Cardio-Selective Beta-Blocker Therapy Improves Survival and Cardiac Function in Experimental Pulmonary Hypertension

M.L. Handoko 1,2 * & F.S. de Man 1,2 *, J. Van Balle goij 2, I. Schalij 1,2, P.E. Postmus 1, N. Westerhof 1,2, J. Van der Velden 2, W.J. Paulus 2, A. Vonk-Noordegraaf 1

1 Departments of Pulmonology and 2 Physiology, VU University Medical Center / Institute for Cardiovascular Research, Amsterdam, The Netherlands

* Both authors contributed equally

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ABSTRACT

Background – Pulmonary arterial hypertension (PH) eventually leads to right heart failure. The use of β-blockers is strongly discouraged in PH, because of their acute negative inotropic and chronotropic effects. However, use of β-blockers in chronic (left) heart failure is safe and significantly reduces mortality. We investigated whether chronic low-dose treatment with bisoprolol (a cardioselective β₁-adrenergic receptor antagonist) has beneficial effects on mortality and cardiac function in experimental PH.

Methods and Results – Progressive PH in rats was induced by a single injection of monocrotaline (60 mg/kg). Pressure-telemetry in PH-rats revealed that 10 mg/kg bisoprolol was the lowest dose that blunted heart rate response during daily activity. Ten days after monocrotaline-injection, echocardiography was performed, and PH-rats were randomized for bisoprolol-treatment (oral gavage; n=7/group). At end-of-study (body mass loss >10%), echocardiography was repeated with additional pressure-volume measurements. After euthanization, heart and lungs were harvested for histomorphological analyses.

Echocardiography confirmed PH-status at start-of-treatment. Bisoprolol delayed disease progression and improved survival (p<0.05). Compared to control, RV systolic pressure and arterial elastance (Ea; measure of vascular resistance) more than tripled in PH. RV afterload was unaffected, however bisoprolol-treatment increased RV contractility (Ees) and elastance (Eed; both p<0.01), and partially restored RV ventriculo-arterial coupling (Ees/Ea) and cardiac output (both p<0.05). Histology revealed significantly less RV fibrosis and less RV myocardial inflammation in bisoprolol-treated PH-rats.

Conclusions – In experimental PH, treatment with bisoprolol improves survival, RV ventriculo-arterial coupling and reduces RV diastolic dysfunction. These promising results suggest a therapeutic role for β-blockers in PH that warrants further clinical investigation.
INTRODUCTION

Pulmonary arterial hypertension (PH) is a fatal disease, characterized by progressive vascular remodeling and increased right ventricular (RV) afterload, which eventually leads to manifest right heart failure and premature death. Current available medical treatments aim to reduce RV afterload, thereby secondarily improving RV function. No treatment is currently available that improves RV function directly, partially because it is not considered a therapeutic target in PH.

Recently, several reports have shown that sympathetic activity is increased in patients with PH. Similar to left heart failure (LHF), it was found that signs of sympathetic over-stimulation, such as blunted baroreflex, reduced heart rate variability, increased muscle sympathetic nerve activity, and “ventricle-specific” down-regulation of β₁-adrenergic receptors, are closely related to disease severity in PH.

Although increased adrenergic activity is a compensatory mechanism to maintain cardiac function by increasing contractility and heart rate, it became apparent that chronic adrenergic over-activity has – in the long run – detrimental effects on cardiac function. This supports use of β-adrenergic blockade in LHF-management, which has been demonstrated to significantly reduce mortality and left ventricular (LV) remodeling.

Nevertheless, and notwithstanding the substantial evidence of their beneficial effects in LHF, the use of β-blockers is currently contra-indicated for patients with PH. This recommendation is partially substantiated by the findings of Provencher et al. that within weeks, exercise capacity improved after β-blocker withdrawal. PH-patients are unable to increase stroke volume during exercise, and as a consequence they are presumed to be highly heart rate dependent to raise cardiac output. Furthermore, in an acute model of PH, it was demonstrated that acute right ventriculo-arterial uncoupling occurs after intravenous β-blocker administration.

However, the β-blockers used in these studies were first generation unselective β-blockers, with more bronchial and vascular side-effects. In addition, the dosages used in these studies were relatively high, whereas a low dose could have sufficed and better tolerated. Furthermore, no data is available on the long-term effects of β-adrenergic blockade in PH. This aspect is important, as the typical time-course of improvement by β-blockers in LHF is preceded by initial functional decline, with significant clinical improvement not to be expected before three months after start of therapy.

Finally, we recently demonstrated that exercise training was detrimental in experimental and progressive PH. The deleterious effects could be related to bouts of exercise-induced sympathetic stimulation. The present study therefore assesses if β-blocker therapy, titrated to blunt heart rate response during daily activity, could favorably alter survival, RV function and RV remodeling in experimental PH.
METHODS

All experiments were approved by the Institutional Animal Care and Use Committee of the VU University, Amsterdam, The Netherlands.

Experimental pulmonary arterial hypertension

Male Wistar rats were used (30 in total, 150-175g; Harlan, Horst, The Netherlands). Progressive PH developing right heart failure was induced by a single subcutaneous injection of monocrotaline (60 mg/kg body mass; Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in sterile saline; the control group was injected with saline only.\textsuperscript{15,16}

Part I – Dose-finding by pressure-telemetry

A group of 8 PH-rats was studied to determine the minimal effective dose of bisoprolol that could blunt heart rate response during daily activity. This strategy was motivated by our previous observations that episodes of increased heart rate during exercise had deleterious effects in progressive PH.\textsuperscript{15} Furthermore, a recent meta-analysis demonstrated that the beneficial effects of β-blockers are related to the degree of heart rate reduction and not to the dosage administered, whereas the adverse effects of β-blockers are dose-dependent.\textsuperscript{17}

For this purpose, rats were equipped with an implantable telemetric pressure-transmitter (TA11PA-C40, Data Science International (DSI), St. Paul MN) fitted with a 10-cm long catheter that was placed in the abdominal aorta, as previously described.\textsuperscript{18,19} Telemetry does not only allow continuous recordings of heart rate and systemic blood pressure, free of artifacts like stress or anesthesia, but also informs on (relative) physical activity of the rats, based on changes in signal strength while the rat is moving through its cage. Telemetry-recordings were analyzed off-line, using Dataquest A.R.T. Analysis software (version 4.2, DSI). Rats were given a post-operative 10-day resting-period, ensuring full recovery, indicated by normalization of body mass, heart rate and blood pressure, and return of their normal circadian rhythm.\textsuperscript{18,19}

After full recovery, PH was induced by monocrotaline-injection, and two weeks later their PH-status was confirmed by echocardiography (see below). Three days later, bisoprolol was given once daily for 3 consecutive days by oral gavage, at start of their active phase (i.e. night: 18:00 – 06:00h): 4 PH-rats received 5 mg/kg bisoprolol once daily and the other four received 10 mg/kg. These dosages were based on results from similar pilot-experiments in control rats. The effect of bisoprolol on heart rate, systemic blood pressure and physical activity were evaluated. After these experiments, all rats were euthanized and their organs examined. No additional measurements were performed.
Part II – “Clinical” effects of bisoprolol-treatment in experimental PH

In the second part of the study, 22 rats were included (no telemetry): 8 control rats and 14 rats treated with monocrotaline. Ten days after the (monocrotaline-)injection, PH-rats were randomized for bisoprolol-treatment (PH+biso; 10 mg/kg) or vehicle/water (PH) by oral gavage (n=7/group). Rats were treated for maximally 3 weeks (day 10 until day 31). Rats that showed clinical signs of manifest right heart failure (defined as >10% loss in body mass and/or respiratory distress, cyanosis, lethargy) were euthanized earlier, in keeping with the protocol, approved by the institutional animal care. Manifest right heart failure was the survival endpoint and recorded as an event in the survival analyses.\textsuperscript{15}

Hemodynamic evaluation

Echocardiography

Rats were evaluated by echocardiography 10 days after (monocrotaline-)injection and at end of the study protocol (when manifest right heart failure developed, or 31 days after injection). Transthoracic echocardiographic measurements (ProSound SSD-4000 system equipped with a 13 MHz linear transducer (UST-5542), Aloka, Tokyo, Japan) were performed on anesthetized but spontaneously breathing rats (isoflurane 2.0% in 1:1 O\textsubscript{2}/air mix; Pharmachemie, Haarlem, The Netherlands), as previously described.\textsuperscript{15,19} Analyses were performed off-line (Image-Arena 2.9.1, TomTec Imaging Systems, Unterschleissheim / Munich, Germany). Measured parameters for RV function were: Doppler-derived stroke volume, cardiac output, and tricuspid annular plane systolic excursion (TAPSE). Parameters for RV remodeling were: RV end-diastolic diameter and RV wall thickness. Pulmonary artery acceleration time normalized for cardiac cycle length (PAAT/cl) was used to a non-invasive estimate for RV systolic pressure (PAAT/cl and RVSP are inversely correlated\textsuperscript{15}). Disease progression of PH during treatment-period was expressed as percentage changes in hemodynamics over time, e.g. change in cardiac output:

\[
\Delta CO = \frac{[CO_{\text{END OF PROTOCOL}}] - [CO_{\text{START OF TRAINING}}]}{[CO_{\text{START OF TRAINING}}]} \times 100\% / [\text{days of training}]
\]

Other parameters for disease progression (change in stroke volume, TAPSE, etc) were calculated similarly.

Invasive RV pressure-volume measurements

Catheterization

At end of the study protocol, open-chest RV catheterization was performed (SPR-869, Millar Instruments, Houston TX) under general anesthesia (isoflurane 2.0% in 1:1 O\textsubscript{2}/air mix) in all 22 rats, as previously described.\textsuperscript{15,20} The rats were sedated by inhalation of isoflurane (induction:
4.0% in 1:1 O$_2$/air mix; maintenance: 2.0% in 1:1 O$_2$/air mix), intubated (16 G Teflon tube) and attached to a mechanical ventilator (Micro-Ventilator, UNO, Zevenaar, The Netherlands; ventilator settings: breathing frequency 75/min, pressures 9/0 cmH$_2$O, inspiratory/expiratory ratio 1:1). The rats were placed on a warming pad to maintain body temperature.

After opening of the thorax, a temporal ligature was placed around the inferior vena cava. Following an apical stab (23G), a combined pressure-volume catheter (SPR-869, Millar Instruments, Houston TX) was inserted into the right ventricle and positioned along its long axis. The signals (processed by MPVS-400, Millar Instruments), obtained at steady state (at least 10s) and during transient vena cava occlusion were digitally recorded (2.0 kHz sampling rate; Chart 5.5.6, ADInstruments, Sydney, Australia) and analyzed off-line, using PVAN 3.6 (Millar Instruments) and custom-made algorithms (programmed in MATLAB R2007b, The MathWorks, Natick MA). Stroke volume (in RVU) derived from the conductance signal was calibrated, using stroke volume (in ml) derived from echo-Doppler as external reference.

**Pressure-volume analyses**

Using custom-made algorithms (programmed in MATLAB 2007b, The MathWorks, Natick MA) RV (peak-)systolic pressures and RV end-diastolic pressures (RVEDP) were automatically determined from steady-state measurements, as well as arterial elastance (Ea), a measure for RV afterload \( E_a = \frac{RV \text{-end-systolic pressure}}{RV \text{-stroke volume}} \).

From occlusion-data, end-systolic and end-diastolic elastance (Ees, Eed) were determined, which represent the slope of the end-systolic and end-diastolic pressure-volume relationships, respectively, and are considered load-independent measures for cardiac contractility (Ees) and relaxation (Eed).

The ratio Ees/Ea was calculated as an estimate for ventriculo-arterial coupling, which is considered a measure for cardiac adaptation, in relation to its (after)load.

**Histomorphometric analyses of heart and lungs**

After the final hemodynamic assessment, all 22 rats were euthanized (by exsanguination under isoflurane), and heart, lungs and other major organs were harvested. Lungs were weighed and the left lobe was subsequently filled by 1:1 mix of saline and cryofixative (Tissue-Tek O.C.T. compound, Sakura, Fintek, Europe, Zoeterwolde, The Netherlands), and snapfrozen in liquid nitrogen. The right lobe was used to measure wet/dry lung mass ratio. The heart was perfused, weighted, dissected and snap-frozen in liquid nitrogen.

**Bright-field microscopy**

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) 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.
Cardiomyocyte cross sectional area:
Haematoxylin & eosin (HE)-stained cardiac cryosections (5 μm) were used to determine LV and RV cardiomyocyte cross sectional area (CSA). Cardiomyocyte size for each ventricle was expressed as the average CSA of minimally twenty transversally cut cardiomyocytes at the level of the nucleus, randomly distributed over the ventricles.

Cardiac fibrosis:
The combination of picrosirius red staining (5 μm) and polarized light was used for analysis of cardiac fibrosis. LV and RV fibrosis were expressed as the percentage tissue area positive for collagen, measured over minimally three randomly chosen areas per ventricle.

Relative wall thickness of pulmonary arterioles:
Pulmonary sections (5 μm) were stained with Elastica von Giessen for morphometric analysis of vascular dimensions. Minimally fifty transversally cut pulmonary arterioles, with an outer diameter between 25 and 100 μm, randomly distributed over the lungs, were measured. Relative wall thickness of pulmonary arterioles (PA) was calculated as:

\[
[PA \text{ wall thickness}] = \frac{2 \times [\text{medial wall diameter}]}{[\text{external diameter}]} \times 100\%
\]

Immunofluorescence microscopy
For the analyses of cardiac capillarization and cardiac inflammation, cardiac cryosections (5μm) were incubated for 60 min with primary CD31- (1:35; sc-1506-R, Santa Cruz Biotechnology, Santa Cruz CA) and CD45-antibodies (1:25; sc-53045, Santa Cruz) for capillary density and leukocyte infiltrations, respectively, followed by appropriate secondary antibody staining as well as WGA (glycocalyx) and DAPI (nuclei) counterstaining. Image acquisition was performed on a Marianas digital imaging microscopy workstation (Intelligent Imaging Innovations (3i), Denver CO). SlideBook imaging analysis software (SlideBook 4.2, 3i) was used to semi-automatically quantify the images.

Myocardial capillary density:
Capillary density was expressed as the number of capillaries per section area, measured in at least three randomly chosen areas per ventricle, where cardiomyocytes were transversally sectioned.

Myocardial leukocyte infiltration:
Leukocyte infiltration was expressed as the number of positive CD45-nuclei per section area, measured over minimally three randomly chosen areas per ventricle.
Statistical analysis

All analyses were performed in a blinded fashion. All data were verified for normal distribution. Data are presented as mean±SEM and analyses were performed on all rats, unless stated otherwise. A p-value < 0.05 was considered significant.

Comparison of telemetric-registrations of PH-rats before and after bisoprolol-treatment was performed by two-way analysis of variance for repeated measurements, the interaction between bisoprolol-treatment and time was tested and reported. One-way analysis of variance was used for the analyses of disease progression, pressure-volume relation and autopsy data, with Bonferroni post-hoc comparison between PH-rats with and without bisoprolol-treatment. Survival estimates were performed by Kaplan-Meier analysis, with post-hoc comparison performed by log-rank test between PH-rats with and without bisoprolol-treatment; Hazards ratio was calculated by the proportional hazards model (SPSS 16.0 for Windows, SPSS, Chicago IL; Prism 5 for Windows, GraphPad Software Inc, San Diego CA).

For the histological data, multilevel analysis was used to correct for the non-independence of successive measurements per animal (MLwiN 2.02.03, Center for Multilevel Modelling, Bristol, UK).15,23

RESULTS

Part I – Minimal effective dose of bisoprolol in PH-rats

Echocardiography confirmed the PH-status of all 8 rats at day of bisoprolol-administration (reduced PAAT/cl, increased RV wall thickness). The effects of 5 and 10 mg/kg bisoprolol were tested: only 10 mg/kg was able to completely blunt heart rate response during daily activity completely (Figure 1A). At a dose of 10 mg/kg, systemic blood pressure (-6.1±3.1 %, n.s.) and physical activity (-1.7±1.3 %, n.s.) were minimally affected, which indicates that this dosage was well-tolerated by PH-rats (Figure 1B,C).

Figure 5.1 Averaged 24-hr registration of PH-rats before and after 10mg/kg bisoprolol

This dosage was able to completely blunt heart rate response during the whole active phase of the rats (A: grey area). In addition, only a moderate effect on systemic blood pressure was observed (B), without an (adverse) effect on daily physical activity (C). This indicates that 10 mg/kg bisoprolol was well-tolerated by the PH-rats.

Data presented as mean±SEM, n=4 (PH / PH+biso). P-values represent interactive term (time*treatment). Abbreviations: PH (solid/black line), vehicle-treated PH-rats; PH+biso (dotted/grey line), bisoprolol-treated PH-rats.

A

B

C
From these experiments, 10 mg/kg bisoprolol once daily was considered the minimal effective dose, and was used for the second part of the study.

Part II – Effects of 10 mg/kg bisoprolol in established PH

***Bisoprolol delayed the progression towards right heart failure***

Ten days after (monocrotaline-)injection, echocardiography in all 14 monocrotaline-treated rats revealed lower PAAT/cl (indicating higher RV systolic pressure\(^{15}\)) and higher RV wall thickness, indicating (moderate) RV hypertrophy (Figure 2A,B). The PH-state before start of bisoprolol-treatment was thereby confirmed in all monocrotaline-treated rats. At this point, no signs of cardiac dysfunction or dilatation were present yet (measured by cardiac output, TAPSE and RV end-diastolic diameters; Figure 2C and Figure S-1).

**Figure 5.2** Confirmed PH status at start of treatment

Before treatment-randomization, all monocrotaline-treated rats (PH, PH+biso: all still bisoprolol-naive) had developed increased pulmonary artery pressures (indicated by lower PAAT/cl; A), and RV hypertrophy (B), without signs of RV dilatation (C), compared to control rats. Data presented as mean±SEM, control: n=8, PH / PH+biso, n=7. \(^{***}\): p<0.001 vs. control. Abbreviations: PAAT/cl, pulmonary artery acceleration time normalized for cardiac cycle length.

Compared to vehicle-treated PH-rats, bisoprolol (PH+biso) improved survival (Figure 3); Even though eventually all PH-rats developed right heart failure, it was significantly delayed by bisoprolol-treatment. This finding was confirmed by serial echocardiography that was used to assess the effects of daily bisoprolol-treatment on disease progression in PH (Figure S-1, Table S-1); Bisoprolol significantly delayed the progression of RV dilatation and reduced the decline in cardiac function, whether measured by TAPSE or cardiac output (ΔRV end-diastolic diameter, ΔTAPSE, Δcardiac output; all p<0.05).

At end-of-study, cardiac function was partially maintained by bisoprolol-treatment (TAPSE, PH+biso 2.4±0.2 vs. PH: 1.4±0.1 mm, p<0.001; cardiac output, PH+biso: 34±1.9 vs. PH 17±1.6 ml/min, p<0.001; also Figure S-1D,E). No differences were observed in RV wall thickness and RV dilatation between bisoprolol- and vehicle-treated PH-rats (Figure S-1B,C).

***Bisoprolol improved cardiac function, without effecting RV afterload***

RV pressure-volume measurements at end-of-study (Figure 4A-C) revealed that RV systolic pressures were significantly elevated in PH-rats compared to control, but no difference was
found between bisoprolol- and vehicle-treated PH-rats (Figure 4D), which is in line with previous echo-findings (Figure 5A,B). Ea (measure of vascular resistance) was also elevated in PH, but again, no difference was observed between bisoprolol- and vehicle-treated PH-rats (Figure 4E). This indicates that bisoprolol-treatment did not affect RV afterload. This finding was confirmed by the equal increase in (wet) lung mass, observed during autopsy, and comparable remodeling of the pulmonary arteries, observed during histological examination (Figure 5A,B; Table S-2).
On the other hand, bisoprolol-treatment significantly increased RV contractility, as measured by Ees (Figure 4F), resulting in partial normalization of the ventriculo-arterial coupling (Ees/Ea; Figure 4G), which is in line with previous echo-findings (Figure S-1D,E). Of note, after normalization of Ees for RV mass, no significant difference was observed anymore between vehicle-treated PH-rats and controls, whereas the difference in contractility between bisoprolol-treated PH-rats and controls remained significant (Table S5-1).
prolol- and vehicle-treated PH-rats remained statistically significant (Ees/RVmass, control: 40.3±6.3, PH: 43.6±12.0, PH+biso: 99.0±10.9 mmHg/ml/g; p=0.02 PH+biso vs. PH). This implies that the increase in RV contractility in PH-rats (vehicle-treated compared to controls) was primarily attributed to RV hypertrophy and remodeling, whereas bisoprolol-treatment further improved RV contractility by enhancement of intrinsic contractile properties of the right ventricle. Furthermore, bisoprolol-treatment reduced RV diastolic dysfunction, demonstrated by a decrease in RV end-diastolic pressures and end-diastolic elastance (Eed; Figure 4H,I). Thus, bisoprolol selectively improved cardiac function in PH-rats, by improving both systolic and diastolic properties of the heart.

**Bisoprolol reduced RV fibrosis and RV myocardial inflammation**

In line with previous echo-findings (Figure S-1B), the right ventricles of PH-rats at end of study protocol were hypertrophied, compared to controls. No differences were observed between bisoprolol- and vehicle-treated PH-rats, whether expressed as RV mass (irrespective
Table S5-2 Autopsy data

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>PH (n=7)</th>
<th>PH+biso (n=7)</th>
<th>p-value</th>
<th>Control vs. PH</th>
<th>PH vs. PH+biso</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (g)</td>
<td>337 ±6</td>
<td>234 ±4</td>
<td>245 ±5</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>BMchange (%/2d)</td>
<td>1.4 ±0.2</td>
<td>-6.1 ±0.9</td>
<td>-6.5 ±0.7</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Tibia length (mm)</td>
<td>36.5 ±0.4</td>
<td>32.1 ±0.4</td>
<td>32.7 ±0.5</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Lungs/tl (g/mm*1000)</td>
<td>33.6 ±1.1</td>
<td>66.8 ±3.0</td>
<td>68.3 ±5.4</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Lung wet/dry ratio</td>
<td>4.7 ±0.1</td>
<td>4.6 ±0.1</td>
<td>4.4 ±0.1</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Heart/tl (g/mm*1000)</td>
<td>34.6 ±0.8</td>
<td>39.0 ±1.4</td>
<td>37.7 ±1.6</td>
<td>&lt;0.05</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>RV mass/tl (g/mm*1000)</td>
<td>5.2 ±0.3</td>
<td>8.8 ±0.3</td>
<td>8.9 ±0.4</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>LV mass/tl (g/mm*1000)</td>
<td>21.9 ±0.7</td>
<td>16.9 ±0.6</td>
<td>16.0 ±0.6</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>RV/(LV+S)</td>
<td>0.24 ±0.02</td>
<td>0.53 ±0.03</td>
<td>0.56 ±0.02</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Liver/tl (g/mm*1000)</td>
<td>367.3 ±8.3</td>
<td>249.4 ±9.7</td>
<td>271.2±10.8</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Spleen/tl (g/mm*1000)</td>
<td>17.5 ±0.5</td>
<td>17.4 ±1.7</td>
<td>16.5 ±1.4</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
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<tr>
<td>Kidneys/tl (g/mm*1000)</td>
<td>60.7 ±1.6</td>
<td>50.0 ±2.0</td>
<td>52.4 ±1.7</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Data presented as mean ±SEM. Abbreviations: BMchange, percentage change in body mass of the last 2 days; …/tl, organ mass normalized for tibia length.

Figure 5.6 Bisoprolol-treatment reduced RV fibrosis and RV inflammation

Histomorphometric analyses revealed significant and selective increase of interstitial fibrosis and myocardial inflammation in the RV myocardium of PH-rats, compared to control (B,C). No difference was observed for RV capillary density between bisoprolol- and vehicle-treated PH-rats (A).

Typical examples are shown of histological sections of the right ventricle of vehicle- (PH: D-F) and bisoprolol-treated PH-rats (PH+biso: G-I), stained for RV capillarization (D,G: endothelin marker CD31 is stained green, cell membranes red; capillaries appear as small yellow/orange dots), fibrosis (E,H: picrosirius red staining, dark grey), and infiltrating inflammatory cells (F,I: lymphocyte-marker CD45 is stained green, cell membranes red, nuclei blue).

Data presented as mean±SEM, control: n=8; PH / PH+biso: n=7. *: p<0.05 PH+biso vs. PH. Abbreviations: Cap., capillaries.
The findings for RV capillary density were similar; compared to control, capillary density was reduced in PH, without a significant difference between the two PH-groups (Figure 6A, D, G).

More interstitial fibrosis was observed when comparing right ventricles of PH-rats and controls; Interestingly, bisoprolol-treatment significantly reduced RV fibrosis (Figure 6B, E, H). Moreover, the presence of (CD45−)-inflammatory cells in RV myocardium of bisoprolol-treated PH-rats was significantly less, compared to vehicle-treated PH-rats (Figure 6C, F, I). Leukocyte infiltration was not observed in the left ventricle: LV values for all groups were low and comparable to control-values of the right ventricle (Table S-3).

### DISCUSSION

To the best of our knowledge, this is the first study that investigated the effects of bisoprolol-treatment in experimental PH, focusing on RV function and remodeling. Using a comprehensive set of physiologic and pathologic endpoints, we have demonstrated that:

1) Chronic low-dosed bisoprolol-treatment was well-tolerated, and delayed the progression towards right heart failure;

2) Bisoprolol-treatment improved cardiac function, by improving RV contractility (Ees), relaxation (Eed), and ventriculo-arterial coupling (Ees/Ea);

3) The cardiac-selective effects of bisoprolol can be attributed to the reduction of RV (interstitial) fibrosis and RV myocardial inflammation.

These results suggest a potential role for β-blockers in PH that warrants further clinical investigation.

Bisoprolol-treatment was well-tolerated

Beta-blockers are currently contra-indicated, because PH-patients are believed not to tolerate the acute (but transient) negative inotropic and chronotropic effects.1,10,13 To address this legitimate argument, we used an approach that was inspired by successful β-blocker use in left heart failure.
Low vs. high dose.

By definition, patients with left heart failure are hemodynamically compromised, and like in PH, their adrenergic system is over-activated as well. To some extent, these two patient-groups are therefore comparable. Interestingly, most left heart failure patients (approximately 85%) enrolled in clinical trials with β-blockers, were able to tolerate short- and long-term treatments with this drug and reached the maximum planned target dose, when β-blockers are introduced at a very low (sub-therapeutical) dose followed by gradually dosage-increase (“start low, go slow”). In addition, whereas the adverse effects of β-blockers are dose-dependent, the beneficial effects are associated with heart rate reduction, which can be achieved by lower dosages. In this study, we used the minimum dose that effectively blunted heart rate response. This was accompanied by only minimal side-effects, and was therefore well-tolerated by the PH-rats (minor effect on blood pressure, no effect on activity). Compared to other rat-studies that used bisoprolol (typically 60 mg/kg), the dose used in this study can be considered low.

Selective vs. unselective β-blocker.

Of all β-blockers, only bisoprolol, carvedilol and (sustained released) metoprolol have been proven to reduce mortality in left heart failure. Of these three, bisoprolol is the most β₁-cardioselective β-blocker. We chose bisoprolol to avoid potential harmful effects of β₂-mediated blockade, as adopted from recent positive experience with cardioselective β-blockers in patients with asthma / COPD, whom were always believed not to tolerate β-blockers either. The β₂-subtype is the predominant β-adrenergic receptor present in the pulmonary vasculature. Blockade of the β₂-receptors may lead to smooth muscle contraction, which could result in a further increase in pulmonary vascular resistance and RV pressures. Selectivity of bisoprolol for the β₁-adrenergic receptor might well explain the absence of any (detrimental) effects on RV afterload and pulmonary vascular remodeling, observed in our study.

Previous observations of poor tolerability to β-blocker by PH-patients might be related to the use of relatively high-dosed unselective β-blockers. Whether the careful approach used in this study also holds in the clinical situation, remains to be validated.

Beneficial effects of bisoprolol

To ease the clinical interpretation of our findings, we used robust and clinical relevant outcome measures to investigate the effects of bisoprolol. We explicitly evaluated pressure-volume relations, because it is considered the gold standard to describe cardiac function, and more specifically, to address the potential risk of ventriculo-arterial uncoupling after β-blocker use in PH, as raised by others. Key observations of this study are that: careful bisoprolol-treatment in experimental PH improved survival, as well as systolic and diastolic function of the right ventricle. Furthermore, in contrast to what was feared, we observed
partial normalization of the ventriculo-arterial coupling, which may be related to chronic opposed to acute drug administration.

Only a few studies have evaluated the (chronic) effects of β-blockers in the context of PH. Usui et al. investigated the effect of carvedilol in monocrotaline-treated rats. They also observed survival benefit with β-blocker, but unfortunately they did not report any measures on cardiac function, and focussed mainly on LV rather than RV remodeling. Also, no information was provided on possible side-effects and tolerability. Recently, Ishikawa et al. reported beneficial effects of arotinolol (an aspecific β-blocker) using the same PH-rat model. However the clinical implications of this study are limited: arotinolol was studied to prevent rather than to treat PH-associated right heart failure, and, unlike bisoprolol, arotinolol is not clinically used or FDA-approved.

There are interesting similarities between our findings (on survival, RV contractility, RV relaxation) and the well-described effects of β-blockers in left heart failure. The CIBIS-II trial convincingly demonstrated beneficial effect of bisoprolol on survival in left heart failure, adding to earlier observations of improved LV function from the preceding trial. Using a “pressure-volume relationship”-like approach, Maurer et al. could demonstrate that for heart failure patients, increase in LV contractility was one of the underlying mechanisms of improved ejection fraction with carvedilol. Furthermore, experimental and clinical studies in left heart failure previously reported beneficial effects of β-blockers on LV diastolic function. The observations of our study are therefore in line with and extend earlier observations on the positive cardiac effects of β-blockers in left heart failure.

Potential mechanisms

In this proof-of-concept study, we did not perform in-depth analysis on cellular and molecular effects of bisoprolol. Nonetheless, our histological analysis may provide some mechanistic insights, based on the experiences with β-blockers in left heart failure.

Although the β-adrenergic system in (left) heart failure is not completely understood, it is well-accepted that the therapeutic effects of β-blockers are (mainly) attributed to blocking of the detrimental consequences of sustained β₁-receptor stimulation. Cardiac over-expression of β₁-receptors in transgenic mice causes cardiomyocyte hypertrophy, followed by (interstitial) fibrosis, myocardial infiltration of inflammatory cells, and eventually heart failure. In line with these findings, we too observed cardiomyocyte hypertrophy, interstitial fibrosis and inflammatory cells in RV myocardium of PH-rats, and reduction of fibrosis and inflammation by bisoprolol-treatment.

A complementary mechanism underlying the therapeutic effects of beta-blockers in heart failure is the resensitization of the cardiac β-receptor system. Beta-blockers can restore the cAMP/PKA signalling-pathway, which results in normalization of PKA-mediated phosphorylation of regulatory proteins, involved in sarcomere contraction and calcium-handling, that are essential for cardiac systolic and diastolic function.
We previously observed that exercise increased myocardial inflammation in experimental PH, and related this to increased RV wall stress during episodes of activity, comparable to what has been described in detail by Sun et al. Prevention of sustained high levels of RV wall stress by heart rate reduction might therefore be an alternative explanation for the observed beneficial effects of bisoprolol therapy.

Future studies are necessary that investigate the relevance of the proposed mechanisms for PH.

Clinical relevance
The model of PH induced by monocrotaline does not fully replicate the pathophysiology and resulting pulmonary and cardiovascular effects of clinical PH. Therefore, this study should be viewed as a seminal analysis of β-blocker therapy in progressive PH from which other (clinical) studies should arise. Nevertheless, this model exhibits alterations in the β-adrenergic system that resemble those in human PH; others have previously demonstrated that in monocrotaline-treated rats with right heart failure, heart rate variability is reduced, plasma norepinephrine levels are increased and β1-adrenergic receptor density of the right ventricle is decreased, similar to clinical PH.

The findings of this study therefore provide a rationale to investigate the role of (cardioselective) beta-blockers as an add-on therapy in the management of clinical PH.

Conclusions
In our PH-rat model, we demonstrated that bisoprolol-treatment was well-tolerated and beneficial. It delayed the progression towards right heart failure, which was attributed to improved RV contractility and compliance, and accompanied by reduced RV fibrosis and inflammation. Future studies are necessary to address the clinical implications of our findings.
REFERENCES


6

Specific Diaphragm Muscle Weakness in Pulmonary Hypertension

F.S. de Man 1,2, H.W.H. van Hees 4, M.L. Handoko 2, H.W. Niessen 3, I. Schalij 1,2, P.E. Postmus 1, N. Westerhof 1,2, G.J.M. Stienen 2, W.J. van der Laarse 2, A. Vonk-Noordegraaf 1, C.A.C. Ottenheijm 2

1Departments of Pulmonology, 2Physiology and 3Pathology, VU University Medical Center / Institute for Cardiovascular Research, Amsterdam, The Netherlands
4Department of Pulmonary Diseases, Radbout University Nijmegen Medical Center, The Netherlands.

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**ABSTRACT**

*Rationale* – Recently it was suggested that patients with pulmonary arterial hypertension (PH) suffer from inspiratory muscle dysfunction. However, the nature of inspiratory muscle weakness in PH remains unclear.

*Objectives* – In the present study we assessed whether alterations in contractile performance and in morphology of the diaphragm, the main muscle of inspiration, underlie inspiratory muscle weakness in PH.

*Methods* – PH was induced in Wistar rats by a single injection of monocrotaline (60 mg/kg). Diaphragm (PH n=8; controls n=7) and extensor digitorum longus (EDL, PH n=5; controls n=7) muscles were excised for determination of *in vitro* contractile properties and cross sectional area (CSA) of the muscle fibers. Furthermore, we compared the CSA of diaphragm and skeletal muscle fibers from two PH patients with those from two controls.

*Main results* – In PH rats, twitch and maximal tetanic force generation of diaphragm strips were significantly lower, and the force-frequency relation was shifted to the right in comparison with controls. Diaphragm fiber CSA was significantly smaller in PH compared with control rats. No significant differences in contractility and morphology of EDL muscle fibers were found between PH and control rats. In line with the data from PH rats, the studies on PH patients revealed significantly reduced CSA of diaphragm muscle fibers compared with controls, with no changes in quadriceps muscle.

*Conclusions* – Selective diaphragm muscle fiber weakness and atrophy were observed in PH rats. Importantly, similar findings were observed in the diaphragm muscle from two patients with PH.
INTRODUCTION

Pulmonary Hypertension (PH) is characterized by excessive pulmonary vascular remodelling, resulting in high pulmonary artery pressures.\(^1\) Eventually, the right ventricle can not adapt to the increased afterload and PH patients die as a consequence of overt right heart failure.\(^2\)

Recently it was speculated that patients with PH suffer from inspiratory muscle dysfunction.\(^3\) This speculation was based on the notion that in PH the inspiratory muscles are weakened and subjected to increased activity. The notion that in PH the inspiratory muscles are more active comes from the observation that PH patients hyperventilate during exercise, but also at rest, and even during sleep.\(^3-5\) This continuous hyperventilation increases respiratory frequency and places an increased demand on the inspiratory muscles. Inspiratory muscle weakness in PH was suggested by recent studies showing that volitionally assessed maximal inspiratory mouth pressures,\(^3\) and non-volitionally assessed transdiaphragmatic pressures during bilateral anterior magnetic phrenic nerve stimulation\(^6\) were markedly lower in PH patients compared with control subjects. Thus, as postulated previously by Naeije\(^7\), patients with PH need to breath more with weaker inspiratory muscles.

This imbalance between the demand placed on the inspiratory muscle on the one hand and the impaired capacity to generate force on the other hand is likely to contribute to the reduced exercise capacity and quality of life of PH patients.\(^6\) In addition, inspiratory muscle weakness is an important determinant of dyspnoea, a symptom which affects the majority of PH patients.\(^8\)

The nature of inspiratory muscle weakness in PH remains to be elucidated. The capacity to generate negative intrathoracic or transdiaphragmatic pressure is an indirect measure of inspiratory muscle strength: the outcome depends not only on muscle fiber function, but on central drive, nerve function, and neuromuscular transmission as well.

In the present study we tested the hypothesis that alterations in contractile performance and in morphology of the diaphragm, the main muscle of inspiration, underlie inspiratory muscle weakness in PH. Obtaining diaphragm muscle biopsies from living PH patients is hampered by ethical difficulties. Therefore, to test our hypothesis, we determined the contractile performance and the morphology of diaphragm muscle fibers in an experimental rat model for PH. To study whether changes in the rat model for PH extrapolate to clinical PH, we performed morphological analyses on two diaphragm specimens from patients who died from PH.
METHODS

This study was approved by the Institutional Animal Care and Use Committee at the VU University and the ethics committee of the VU University Medical Center, Amsterdam, The Netherlands.

Experimental pulmonary hypertension

Male Wistar rats were used (150-175g; Harlan, Horst, the Netherlands). Pulmonary hypertension was induced by a single subcutaneous injection of 60 mg/kg monocrotaline (MCT; Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in sterile saline. The control group was injected with saline only. When right heart failure developed (defined as > 5% loss of body mass per day, and/or respiratory distress, cyanosis, lethargy), animals were hemodynamically evaluated by echocardiography and invasive right ventricular (RV) pressure measurements, as described before. Subsequently, diaphragm and extensor digitorum longus (EDL) muscle were excised for determination of contractile performance and morphology as described below.

Quantification of breathing frequency by telemetry

Breathing gives small variations in intra-thoracic pressures, which can be detected as small baseline fluctuations in RV pressure recordings. We used these fluctuations in RV pressure recordings to determine the breathing frequency in freely moving rats. For this purpose, 10 rats (5 PH, 5 control) were equipped with a radio-transmitter fitted with a 10-cm long catheter (TA11PA-C40, Data Science International, St. Paul MN, USA), as previously described in detail. Pressure recordings were analyzed off-line, using Dataquest A.R.T. Analysis version 4.20 (DSI).

Intact muscle contractility measurements

For intact muscle experiments, diaphragm (8 PH, 7 control) and EDL (5 PH, 7 control) muscles were quickly dissected and mounted vertically in a tissue bath between a dual-mode lever arm and a fixed hook (1200A Intact Muscle Test System, Aurora Scientific Inc, Canada) as described before. Muscles were bathed in continuously oxygenated (95% O₂, 5% CO₂) mammalian Ringer solution with pH 7.40 at 30°C, and stimulated directly by using platinum electrodes placed in close apposition to the muscle. Muscle preload force was adjusted until optimal fiber length for maximal force was achieved, and the stimulation current was adjusted for maximal force generation.

Twitch and tetanus force:

First a single twitch was induced followed, after 20 seconds, by a 2 second tetanus using a stimulation frequency to induce maximal tetanic force (100 Hz for diaphragm, 200 Hz for EDL). From these data maximal twitch and tetanic force were determined.
**Force frequency protocol:**

Five minutes after completion of the twitch and tetanus measurements, the muscle was stimulated at various incremental stimulation frequencies (Diaphragm: 1, 5, 10, 20, 30, 40, 60, 80, 100 Hz; EDL: 1, 5, 10, 20, 40, 60, 80, 100, 150, 200 Hz).

After completion of the contractility measurements, length and weight of the muscle preparations were determined. Cross-sectional area (in mm²) was calculated by dividing muscle weight (g) by muscle length (cm) multiplied by specific density (1.056 g/ml) x 100. Force was normalized to muscle cross-sectional area (in mN/mm²).

**Histological analyses**

Antibody clones brd-5 and sc-71 were used to assess cross sectional area (CSA) of slow and fast muscle fibers, respectively, as previously described. In short, cryosections (10 µm thick) of 10 rats (5 PH, 5 control) were rehydrated for 10 minutes in phosphate buffer and subsequently blocked with phosphate buffer containing 0.3% (w/v) bovine serum albumine. Subsequently, cryosections were incubated with primary brd-5 (slow) or sc-71 (fast) antibody, followed by appropriate fluorescent ALEXA labeled secondary antibodies (Molecular Probes, Eugene, Oregon, USA). Following each incubation, cryosections were washed three times for 5 minutes with phosphate buffer. Finally, the sections were embedded in Mowiol (10% (w/v) in 0.1M Tris-HCL, pH 8.5 / 25% (v/v) glycerol / 2.5% (w/v) NaN₃). Image acquisition was performed on a Marianas digital imaging microscopy workstation (Intelligent Imaging Innovations (3i), Denver CO). SlideBook imaging analysis software (SlideBook 4.2, 3i) was used to quantify the images. CSA of 50 cells per rat/patient were quantified.

**Analysis of proteasome activity**

Proteasome activity was determined as described previously. ¹³-¹⁵ In short, the proteolytic activity of 20S proteasomes was determined by measuring the activity against the fluorogenic substrates succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY) and N-carbenzoxo-Leu-Leu-Glu-7-amido-4-methylcoumarin (LLE) (Sigma). The generation of the fluorogenic cleavage product (amido-4-methylcoumarylamide) was measured at 380 nM excitation wavelength and 440 nM emission wavelength with a spectrophotometer. Standard curves were established for the fluorogenic product, and peptidase activity was expressed as picomoles per microgram protein per minute. Addition of MG-132 (a specific proteasome inhibitor) to the reaction resulted in complete inhibition of amido-4-methylcoumarylamide production, indicating the successful isolation of proteasomes without the presence of significant amounts of other proteases.

**Analysis of proteasome content and Akt phosphorylation levels**

For determining proteasome content and protein kinase B (Akt) phosphorylation levels diaphragm samples were homogenized in 100 volumes 62.5 mM Tris-HCl (pH6.8), 2% SDS,
10% glycerol, 50 mM DTT, 0.01% Brome phenol blue and protease and phosphatase inhibitor cocktails (Sigma Aldrich, Zwijndrecht, the Netherlands). Homogenates were centrifuged at 10,000 \( g \), 4°C for 10 min. The resulting supernatants were subjected to routine Western blotting using polyacrylamide SDS-gels and specific antibodies: anti-20S proteasome subunit C8 (Affiniti, Gorinchem, the Netherlands), anti-Akt and anti-phospho-Akt (Ser473) antibodies (Cell signaling Technology Inc., Beverly, MA, USA), anti GAPDH (Cell signaling Technology Inc., Beverly, MA, USA) was used to check for equal loading of the lanes. After washing, proteasome blots were incubated with an IR Dye-800-labeled goat anti-mouse secondary antibody (LiCor Biosciences, Bad Homburg, Germany) and subsequently scanned and quantified using Odyssey Infra-red Laser Scanner and software (LiCor Biosciences, Bad Homburg, Germany). Blots for analysis of Akt and phospho Akt levels were incubated with anti rabbit peroxidase (Pierce, Rockford, IL, USA) for subsequent chemiluminiscent detection (GE Healthcare, Zeist, the Netherlands) and quantification with optical densitometry software (GeneTools, Syngene, UK).

Diaphragm and quadriceps biopsies of patients with pulmonary hypertension

Biopsies were obtained by autopsy from two patients who died from right heart failure secondary to severe pulmonary arterial hypertension at the age of 84 and 58 years respectively (PH1: female, echo-derived tricuspid insufficiency peak gradient (TIPG): 50 mmHg (guidelines for diagnosis of PH\textsuperscript{16}: TIPG ≥ 32.1 mmHg); PH2: female, catheterization-derived mean pulmonary artery pressure (mPAP) 49 mmHg (guidelines for diagnosis of PH\textsuperscript{16}: mPAP > 25 mmHg)). For controls, we used specimens of two subjects who died of sudden cardiac arrest, without a history of pulmonary, cardiac or muscle disease (CON1: female, 71 years old; CON2: male, 59 years old). Tissue slides were stained for haematoxylin & eosin (HE) to assess CSA of the muscle fibers.

Statistical analyses

All data are presented as mean ± SEM and were verified for normal distribution. A p-value < 0.05 was considered significant.

Differences between control and PH rats in hemodynamics, diaphragm contractile measurements and molecular alterations were analyzed by an independent t-test. Two way repeated measure ANOVA was used to analyze the differences in respiratory rate and force frequency between control and PH, with Bonferroni post-hoc analyses. Muscle fiber cross sectional area was analyzed by multilevel analyses to correct for non-independence of successive measurements\textsuperscript{9,17}. 
RESULTS

General characteristics of pulmonary hypertensive rats

Compared with controls, rats with PH had clear signs of pulmonary vascular remodeling (increased RV systolic pressure and pulmonary vascular resistance), RV dysfunction (decreased cardiac output, tricuspid annular plane systolic excursion) and increased RV remodeling (increased RV wall thickness, RV end-diastolic diameter) (Table 1). In addition, at end of protocol, breathing frequencies of PH rats were significantly elevated in comparison with controls (CON: 82.4 ± 2.2 vs. PH: 157.8 ± 8.2 rpm, p<0.001; Figure 1).

Table 6.1 Hemodynamic characteristics of rats with Pulmonary Hypertension

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pulmonary Hypertension</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td><strong>Catheterization</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RVSP (mmHg)</td>
<td>28.1 ±1.2</td>
<td>70.6 ±4.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RVEDP (mmHg)</td>
<td>3.6 ±0.4</td>
<td>8.8 ±1.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RVWT (mm)</td>
<td>0.87 ±0.02</td>
<td>1.34 ±0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RVEDD (mm)</td>
<td>3.68 ±0.05</td>
<td>7.31 ±0.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CO (mL/min)</td>
<td>102 ±9</td>
<td>32 ±5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TAPSE (mm)</td>
<td>3.8 ±0.1</td>
<td>1.7 ±0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PVR (mmHg/ml/min)</td>
<td>0.33 ±0.05</td>
<td>2.05 ±0.27</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

All monocrotaline-treated animals had developed PH with right heart failure at end of study protocol, as characterized by increased pulmonary vascular remodeling, right ventricular dysfunction and right ventricular remodeling.

Data presented as mean ±SEM. Abbreviations: RVSP, right ventricular systolic pressure; RVEDP, right ventricular end-diastolic pressure; RVWT, right ventricular wall thickness; RVEDD, right ventricular end-diastolic diameter; CO, cardiac output; TAPSE, tricuspid annular plane systolic excursion; PVR, pulmonary vascular resistance.

Figure 6.1 Elevated breathing frequencies in rats with Pulmonary Hypertension

Telemetry revealed that at the end of protocol, PH rats have severely elevated breathing frequencies in comparison with controls. A) Typical pressure registration by telemetry of a rat that is injected with monocrotaline at day 7 and has developed overt right heart failure at day 35. B) Breathing frequencies increased significantly during the development of right heart failure in PH rats in comparison to controls.
Diaphragm muscle weakness in PH

To determine whether diaphragm muscle function is impaired in PH, we measured the contractile performance of intact diaphragm muscle strips. In PH, both twitch force (PH: $50.7 \pm 7.5$ vs. CON: $74.7 \pm 3.1$ mN/mm$^2$, $p=0.0149$) and maximal tetanic force (PH: $187.1 \pm 20.1$ vs. $257.0 \pm 8.6$ mN/mm$^2$, $p=0.096$) were significantly reduced in comparison with controls (Figure 2 C,D).

Figure 6.2 Diaphragm weakness in rats with Pulmonary Hypertension

Diaphragm muscle contractility measurements revealed significant weakness in rats with PH. A) Typical force tracing in response to twitch (submaximal) stimulation of diaphragm strips from control (black) and PH (grey) rats. B) Typical force tracing in response to tetanic (maximal) stimulation of diaphragm strips from control (black) and PH (grey) rats. C) Twitch force was significantly reduced in PH rats. D) Maximal tetanic force was significantly reduced in PH rats. E) Absolute force-frequency relation demonstrated reduced force generating capacity in PH rats (grey), at all stimulation frequencies, in comparison to control (black) rats. F) Normalized force-frequency relation was shifted to the right in PH rats (grey) indicating impaired relative force generation in PH rats in comparison with control (black).

Data presented as mean ±SEM. Control n=7, PH n=8. Abbreviations: CON, control; PH, pulmonary hypertension; freq, frequency.
In vivo, the diaphragm mainly undergoes submaximal, rather than maximal activation. Therefore, we also determined the force generating capacity of the diaphragm at various submaximal stimulation frequencies. The absolute force-frequency relation clearly demonstrates reduced force generating capacity in PH rats, at all stimulation frequencies, in comparison with controls (p<0.001; Figure 2E). As shown in Figure 2F, the normalized force-frequency relation was shifted to the right in PH rats (p<0.05), indicating impaired relative force generation in PH rats.

To determine whether the changes in muscle function in PH are specific for the diaphragm, rather than being part of a generalized muscle weakness, we also studied the contractile performance of the extensor digitorum longus (EDL), a lower leg muscle. The EDL is widely used for studies on intact skeletal muscles, mainly due to its relative small size, which facilitates oxygenation, and its well-defined tendons. No differences were observed in twitch and tetanic force in rats with PH in comparison to control (Figure 3A,B). In addition, no changes

Figure 6.3 No change in EDL muscle function in rats with Pulmonary Hypertension
Intact muscle contractility measurements revealed no change in EDL muscle function in rats with PH. A) No change in twitch force of the EDL muscle in PH. B) No change in maximal tetanic force of the EDL muscle in PH. C) Absolute force-frequency relation of the EDL muscle was not altered in PH. D) No change in normalized force-frequency relation of the EDL muscle in PH rats.

Data presented as mean ±SEM. Control n=7, PH n=5. Abbreviations: CON, control; PH, pulmonary hypertension.
were found in the force-frequency relation between PH and control rats (Figure 3C,D). Thus, the force generating capacity of EDL muscle from PH rats is not different from that of control rats.

**Atrophy of diaphragm muscle fibers from PH rats**

To assess whether the reduced force generating capacity of the diaphragm is accompanied by histological alterations, we determined the CSA of diaphragm muscle fibers in control and PH rats. Because the response to PH may be differential for slow and fast muscle fibers, we analyzed both fiber types separately by the use of specific antibodies. As shown in Figure 4B, the CSA of both slow (CON: 1817 ± 103 vs. PH: 1012 ± 44 µm²) and fast (CON: 1850 ± 75 vs. PH: 1192 ± 102 µm²) diaphragm fibers are significantly smaller in PH rats than in controls. These findings indicate manifest atrophy of diaphragm muscle fibers in PH.

**Figure 6.4 Diaphragm atrophy in rats with Pulmonary Hypertension**

Histological analyses of the diaphragm revealed clear atrophy of both slow and fast muscle fibers in PH rats. A) Typical example diaphragm muscle sections of control and PH rats stained for slow (antibody brd-5) and fast muscle fibers (antibody sc-71). B) Cross sectional area of diaphragm muscle fibers from PH rats was significantly reduced, irrespective of fiber type.

Data presented as mean ±SEM. Control n=5, PH n=5. ***: p<0.001. Abbreviations: PH, pulmonary hypertension.

To test whether this fiber atrophy is also present in EDL muscle, we performed fiber CSA measurements in EDL from control and PH rats. In contrast to the diaphragm, the CSA of EDL muscle fibers was not different between control and PH rats (slow fibers: 1334 ± 70 vs. 1366 ± 73 µm²; fast fibers: 3079 ± 164 vs. 2857 ± 134 µm², control vs. PH, respectively).

**Proteasome activity and Akt phosphorylation in the diaphragm of PH rats**

Muscle fiber atrophy is caused by an imbalance between the rate of protein synthesis and proteolysis.\(^{18}\) We found that the activity of proteasomes, the major cellular proteolytic system, was not different between control and PH rats (Fig 5A). Moreover, Western blot analysis showed that the amount of proteasomes is not different between control and PH (Fig 5B). Since phosphorylation of Akt strongly promotes protein synthesis, we measured Akt phosphorylation to investigate whether PH modulates protein synthesis in the diaphragm muscle.
The ratio of phosphorylated Akt over non-phosphorylated Akt, was not different between diaphragms from control vs. PH rats (Figure 5C).

Severe diaphragm fiber atrophy in two patients with PH

To determine whether the findings in PH rats extrapolate to clinical PH, we compared the CSA of diaphragm fibers from two PH patients with those from and two subjects without PH. A typical example of diaphragm fiber cross sections is shown in Figure 6A. Note the marked reduction in CSA of diaphragm muscle fibers from PH patients (CON: 1585 ± 39 vs. PH: 430 ± 19 µm²), indicating severe diaphragm fiber atrophy in patients with PH.

To evaluate whether this fiber atrophy was specific for the diaphragm, we also analysed the CSA of quadriceps muscle fibers. As can be observed in Figure 6B, the average CSA of quadriceps muscle fibers was preserved in PH patients when compared to control subjects. These findings are in line with the aforementioned preclinical results from our rat model, and indicate specific diaphragm muscle alterations in PH patients.

**DISCUSSION**

This is the first study demonstrating diaphragm muscle fiber weakness and atrophy in a rat model for PH. Our data suggest that this weakness is not part of a generalized muscle weakness, but is specific for the diaphragm muscle. Importantly, similar findings were observed in the diaphragm muscle from two patients with PH.

Selective diaphragm muscle weakness in Pulmonary Hypertension

We found an approximately 30% reduction in the maximal force generating capacity of diaphragm muscle strips from rats with PH, while in these rats EDL muscle function was
Preserved. This suggests that diaphragm muscle weakness in PH results from a local process, and is not part of a generalized muscle weakness.

As force was normalized to the CSA of the muscle strip, and since we observed no changes in extracellular area fraction (data not shown), our findings suggest that in PH the intrinsic capacity of diaphragm fibers to generate force is impaired. The nature of this intrinsic diaphragm fiber weakness remains to be established, but could involve sarcomeric injury, or loss of myosin, the main contractile protein. Both have been reported to occur in animal models for CHF and COPD, as well as in diaphragm fibers of patients with COPD.

In addition to intrinsic diaphragm fiber weakness, we observed marked atrophy of diaphragm muscle fibers in PH rats. As the force generating capacity of a muscle fiber is proportional to its CSA, these findings suggest that, on top of the intrinsic diaphragm fiber weakness, diaphragm strength is further impaired by fiber atrophy. Muscle fiber CSA depends on the balance between the rates of protein synthesis and degradation. During atrophy, the bulk of sarcomeric protein degradation occurs via the ubiquitin–proteasome pathway. Increased activity of the proteasome has been used widely as a marker for elevated proteolytic activity, as it is typically associated with muscle fiber atrophy. However, despite the marked

Figure 6.6 Selective diaphragm fiber atrophy in patients with Pulmonary Hypertension

Selective diaphragm muscle atrophy was observed in two patients who died of PH in comparison with control. A) Left: Typical examples of diaphragm muscle fiber cross sections of a control subject and a patient with PH. Right: Average cross sectional area of diaphragm muscle fibers (n=50 / subject) for two control subjects and two PH patients. B) In contrast to the diaphragm, quadriceps muscle fiber cross sectional area was preserved. Left: Typical examples of quadriceps fiber cross sections of a control subject and a patient with PH. Right: Average cross sectional area of quadriceps muscle fibers (n=50 / subject) from two control subjects and two PH patients.
atrophy, we were unable to demonstrate changes in activity or amount of proteasomes in the diaphragm from PH rats. To test whether changes in protein synthesis are involved, we also measured the ratio of phosphorylated Akt over non-phosphorylated Akt; phosphorylated Akt is an upstream activator of pathways involved in muscle protein synthesis. Since we found no reduction of p-Akt/Akt ratio in the diaphragm of PH rats, Akt-mediated protein synthesis seems not to play a major role.

Although diaphragm weakness was apparent across a wide range of stimulation frequencies, it was most pronounced at submaximal stimulation frequencies, as reflected by the rightward shift of the force-frequency relation (Figure 1). This rightward shift was most evident at a stimulation frequency of 30Hz, which is close to the normal firing rate of the phrenic motorneurons activating the diaphragm muscle. The mechanisms underlying this rightward shift of the force-frequency relation in PH rats should be addressed by future studies, but might involve changes in calcium handling by the sarcoplasmic reticulum and/or reduced myofilament calcium sensitivity.

Diaphragm atrophy in rats extrapolates to clinical Pulmonary Hypertension

An important finding of the present study was that we also observed severe diaphragm fiber atrophy in two patients with PH (see Figure 6). These findings likely provide a cellular basis for the impaired inspiratory muscle function in PH patients that has been described before. The magnitude of diaphragm fiber atrophy was even more pronounced in the patients with PH (~70% in patients) when compared to the magnitude of atrophy in PH rats (~40% in rats). The reason for this discrepancy is unclear but might involve the difference in duration to PH exposure. Muscle fiber remodeling such as atrophy requires time, and, clearly, patients with PH had more time for diaphragm remodeling to occur than PH rats, who had only ~25 days between induction of PH and death.

In line with the rat model for PH, the diaphragm atrophy in PH patients was not part of a generalized process, as quadriceps muscle fibers from PH patients showed no signs of atrophy. Data describing skeletal muscle function and structure in PH patients are scarce. In line with our findings, Mainguy et al reported no significant changes in quadriceps muscle fiber size in patients with PH compared with control subjects. Nevertheless, they did observe a mild decrease in in vivo quadriceps strength, which might be caused by intrinsic fiber alterations.

What triggers the selective diaphragm weakness in Pulmonary Hypertension?

It has been reported that PH patients, as well as rats with monocrotaline-induced PH, have elevated levels of circulating proinflammatory cytokines, such as interleukin-1, interleukin-6, and tumor necrosis factor-α. Animal studies have shown that cytokines initiate striated muscle injury, impair contractile protein function, and stimulate proteolysis. Thus, systemic inflammation might be involved in the development of diaphragm weakness in PH.
However, if such a systemic etiology is at play, alterations in the diaphragm and peripheral skeletal muscles are expected to share a high degree of similarity and, to a certain extent, develop simultaneously. This notion is based on the assumption that the different contractile pattern of these muscles does not affect their response to a systemic trigger. As our findings suggest that the diaphragm and peripheral muscles are affected differentially in PH, a major role for circulating cytokines seems unlikely.

Instead, we propose that the increased activity of the inspiratory muscles in PH plays a role (see Figure 7 for a schematic overview). Recent studies suggest that patients with PH hyperventilate during exercise, at rest, and even during sleep.\(^3\)\(^-\)\(^5\) This continuous and unrelenting hyperventilation places an increased demand on the inspiratory muscles. We observed an approximately 90% increase in breathing frequency in PH rats, suggesting a marked increase in diaphragm activity. The diaphragm is remarkably sensitive to changes in activity: elevated inspiratory muscle activity in COPD and CHF causes profound cellular and functional alterations in the diaphragm, such as fiber atrophy and weakness.\(^12\)\(^-\)\(^15\);\(^22\);\(^33\)\(^-\)\(^37\) Moreover, the observation that diaphragm unloading in patients with CHF restores strength, and, importantly, alleviates symptoms of dyspnoea\(^38\), further illustrates the sensitivity of the diaphragm to changes in activity.

Thus, future research should focus on unraveling the exact mechanisms underlying diaphragm weakness in PH. In our opinion, the hyperventilation-induced elevated diaphragm activity in PH is a prime candidate, and modulation of respiratory frequency in PH rats might elucidate a causal relation.
In summary, this is the first study revealing diaphragm muscle fiber weakness in PH. We propose that this weakness is not part of a generalized muscle weakness but is specific for the diaphragm, and we speculate that it is at least partly caused by the chronic increase of diaphragm activity in PH. The identification of the pathogenesis of diaphragm muscle weakness may provide new therapeutic targets to reduce the sensation of dyspnoea and eventually improve the quality of life of patients with pulmonary hypertension.
REFERENCES


