construct the diastolic PV relation was set at \(0_m\), because prolonging the PV curves to volumes lower than the intercept volume does not modify the exponential term \(\beta\) and the curve constant \(t\) (see the online-only Data Supplement). Because of the large variation in EDV in control patients compared with PAH patients, the EDV was normalized to the maximal EDV recorded among the patients. Consequently, the BDV was calculated by subtracting the corresponding SV from the normalized EDV. To avoid measurement errors caused by the positioning of the RV catheter, BDP was normalized at 1 mm Hg, whereas the EDP was calculated with the following formula:

\[
EDP_{normalized}=EDP_{initial}+1+EDP_{initial}-BDP_{initial}.
\]

To account for covariance in \(t\) and \(\beta\), derived from each individual subject were used to calculate the \(V\) at a common \(P\) of 20 mm Hg (\(V_{20}\)).

Experimental support for the use of a single-beat instead of a multiple-beat RV diastolic PV relation was obtained in rats with PAH-induced right heart failure undergoing right heart catheterization with a conductance catheter and echocardiography. For details, see the online-only Data Supplement.

Assessment of RV End-Systolic Elastance

The slope of the end-systolic PV relation (end-systolic elastance \([Ees]\)) was calculated as previously described: \(Ees=\frac{(P_{eso}-mPAP)}{SV}\). The isovolumic pressure \((P_{eso})\) was obtained by fitting an inverted cosine wave over the RV pressure curve using the isovolumic contraction period (from end diastole to the point of maximal rate of pressure rise \([dP/dtmax]\)) and the isovolumic relaxation period (from minimal \(dP/dt\) to the start of diastole) by a semiautomatic Matlab R2008a program (The MathWorks, Natick, MA).

RV Histological Analyses

Explanted RV tissue samples were collected from PAH patients undergoing heart/lung transplantation (n=10). Control RV tissue was obtained from nonfailing donors (n=9). Written informed consent was obtained, and the study protocol was approved by the local ethics committees. All samples were immediately frozen and stored in liquid nitrogen.

The degree of RV hypertrophy was analyzed on 5-μm-thick tissue sections stained with antibodies against the extracellular protein laminin (1:200; L9393, Sigma-Aldrich). Minimally, 40 cells per sample were used to calculate cross-sectional area. Cardiomyocytes with nontransversal cross sections were not included in the analysis.

RV fibrosis was determined on 5-μm-thick tissue sections stained with picrosirius red and analyzed under double-polarized light. Images were collected by the use of a Leica DMRB microscope (Wetzlar,
Germany), a Sony XC-77CE camera (Towada, Japan), and a LG-3 frame grabber (Scion, Frederick, MD). For each PAH and control sample, a minimum of 10 pictures obtained from different areas were analyzed. ImageJ for Windows 1.42 software (National Institutes of Health, Bethesda, MD) was used for image analysis, taking the pixel-to-aspect ratio into account. Collagen content was quantified as area percentage of the recorded images under a microscopy magnification of ×20.

**RV Cardiomyocyte Force Measurements**

Tissue pieces were defrosted in relaxing solution, and single cardiac cells were isolated mechanically as described before (7 control and 7 PAH samples). A minimum of 3 cells per sample was measured, and the average total tension, active tension, and passive tension were calculated. Cardiomyocytes were incubated for 5 minutes in relaxing solution containing 1% Triton X-100 to permeabilize membranes. To remove Triton, the cardiomyocyte solution was washed 6 times with relaxing solution, after which a single cell was attached with silicone adhesive between a force transducer and a piezoelectric motor. Force measurements were performed at 1.8- and 2.2-μm sarcomere length in activating solutions with maximal and submaximal calcium concentrations ranging from 1 to 30 μmol/L. After maximal force development in activating solutions, the cell was shortened to 70% of its original length to determine total force development (F_total). A similar shortening was performed in the relaxing solution to record passive tension (F_passive). Active force (F_active) was calculated by subtracting F_passive from F_total. Force values at submaximal [Ca]2+ were normalized to the maximal force value obtained at 30 μmol/L Ca2+ to determine the Ca2+ sensitivity of the myofilaments expressed as EC50, that is, the [Ca]2+ at which 50% of maximal force was obtained. Steady-state F_passive measurements were performed at increasing sarcomere lengths (1.8–2.6 μm).

To determine tension, we corrected for differences in RV cross-sectional area between control and PAH. Individual force values were normalized for the cardiomyocyte width and depth recorded at 2.2-μm sarcomere length.

The contribution of actomyosin interaction to passive tension was determined by incubating skinned cardiomyocytes with the actomyosin inhibitor 2,3-butanedione monoxime (BDM; 25 mmol/L) at 15°C for 10 minutes. After 10 minutes, active tension was measured in maximal activation solution to determine the efficiency of the compound. Subsequently, passive tension was recorded at increasing sarcomere lengths (1.8–2.4 μm) and compared with passive tension recorded before BDM incubation.

**Figure 1.** Increased right ventricular (RV) diastolic stiffness in pulmonary arterial hypertension (PAH) patients. A, Illustration of the methodology. End-diastolic volume (EDV) was combined with end-diastolic pressures (EDP), and end-systolic volume (ESV) was combined with begin-diastolic pressures (BDP). Pressure-volume (PV) relations were constructed by fitting a nonlinear exponential curve through the diastolic PV points using the following formula: P=α(e^β−1), where P is pressure, α is a curve-fitting constant, β is a diastolic stiffness constant, and V is volume. B, Average diastolic PV relations in control and PAH patients show a steeper curvature in PAH patients vs control subjects. C, Diastolic stiffness coefficient (β) measured by the single-beat protocol is significantly increased in PAH patients vs control subjects. D, Noninvasive assessment of RV early (E) and atrial (A) induced peak filling rate (E/A ratio) confirmed increased RV diastolic dysfunction in PAH patients vs control subjects. E, RV diastolic stiffness coincided with increased RV end-systolic elastance (Ees) in PAH patients. Data are presented as means±SEM; n=21 PAH patients, n=7 control subjects. *P<0.05, **P<0.001.
Titin Isoform Composition and Phosphorylation
Frozen RV tissue samples were weighed and pulverized in liquid nitrogen with the use of a mortar and pestle. Tissue powder was solubilized in 8 mol/L urea buffer with dithiothreitol and 50% glycerol solution with protease inhibitors (4x Leupeptin, E-64, and phenylmethylsulfonyl fluoride). Equal dilutions were calculated on the basis of myosin heavy chain content; protein homogenate samples were loaded on custom-made 1% agarose gels. Solubilized human soleus muscle was used as a reference. Gels were washed overnight in presoak solution, stained with Coomassie Blue, and destained. Protein composition was determined with the 1D-Scan software program. Titin N2B, N2BA, degradation products, and myosin heavy chain were quantified, and the titin N2B/N2BA ratio was determined.

To quantify titin phosphorylation, gels were stained for 2 hours with ProQ diamond (Molecular Probes). Thereafter, the gels were washed and subsequently stained with SYPRO Ruby (Molecular Probes).22

Statistical Analyses
Statistical analyses were performed with Prism 5 for Windows (GraphPad Software Inc, San Diego, CA). Normal distribution was tested, and logarithm transformation was performed if necessary. Values of P<0.05 were considered significant.

Changes in patient characteristics and diastolic stiffness were tested for significance with unpaired Student t tests or nonparametric Mann-Whitney U test (RAP, NT-proBNP). The relations between diastolic stiffness and several variables for disease severity (SV, 6-minute walking distance, RAP, and NT-proBNP levels) were tested with the Pearson correlation. To adjust for possible confounding by body surface area, age, treatment duration, and PVR, multivariable regression analyses were performed. Histological data were analyzed using multilevel analysis to correct for nonindependence of successive measurements per patient (MLwiN 2.02.03, Center for Multilevel Modeling, Bristol, UK).15-18,23 Changes in cardiomyocyte maximal tension, Ca2+ sensitivity, and passive stiffness were tested for significance by repeated measures ANOVA followed by the Bonferroni post hoc test.

Results
Assessment of RV Diastolic Stiffness
RV diastolic stiffness was calculated in PAH patients (n=21) and control subjects (n=7). The clinical characteristics of the patients enrolled in this part of the study are described in Table 1. The majority of PAH patients were women (20 of 21) with an average age of 45 years. Control subjects matched in terms of age, sex, and body mass index. Compared with control subjects, PAH patients had significantly increased mPAP and PVR and normal PCWP. RAP and NT-proBNP levels were significantly higher in PAH patients compared with control subjects. Furthermore, RV ejection fraction was lower in PAH patients, as well as CO. PAH patients were in a relatively good functional state (New York Heart Association class II, 17 of 21; 6-minute walking distance comparable to that of control subjects), presumably related to intensive treatment (multiple therapy, 16 of 21).

As shown in the online-only Data Supplement, multiple-beat–derived RV diastolic stiffness (\(\beta_{\text{multiple}}\)) and single-beat–derived RV diastolic stiffness (\(\beta_{\text{single}}\)) were closely correlated in rats (\(R^2=0.94, P<0.001\)). Therefore, we used the single-beat method to calculate RV diastolic stiffness in PAH patients and control subjects. The average PAH patients’ and control subjects’ diastolic curves are presented in Figure 1B. Compared with the control subjects’ curve, the steeper curve for the PAH patients indicates increased stiffness of the myocardium. On average, PAH patients had an almost 2-fold increase in the RV diastolic stiffness parameter \(\beta\) (Figure 1C) and a reduced curve constant \(\alpha\) (Table 2). After the covariance of \(\alpha\) and \(\beta\) was controlled for, RV diastolic stiffness remained significant in PAH patients compared with control subjects (\(V_{20}\); Table 2). Noninvasive assessment of diastolic dysfunction by measures of MRI-obtained E/A ratio confirmed the observed increase in RV diastolic stiffness in PAH patients (Figure 1D). In addition, the RV diastolic stiffness measurements \(\beta\) and \(V_{20}\) were both modestly correlated to E/A ratio (\(r_{\text{E/A versus } \beta}=-0.41; r_{\text{E/A versus } V_{20}}=0.48; \) both \(P<0.05\)). Increased RV diastolic stiffness coincided with increased RV Ees in the same PAH patients (Figure 1E).

<table>
<thead>
<tr>
<th>Table 1. Patient Characteristics</th>
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<td></td>
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<tr>
<td>Age, y</td>
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<tr>
<td>Sex, F/M</td>
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<tr>
<td>BMI, kg/m²</td>
</tr>
<tr>
<td>NYHA class II/III/IV, n</td>
</tr>
<tr>
<td>6MWD, m</td>
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<tr>
<td>mPAP, mm Hg</td>
</tr>
<tr>
<td>CO, L/min</td>
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<tr>
<td>PVR, dynes·s/cm²</td>
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<tr>
<td>RVEF, %</td>
</tr>
<tr>
<td>PCWP, mm Hg</td>
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<tr>
<td>RAP, mm Hg</td>
</tr>
<tr>
<td>HR, bpm</td>
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<tr>
<td>NT-proBNP, pg/L</td>
</tr>
<tr>
<td>Time on treatment, y</td>
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<td>Monotherapy, n/N</td>
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<td>Multiple drug therapy, n/N</td>
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<tr>
<td>Treatment strategies, n/N</td>
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<tr>
<td>Sildenafil</td>
</tr>
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<td>Bosentan</td>
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<tr>
<td>Epoprostenol</td>
</tr>
<tr>
<td>Terugrude</td>
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<tr>
<td>Treprostinil</td>
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<tr>
<td>Sitaxentan</td>
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</tbody>
</table>

Data are presented as mean±SD when appropriate. BMI indicates body mass index; CO, cardiac output; HR, heart rate; mPAP, mean pulmonary arterial pressure; NT-proBNP, N-terminal prohormone brain natriuretic peptide; NYHA, New York Heart Association; PAH, idiopathic pulmonary arterial hypertension; PCWP, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance; RAP, right atrial pressure; RVEF, right ventricular ejection fraction; and 6MWD, 6-minute walking distance.

<table>
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<tr>
<th>Table 2. Diastolic Pressure Volume Parameters</th>
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<tr>
<td></td>
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<tr>
<td>(\alpha)</td>
</tr>
<tr>
<td>(\beta)</td>
</tr>
<tr>
<td>(V_{20}), mL</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. \(\alpha\) indicates curve-fitting constant; \(\beta\), diastolic stiffness constant; and \(V_{20}\), calculated volume at a common pressure of 20 mm Hg based on individually derived \(\alpha\) and \(\beta\) (see the equation in the text).
To investigate whether RV diastolic stiffness is also present in other forms of pulmonary hypertension, we included an additional group of patients with chronic thromboembolic pulmonary hypertension (n=24). Similar to PAH, RV diastolic stiffness was significantly increased in chronic thromboembolic pulmonary hypertension patients compared with control subjects ($\beta$: chronic thromboembolic pulmonary hypertension patients, 0.054±0.005; PAH patients, 0.050±0.005; control subject, 0.029±0.003; $P$<0.05).

RV diastolic stiffness, characterized by the curve constant $\beta$, significantly correlated with PAH disease severity. SV and 6-minute walking distance were significantly and inversely correlated with RV diastolic stiffness, suggesting that increased RV cardiomyocyte stiffness is associated with reduced cardiac function and exercise capacity (Figure 2A and 2B). In addition, RV diastolic stiffness was closely correlated to RAP and NT-proBNP, both markers of increased RV stiffness and wall stress (Figure 2C and 2D). These correlations remained significant after correction for the possible confounding effects of age, sex, body surface area, treatment duration, and PVR (Table 3).

**RV Histology Analyses**

To perform histological analyses, RV tissue samples were obtained from PAH patients (n=10) and control subjects (n=9). Patient characteristics are shown in Table 4. A 2-fold increase in RV cardiomyocyte cross-sectional area in PAH was found compared with control cardiomyocytes (PAH, 531±34 $\mu$m²; control, 256±24 $\mu$m²; $P$<0.001; Figure 3A). In addition, a significant increase in collagen content was found in PAH tissue sections compared with control (PAH: 9.6±0.7%; control, 7.2±0.6%; $P$<0.01; Figure 3B).

**RV Cardiomyocyte Force Measurements**

To investigate the contribution of sarcomeric stiffening on RV diastolic stiffness in PAH, we isolated and membrane-permeabilized single RV cardiomyocytes of RV tissue from PAH patients (n=7) and control subjects (n=7). The advantage of the single RV cardiomyocyte approach is that RV sarcomeric function (the contractile apparatus of the RV cardiomyocytes) can be investigated in detail without the confounding effects of hypertrophy, fibrosis, or calcium handling. First, we investigated overall sarcomeric function in PAH and control RV cardiomyocytes. A similar length-dependent increase in $F_{\text{active}}$ was found in both groups with increasing sarcomere lengths from 1.8 to 2.2 $\mu$m. Interestingly, maximal $F_{\text{active}}$ was higher in PAH compared with control cardiomyocytes at both 1.8 and 2.2 $\mu$m, although the difference was significant only at the 2.2-$\mu$m sarcomere length (Figure 4A). Normalized tension-calcium relations were constructed to determine myofilament Ca²⁺ sensitivity. The length-dependent increase in myofilament Ca²⁺ sensitivity ($\Delta$EC₅₀, ie, the difference between EC₅₀ values at 1.8 and 2.2 $\mu$m) did not differ between PAH patients and control subjects, indicating preserved Frank-Starling mechanism in PAH patients (Figure 4B). No significant changes in Ca²⁺ sensitivity were

**Table 3. Multivariable Linear Regression Corrected for Body Surface Area, Age, Sex, Treatment Duration, and Pulmonary Vascular Resistance**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression Coefficient</th>
<th>95% CI for $\beta$</th>
<th>$R^2$</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroke volume</td>
<td>−2.92</td>
<td>−4.34 to −1.50</td>
<td>0.71</td>
<td>0.001</td>
</tr>
<tr>
<td>6MWD</td>
<td>−11.8</td>
<td>−20.0 to −3.9</td>
<td>0.59</td>
<td>0.009</td>
</tr>
<tr>
<td>RAP</td>
<td>1.01</td>
<td>0.52 to 1.51</td>
<td>0.62</td>
<td>0.001</td>
</tr>
<tr>
<td>Ln NT-proBNP</td>
<td>0.15</td>
<td>0.02 to 0.27</td>
<td>0.59</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Multivariable regression analyses of right ventricular diastolic stiffness (independent variable) and markers of disease severity (dependent variable). Regression coefficients present 0.005-unit change in right ventricular diastolic stiffness. CI indicates confidence interval; Ln NT-proBNP, log-transformed N-terminal prohormone brain natriuretic peptide; RAP, right atrial pressure; and 6MWD, 6-minute walking distance.
observed between PAH patients and control subjects, although the averaged tension-calcium curve was shifted slightly to the left in PAH patients (Figure 4C). Overall, RV cardiomyocytes in PAH had a significantly higher total tension compared with control cardiomyocytes over a broad range of calcium concentrations (Figure 4D).

Second, we determined cardiomyocyte passive tension (measure of sarcomeric stiffness) in relaxing solution at increasing sarcomere lengths (1.8–2.6 μm). A significantly higher cardiomyocyte passive tension at different sarcomere lengths was observed in PAH compared with control cardiomyocytes (200%; Figure 5A). The relative increase in passive tension observed in PAH compared with control is shown in Figure 5B. To determine the role of the actin-myosin interaction component in generating passive tension, RV cardiomyocytes were incubated with BDM, and passive tension was measured before and after incubation; no change in passive tension was observed (Figure 6A–6C), only a reduction in total tension (Figure 6D). This indicates that the increase in RV passive tension in PAH cardiomyocytes is not a consequence of residual actin-myosin interactions but a consequence of increased RV sarcomeric stiffness derived from passive structures (titin).

**Titin Isoform Expression and Phosphorylation**

To investigate the underlying molecular mechanism accounting for RV diastolic stiffness in PAH, we analyzed titin isoform composition and phosphorylation. Titin is a giant sarcomeric protein that regulates sarcomere compliance. Titin consists of 2 isoforms, the stiff N2B isoform and the compliant N2BA isoform. In RV samples of PAH patients and control subjects, we did not observe a difference in the ratio between N2B and N2BA isoform expression (Figure 7A). However, we did observe reduced titin phosphorylation in RV samples of PAH patients (Figure 7B), indicating that the observed RV sarcomeric stiffening was associated with reduced titin phosphorylation.

### Table 4. Patient Characteristics of RV Tissue Samples

<table>
<thead>
<tr>
<th>RV Sample</th>
<th>Diagnosis</th>
<th>NYHA Class</th>
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<tr>
<td>1</td>
<td>Idiopathic PAH</td>
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<td>Female</td>
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<td>IV</td>
<td>Female</td>
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<td>PAH-Eisenmenger</td>
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<td>Female</td>
<td>50</td>
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NYHA indicates New York Heart Association; PAH, pulmonary arterial hypertension; and RV, right ventricular.
Discussion

By combining in vivo measurements of RV function in PAH patients with functional and histological analyses of RV tissue derived from PAH patients, we were able to demonstrate the following:

1. RV diastolic stiffness is increased in PAH patients and is closely associated with markers of disease severity.
2. RV hypertrophy and collagen deposition are increased in RV tissue of PAH patients compared with control subjects.
3. RV cardiomyocyte passive tension at different sarcomere lengths was significantly higher in PAH cardiomyocytes than in control cardiomyocytes; RV cardiomyocytes exhibited preserved length-dependent activation and generated higher total tension compared with control RV cardiomyocytes over a broad range of calcium concentrations.
4. Titin phosphorylation was significantly reduced in RV tissue of PAH patients compared with control subjects.

RV Diastolic Stiffness in PAH

Diastolic dysfunction is characterized by altered filling patterns, prolonged relaxation, and intrinsic diastolic stiffness. Several epidemiological studies have demonstrated elevated RAP in PAH patients. In concordance, RV imaging studies revealed altered RV filling patterns characterized by increased atrium-induced filling (atrial kick). In addition, prolonged RV isovolumic relaxation time has been described in PAH patients. However, previously used measurements of diastolic function are all highly load dependent; therefore, it is still unclear whether PAH patients suffer from true RV diastolic dysfunction.
impairment or whether the observed changes in filling and relaxation are merely a reflection of increased RV afterload.4,25

Therefore, we investigated the presence of RV diastolic impairment in PAH patients both in vivo by single-beat PV analyses and by measuring RV diastolic stiffness directly in RV cardiomyocytes. Diastolic stiffness is ideally quantified from the diastolic PV relationship constructed from multiple PV loops at different loading conditions. As a result of cardio-pulmonary compromise, this procedure is highly undesirable and considered too invasive in PAH. Therefore, we used the single-beat approach, a technique that has been used successfully in studies of left heart failure.5,6 In our experimental PAH model, we observed an excellent correlation between RV diastolic stiffness derived by single- and multiple-beat approaches and therefore considered the single-beat approach an appropriate, less invasive alternative for our patients. In addition, the finding of altered early and atrium-induced RV peak filling rate further confirmed increased RV diastolic stiffness in PAH.

Interestingly, RV diastolic stiffness in PAH coincided with increased RV contractility (Ees) and force-generating capacity of RV cardiomyocytes (active force). This finding is somewhat unexpected because it is well known that PAH is associated with severe RV systolic dysfunction. It is also in contrast to earlier observations of diastolic left heart failure (or heart failure with preserved ejection fraction), in which increased passive stiffness was accompanied by reduced active tension.19 In a previous study in PAH rats, we observed an increase in both diastolic stiffness and RV contractility, consistent with our findings in cardiomyocytes of PAH patients.17,18 However, the increase in RV contractility in rats did not result in an improved RV arterial coupling in rats, suggesting that the increase in RV contractility was insufficient to cope with the higher increase in RV afterload.17 Therefore, the observed increase in force-generating capacity may be a compensatory mechanism attempting to cope with the increased RV afterload.26

Figure 6. Role of actin-myosin interaction in right ventricular (RV) cardiomyocyte passive stiffness. No change in RV passive tension was observed on 2,3-butanedione monoxime (BDM) incubations in control (Con) subjects (A and C) and pulmonary arterial hypertension (PAH) patients (B and C), indicating that the higher passive tension in PAH cardiomyocytes is not a consequence of residual actin-myosin interactions. BDM incubation resulted in a significant reduction in total tension in both PAH patients and control subjects (D). Data are presented as mean±SEM; n=7 PAH, n=7 controls. *P<0.05, ***P<0.001.

Figure 7. Titin isoform composition and phosphorylation. A. Expression of the stiff N2B and compliant N2BA titin isoform was similar in right ventricular (RV) tissue of pulmonary arterial hypertension (PAH) patients and control (Con) subjects, indicating no alterations in titin isoform composition. B. Reduced titin phosphorylation in RV tissue of PAH patients vs control subjects. The typical example of the gel electrophoresis illustrates reduced titin phosphorylation in RV tissue of PAH patients. Data are presented as mean±SEM; n=10 PAH, n=9 controls. *P<0.05.
This compensatory mechanism might negatively affect the normal relaxation pattern. The “hypercontractile” sarcomeres, which are evident after combining the increase in maximal force-generating capacity with the higher myofilament Ca\(^{2+}\) sensitivity and increased passive stiffness (Figure 5D), may limit myocardial relaxation during the diastolic phase and contribute to impaired diastolic function in PAH-induced right heart failure.

**Possible Mechanisms Causing RV Diastolic Stiffness in PAH**

RV diastolic stiffness not only was observed in idiopathic PAH patients but also was prevalent in patients with chronic thromboembolic pulmonary hypertension. This indicates that RV diastolic stiffness is not specific for PAH but could also be expected in other syndromes with increased RV pressures. Thus, increased RV pressure overload could be an initial trigger for RV diastolic impairment in PAH. Nevertheless, other factors also could explain RV diastolic stiffness in PAH in vivo. We observed a 3-fold higher RV sarcomeric stiffness over the whole range of sarcomere lengths in PAH patients compared with control subjects. By repeating RV sarcomeric stiffness measurements after incubation with the cross-bridge inhibitor BDM, we could rule out a contribution of remaining cross-bridge interactions on RV diastolic stiffness. A remaining factor likely to contribute to the high cardiomyocyte stiffness is the sarcomeric protein titin. Titin is a molecular spring that spans the half-sarcomere and determines muscle stiffness in diastole. Phosphorylation and isoform composition of titin determine the elasticity of the protein and thus the passive (diastolic) stiffness of the cardiomyocytes. In this study, we revealed that titin isoform composition was unaltered in PAH cardiomyocytes but that titin phosphorylation was significantly reduced in PAH patients compared with control subjects. In addition, extracellular factors such as RV collagen deposition might contribute to diastolic impairment, although we observed only a relatively modest increase in RV collagen deposition, which is in line with previous preclinical studies.16,17,27

**Clinical Implications**

RV diastolic stiffness was closely associated with markers of disease progression. This finding suggests that RV diastolic stiffness may be a contributing factor involved in disease worsening and not a benign compensatory mechanism associated with increased afterload. Future therapeutic strategies targeting the reduced titin phosphorylation and increased RV collagen deposition will reveal the clinical implication of increased RV diastolic stiffness.

**Study Limitations**

RV diastolic stiffness only weakly correlated with RV peak filling rate. This is comparable to earlier data in patients with heart failure with preserved ejection fraction in whom the direct comparison of E/A ratio (echocardiography) with the diastolic stiffness parameter β (conductance catheterization) showed a similar weak correlation.28 A possible explanation for this finding is that E/A measurements by echocardiography or MRI are highly sensitive to the confounding effects of increased preload and afterload. This also indicates that other factors besides RV myocardial stiffness are associated with the reduction in E/A ratio.

The majority of RV samples used in this study were from patients with PAH secondary to congenital heart disease. RV samples of patients with idiopathic PAH are difficult to procure because these patients often undergo only lung transplantation. There may be important differences in myocardial structure and function between the RV of a formerly normal adult who develops idiopathic PAH and that from a patient with congenital heart disease. However, both idiopathic PAH and congenital heart disease patients were in end-stage right heart failure (New York Heart Association class IV) at the time of heart/lung transplantation. More important, subgroup analyses revealed that the increases in active force and cardiomyocyte stiffness were comparable between RV samples of patients with idiopathic PAH and congenital heart disease.

The sample size of this study was relatively small, which may have led to type I errors; therefore, nominally significant P values should be interpreted with caution. However, our main finding has been confirmed by several clinical and experimental observations. Therefore, RV diastolic stiffness in PAH is not only a statistically significant finding but also physiologically plausible.

**Conclusions**

We demonstrated that patients with PAH have increased RV diastolic stiffness. Furthermore, we observed significant correlations between increased diastolic stiffness and disease severity. We revealed that alterations in the extracellular matrix and cardiomyocyte sarcomeres are important contributors to increased RV diastolic stiffness in PAH patients and may represent future treatment targets.

**Acknowledgments**

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**Disclosures**

None.

**References**

Patients with pulmonary arterial hypertension (PAH) suffer from right ventricular (RV) dysfunction, characterized by reduced RV ejection fraction and low cardiac output. However, little is known about the contribution of RV diastolic stiffness in PAH pathophysiology. In the present study, we could demonstrate that RV diastolic stiffness was significantly increased in PAH patients and was closely associated with markers of disease progression. Increased sarcomeric stiffening in PAH was further explained by posttranslational modification of the giant sarcomeric protein titin. Impaired RV diastolic function is a potentially clinically meaningful and as-yet unrecognized aspect of PAH pathophysiology. Until now, RV diastolic stiffness in PAH patients was difficult to quantify. We have shown that with the single-beat approach, a reliable and relatively simple measure of RV diastolic stiffness could be obtained in the clinical setting. This will provide the opportunity to assess RV diastolic stiffness over time and to explore its diagnostic and prognostic value. In addition, future studies should determine whether RV diastolic stiffness is a compensatory mechanism or whether it accelerates the progression to right heart failure. Finally, therapeutic strategies targeting the reduced titin phosphorylation or increased RV collagen are necessary to study the potential of RV diastolic stiffness as a therapeutic target in PAH.
Right Ventricular Diastolic Impairment in Patients With Pulmonary Arterial Hypertension


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SUPPLEMENT MATERIAL

Right Ventricular Diastolic Impairment in Patients with Pulmonary Arterial Hypertension

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Supplemental METHODS

Single-beat method

**Experimental Model.** To experimentally support the single-beat method we performed studies in a rat model of PAH. All animal experiments were approved by the Institutional Animal Care and Use Committee of the VU University Amsterdam, The Netherlands. The study was performed in 15 Male Wistar rats. Pulmonary Arterial Hypertension (PAH) was induced by a single dose monocrotaline (60mg/Kg) subcutaneously injected (n= 9). Rats used as controls received a saline injection (n=6). The study was ended 31 days after monocrotaline or saline injection or after development of manifest right heart failure.\(^{51}\)

**Echocardiography.** Transthoracic Doppler Ultrasound (ProSound SSD-4000 system equipped with 13MHz linear transducer (UST-5542), Aloka, Tokyo, Japan) was performed in all spontaneously breathing rats under general anesthesia at the end of the study (isoflurane 2.0% in 1:1 O\(_2\)/air mix, Pharmachemie, Haarlem, The Netherlands).

Right ventricular function was measured by the following parameters: Doppler derived stroke volume, cardiac output and tricuspid annular plane systolic excursion (TAPSE). Right ventricular morphology was assessed by the RV end diastolic diameter and RV wall thickness.\(^{52}\)

**Invasive RV pressure-volume analysis.** After transthoracic echocardiography, cardiac function was assessed invasively by performing right heart catheterization with dual pressure – volume catheters. Rats underwent general anesthesia by isoflurane inhalation (induction: 4.0% in 1:1 O\(_2\)/air mix; maintenance: 2.0% in 1:1 O\(_2\)/air mix), were intubated (16G Teflon tube) and mechanically ventilated with a frequency of 75/min, at a pressure of 9/0 cmH\(_2\)O and 1:1 inspiratory/expiratory ratio (Micro-Ventilator, UNO, Zevenaar, The Netherlands). During the procedure body temperature was maintained at normal values by placing the rats on warming pads. The thorax was then open and the inferior vena cava was encircled by performing a lose ligature around its trunk. The apex of the heart was then pierced with a needle (23G), a cotton swap was used to stop the hemorrhage and the combined pressure-volume catheter (SPR-869, Millar Instruments, Houston TX) was inserted into the right ventricle.\(^{53}\)
Pressure-volume loops were recorded at rest and after preload reduction secondary to vena cava gradual occlusion (VCO). Analysis was made off line using custom-made algorithms (programmed in MATLAB 2007b, The MathWorks, Natick MA).

Doppler ultrasound derived stroke volume was used to convert catheter volume units in milliliters. One catheterization unit was calculated to its corresponding volume (ml) by dividing the ultrasound obtained stroke volume (ml) by the catheterization stroke volume (units). Catheterization stroke volume was previously obtained from subtracting the end systolic volume (units) from the end diastolic volume (units). Due to procedure limitations to record absolute RV volumes (End Systolic Volume and End Diastolic Volume), only changes in volume could be measured, all end diastolic volumes were normalized at 1.5ml and end systolic volumes were calculated by subtracting the stroke volume (ml) from the reference point (1.5ml).

To quantify RV diastolic stiffness, multiple pressure-volume loops were recorded with the pressure-volume catheter placed in the right ventricle, both at steady state and during vena cava occlusion. The diastolic pressure-volume relation was then constructed using an exponential fit (Equation 1: \( P=\alpha (e^{\beta V}-1) \)) through the decreasing pressure-volume points (after vena cava occlusion) and the diastolic stiffness factor \( \beta_{\text{multiple}} \) was calculated. The same equation was used to calculate RV diastolic stiffness \( \beta_{\text{single}} \) from a single beat pressure-volume loop (recorded before vena cava occlusion was started, at steady-state). For this exponential fit only 3 points were used: 1) \( 0_{\text{pressure},0_{\text{volume}}} \) point, 2) begin diastolic point and 3) end diastolic point.

The classical pressure-volume relation implies the construction of an exponential pressure-volume curve through decreasing pressure-volume points. Furthermore, the pressure-volume relation is considered to intersect the volume axes at pressure=0mmHg and a certain intercept volume \( (V_d) \). To calculate \( \beta_{\text{single}}, V_d \) was set to 0. Although physiologically inexact, we considered the \( 0_{\text{pressure},0_{\text{volume}}} \) point as a satisfactory substitute for the intercept since:

1. \( 0_{\text{volume}} \) is always lower or equal to \( V_d \)
2. Extending the diastolic exponential pressure-volume curve to volumes lower than the
$V_d$ (undetermined value) does not modify the exponential term $\beta$ (further used to
quantify RV diastolic stiffness). See also Fig.S1

**Supplemental RESULTS**

**RV diastolic stiffness**

RV Diastolic stiffness obtained with the classical method using multiple pressure-volume loops
($\beta_{\text{multiple}}$) was compared to diastolic stiffness obtained from a single beat steady-state loop
($\beta_{\text{single}}$). Since no significant difference was found between the methods (Fig. S2), we further
used the single beat approach for the clinical setting, where vena cava occlusion and multiple
pressure-volume loops recording are contraindicated.

**Right ventricular cardiomyocyte force measurements**

To investigate whether resting sarcomere length was different between PAH and control tissue
samples, we randomly selected 10 isolated RV cardiomyocytes for each control and PAH tissue
sample. Resting sarcomere length was optically determined in at least two distinct areas of the
cell and the average cellular sarcomere length was calculated. No significant difference in resting
sarcomere length was found between control and PAH cardiomyocytes (Fig. S3)

To investigate whether our findings of increased RV active force and cardiomyocyte stiffness
differ between RV samples obtained from patients with idiopathic PAH or PAH secondary to
congenital heart disease, we performed a subgroup analyses. As can be observed in Fig. S4, the
increase in RV active force and RV cardiomyocyte stiffness was comparable among the groups.
Supplemental FIGURE LEGENDS

Figure S1

A&C. Example of control and PAH diastolic pressure-volume relation constructed from multiple pressure-volume loops (after vena cava obstruction). Diastolic stiffness $\beta$ is obtained by fitting an exponential curve (see Equation 1) through multiple decreasing pressure-volume points.

B&D. Example of control and PAH diastolic pressure-volume relation constructed from multiple pressure-volume loops (after vena cava obstruction). Diastolic stiffness $\beta$ is obtained by fitting an exponential curve (see Equation 1) through multiple decreasing pressure-volume points and the pressure=0mmHg – volume=0ml point.

Figure S2

A&B. Example of control and PAH diastolic pressure-volume relation constructed from multiple pressure-volume loops (continuous line) and from a single-beat steady-state pressure-volume loop (dotted line). Diastolic stiffness $\beta$ is obtained by fitting an exponential curve (see Equation 1) through multiple decreasing pressure-volume points ($\beta_{\text{multiple}}$), respectively through the end-diastolic, end-systolic and pressure=0-volume=0 points ($\beta_{\text{single}}$).

C. Method correlation between diastolic stiffness $\beta_{\text{multiple}}$ and $\beta_{\text{single}}$ in control rats and PAH rats.

Figure S3

Right ventricular resting sarcomere length determined in control and PAH isolated skinned cardiomyocytes. p=0.56

Figure S4

Measures of right ventricular active force and stiffness do not differ between right ventricular cardiomyocytes obtained from patients with idiopathic pulmonary arterial hypertension or congenital heart disease.

Data presented as mean ± SEM. iPAH, idiopathic pulmonary arterial hypertension; CHD, congenital heart disease.
Supplemental FIGURES
Figure S1

Control Rat

A. β=1.22±0.07  
R²=0.97

B. β=1.22±0.07  
R²=0.99

PAH Rat

C. β=6.68±0.01  
R²=0.97

D. β=6.68±0.01  
R²=0.99
Figure S2

A. Diastolic Pressure Volume Relation in Control Rat

B. Diastolic Pressure Volume Relation in PAH Rat

C. Diastolic Pressure Volume Relation

\[ \beta_{\text{multiple}} = 2.13 \]
\[ \beta_{\text{single}} = 2.08 \]

\[ \beta_{\text{multiple}} = 10.10 \]
\[ \beta_{\text{single}} = 10.75 \]

\[ R^2 = 0.94 \]
Slope = 1.1 (0.95 - 1.29)

p < 0.001
Figure S3

**RV cardiomyocyte resting sarcomere length**

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S. Rain – RV diastolic impairment in PAH
Figure S4

Active Tension
CHD vs. iPAH

RV cardiomyocyte stiffness
iPAH vs. CHD

Interaction sarcomere length*disease
p=0.417

Interaction sarcomere length*disease
p=0.997
Supplemental REFERENCES


