CARDIOSELECTIVE BETA-BLOCKER THERAPY IMPROVES SURVIVAL AND CARDIAC FUNCTION IN EXPERIMENTAL PULMONARY HYPERTENSION

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ABSTRACT

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.

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.

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.¹ No treatment is currently available that improves RV function directly, partially because it is not considered a therapeutic target in PH.²

Recently, several reports have shown that sympathetic activity is increased in patients with PH.³ Similar to left heart failure (LHF), it was found that signs of sympathetic over-stimulation, such as blunted baroreflex,⁴ reduced heart rate variability,⁵ increased muscle sympathetic nerve activity,⁶ and “ventricle-specific” down-regulation of β₁-adrenergic receptors,⁷ 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.⁸ This supports use of β-adrenergic blockade in LHF-management, which has been demonstrated to significantly reduce mortality and left ventricular (LV) remodeling.⁹

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.¹ This recommendation is partially substantiated by the findings of Provencher et al. that within weeks, exercise capacity improved after β-blocker withdrawal.¹⁰ 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.¹¹,¹² Furthermore, in an acute model of PH, it was demonstrated that acute right ventriculo-arterial uncoupling occurs after intravenous β-blocker administration.¹³

However, the β-blockers used in these studies were first generation unselective β-blockers,¹⁰,¹³ with more bronchial and vascular side-effects.⁸ 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.¹⁴

Finally, we recently demonstrated that exercise training was detrimental in experimental and progressive PH.¹⁵ 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 monocrotableine (60 mg/kg body mass; Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in sterile saline; the control group was injected with saline only.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.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.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.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.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 random-
ized 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.

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 O2/air mix; Pharmachemie, Haarlem, The Netherlands), as previously described. 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). Disease progression of PH during treatment-period was expressed as percentage changes in hemodynamics over time, e.g. change in cardiac output (CO):

\[ \Delta CO = \left( \frac{CO_{END} - CO_{START}}{CO_{START}} \right) \times 100\% \]

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

Invasive RV pressure-volume measurements

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 O2/air mix) in all 22 rats, as previously described (for details, see also Supplement). 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 (Ea = RV end-systolic pressure / stroke volume). From occlusion-data, end-systolic and end-diastolic elastance (Ees, Eed) were determined. These parameters 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).
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.\textsuperscript{13,21}

**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.

The determination of cardiomyocyte cross sectional area, cardiac fibrosis, relative wall thickness of pulmonary arterioles (PA), myocardial capillary density (using CD31-antibodies) and myocardial inflammation (using CD45-antibodies) were performed, as previously described (for details, see Supplement).\textsuperscript{15,23}

**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, 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).\textsuperscript{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).

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
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cardiac dysfunction or dilatation were present yet (measured by cardiac output, TAPSE and RV end-diastolic diameters; Figure 2C and Supplement: Figure S-1).

Figure 2

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 (Supplement: 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 S-1A,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- 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.
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Figure 4

Pressure-volume analyses. Typical examples of the pressure-volume relation are shown for control, PH and PH+biso (A-C: line indicates end-systolic pressure-volume relationship). RV systolic pressures (RV SP) and arterial elastance (Ea; measure of RV afterload) were equally increased in both PH-groups (D,E). However, RV contractility (end-systolic elastance; Ees) was significantly increased by bisoprolol-treatment (F), resulting in partial normalization of ventriculo-arterial coupling (Ees/Ea; G). Bisoprolol-treatment also partially restored RV diastolic function, measured by RV end-diastolic pressure (RV EDP) and Eed (H,I).

Data presented as mean ±SEM, control: n=8, PH / PH+biso, n=7. *: p<0.05, **: p<0.01, ***: p<0.001 PH+biso vs. PH.

**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 of normalization; Table S-2), RV / (LV+S) ratio, or RV cardiomyocyte cross sectional area (Figure 5C,D). 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-positive) 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).

Additional autopsy and (LV) histology data can be found in the Supplement (Tables S-2, S-3).
Chapter 4

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-blocker 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.\textsuperscript{1,10,13} To address this legitimate argument, we used an approach that was inspired by successful β-blocker use in left heart failure.
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 Bisoprolol-treatment reduced RV fibrosis and RV myocardial inflammation. Histomorphometric analyses revealed significant and selective increase of interstitial fibrosis and myocardial inflammation in the RV myocardium of PH-rats, compared to control. Bisoprolol-treatment reduced RV fibrosis and RV myocardial 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 bisopropol- and vehicle-treated PH-rats (A). Typical examples are shown of histological sections of the right ventricle of vehicle- (PH: D-F) and bisopropol-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.
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. 26

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. 9 Of these three, bisoprolol is the most β₁-cardioselective β-blocker. 8 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. 27,28 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. 29 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 PAH-patients might be related to the use of relatively high-dosed unselective β-blockers. 10,13 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, 21,24 and more specifically, to address the potential risk of ventriculo-arterial uncoupling after β-blocker use in PH, as raised by others. 13 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. 10 Usui et al. investigated the effect of carvedilol in monocrotaline-treated rats. 30 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. 31 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 result 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.

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Disclosures
None.

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tricular hypertrophy plus neurohumoral activation is necessary to alter the cardiac beta-adrenoceptor
SUPPLEMENT:

EXPANDED METHODS

RV catheterization

The rats were sedated by inhalation of isoflurane (induction: 4.0% in 1:1 O₂/air mix; maintenance: 2.0% in 1:1 O₂/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 cmH₂O, 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.

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 Giesson 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:

$$PA \text{ wall thickness} = \frac{2 \times \text{medial wall thickness}}{\text{outer diameter}} \times 100\%.$$  

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.
Disease progression during treatment-period. Echocardiography could confirm the PH-status at start of treatment (A, B: first time-point, lower PAAT/cl and higher RV wall thickness). Bisoprolol-treatment (PH+biso: dotted line) delayed RV dilatation (C: arrow) and reduced the decline in cardiac function (D, E: arrows), compared to vehicle-treated PH-rats (PH: solid line), numeric data is found in Table S-1. In addition, at end-of-study cardiac function was better maintained in bisoprolol-treated PH-rats.

Data presented as mean±SEM, control: n=8; PH / PH+biso: n=7. **: p<0.01 PH / PH+biso vs. control; *: p<0.05, **: p<0.001 PH+biso vs. PH. Abbreviations: PAAT/cl, pulmonary artery acceleration time normalized for cardiac cycle length (inversely correlated with RV systolic pressure); RVEDD, RV end-diastolic diameter; TAPSE, tricuspid annular plane systolic excursion.
**SUPPLEMENTAL TABLES**

### Table S-1 Disease progression, assessed by serial echocardiography

<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>∆PAAT/cl (%/day)</td>
<td>0.0 ±0.2</td>
<td>-4.0 ±0.7</td>
<td>-2.9 ±0.5</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>∆RV wall thickness (%/day)</td>
<td>0.2 ±0.1</td>
<td>2.8 ±0.3</td>
<td>2.6 ±0.5</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>∆RV end-diastolic diameter (%/day)</td>
<td>0.2 ±0.1</td>
<td>10.0 ±1.9</td>
<td>6.0 ±0.5</td>
<td>&lt;0.001</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>∆TAPSE (%/day)</td>
<td>0.2 ±0.1</td>
<td>-6.2 ±0.8</td>
<td>-2.1 ±0.6</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>∆Cardiac output (%/day)</td>
<td>0.2 ±0.1</td>
<td>-8.0 ±0.8</td>
<td>-3.9 ±0.6</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

Data presented as mean ±SEM. Abbreviations: ∆PAAT/cl, daily percentage change in pulmonary artery acceleration time normalized for cardiac cycle length; ∆RV wall thickness, and so on, daily percentage change in RV wall thickness, etcetera; PH, PH-rats (treated with vehicle); PH+biso: PH-rats treated with 10 mg/kg bisoprolol once daily from day 10; n.s.: not significant.

### Table S-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></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></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></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></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></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></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></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></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></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;0.01</td>
<td>n.s.</td>
<td></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></td>
</tr>
<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></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.

### Table S-3 Additional histological data on the left ventricle

<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>LV CSA (μm²)</td>
<td>488 ±16</td>
<td>377 ±18</td>
<td>393 ±18</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>LV capillary density (/mm² *1000)</td>
<td>2.71 ±0.09</td>
<td>3.49 ±0.24</td>
<td>3.12 ±0.32</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>LV inflammation (CD45+-nuclei/mm²)</td>
<td>28.7 ±3.0</td>
<td>59.5 ±6.7</td>
<td>53.6 ±5.7</td>
<td>0.01</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>LV fibrosis (area %)</td>
<td>8.4 ±0.6</td>
<td>11.3 ±0.1</td>
<td>10.8 ±0.8</td>
<td>0.01</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>

Data presented as mean ±SEM. Abbreviations: CSA, cardiomyocyte cross sectional area.