Bisoprolol Delays Progression TowARDS Right Heart Failure in Experimental Pulmonary Hypertension

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Background—In pulmonary arterial hypertension (PH), sympathetic adrenergic activity is highly elevated. Sympathetic overactivity is a compensatory mechanism at first, but might be detrimental for cardiac function in the long run. We therefore investigated whether chronic low-dose treatment with bisoprolol (a cardioselective β-blocker) has beneficial effects on cardiac function in experimental PH.

Methods and Results—PH was induced in rats 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) or vehicle (n=7/group). At end of study (body mass loss >5%), echocardiography was repeated, with additional pressure-volume measurements and histomolecular analyses. Compared with control, right ventricular (RV) systolic pressure and arterial elastance (measure of vascular resistance) more than tripled in PH. Bisoprolol delayed time to right heart failure (P<0.05). RV afterload was unaffected, however, bisoprolol treatment increased RV contractility and filling (both P<0.01), and partially restored right ventriculo-arterial coupling and cardiac output (both P<0.05). Bisoprolol restored RV β-adrenergic receptor signaling. Histology revealed significantly less RV fibrosis and myocardial inflammation in bisoprolol treated PH rats.

Conclusions—In experimental PH, treatment with bisoprolol delays progression toward right heart failure, and partially preserves RV systolic and diastolic function. These promising results suggest a therapeutic role for β-blockers in PH that warrants further clinical investigation. (Circ Heart Fail. 2012;5:97-105.)

Key Words: pulmonary hypertension ■ right ventricular dysfunction ■ β-adrenergic receptor blocker ■ pressure-volume relationship ■ Wistar rats

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 (RHF) 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 was not considered a therapeutic target in PH.2 Recently, several reports have shown that sympathetic activity is increased in patients with PH.3-7 Similar to left heart failure (LHF), “ventricle-specific” down regulation of β1-adrenergic receptors was observed in RV samples of PH patients.8 In addition, we recently demonstrated that exercise training was detrimental in experimental and progressive PH.9 The deleterious effects could be related to bouts of exercise-induced sympathetic stimulation.

Although increased adrenergic activity is a compensatory mechanism to maintain cardiac function by increasing contractility and heart rate, it became apparent that chronic adrenergic overactivity has, in the long run, detrimental effects on cardiac function.10 This supports the use of β-adrenergic blockade in LHF management, which has been demonstrated to significantly reduce mortality and left ventricular (LV) remodeling.11 Nevertheless, and notwithstanding the substantial evidence of their beneficial effects in LHF, the use of β-blockers currently is not recommended for patients with PH.1 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.\(^\text{12}\) Furthermore, in an acute model of PH, it was demonstrated that right ventriculo-arterial uncoupling occurs after intravenous \(\beta\)-blocker administration.\(^\text{13}\)

However, the \(\beta\)-blockers used in these studies were first generation unselective \(\beta\)-blockers,\(^\text{14,13}\) with more bronchial and vascular side effects.\(^\text{10}\) In addition, the dosages used in these studies were relatively high, whereas a low dose could have sufficed and been tolerated better. Furthermore, no data are available on the long-term effects of \(\beta\)-adrenergic receptor blockade in PH patients. This aspect is important, as the typical time course of improvement by \(\beta\)-blockers in LHF is preceded by initial functional decline, with significant clinical improvement not to be expected before 3 months after start of therapy.\(^\text{15}\)

Recently, Bogaard and coworkers\(^\text{16}\) provided some hemodynamic and molecular insights in effects of carvedilol (an unselective \(\beta\)-blocker) in PH rats. However, in the absence of load independent measurements of RV function, it is unclear whether the beneficial effects are RV-specific or related to the \(\alpha\)-associated pulmonary vascular effects of carvedilol.\(^\text{17}\) In addition, it remains uncertain whether \(\beta\)-blockers can restore \(\beta\)-adrenergic receptor (\(\beta\)AR) signaling and delay progression of right heart failure. In the present study, we therefore (1) assessed the chronic effect of bisoprolol (a cardio-selective \(\beta\)-blocker) on disease progression by sequential echocardiographic measurements, (2) evaluated RV function using load independent parameters derived from pressure-volume analyses, and (3) studied the \(\beta\)AR- signaling, assessing its direct downstream targets.

### Methods

All experiments were approved by the Institutional Animal Care and Use Committee of the VU University, Amsterdam, The Netherlands. Male Wistar rats were used (150 to 175 g, 30 in total), and experimental PH was induced by monocrotaline (60 mg/kg).\(^\text{9,18}\)

#### Part I: Dose Finding by Pressure Telemetry

The minimal effective dose of bisoprolol that could blunt heart rate response during daily activity (\(>10\%\)) was determined by telemetry (TA11PA-C40, Data Science International [DSI], St. Paul, MN).\(^\text{19}\)

Ten days after PH induction, bisoprolol was given once daily for 3 consecutive days by oral gavage, at start of their active phase (ie, night: 18:00 to 06:00 hours); 4 PH rats received 5 mg/kg bisoprolol once daily and the other 4 received 10 mg/kg. The effect of bisoprolol on heart rate, systemic blood pressure, and physical activity were evaluated (for details, see the online-only supplement).

#### 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 PH induction, 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 RHF (defined as \(>5\%\) loss in body mass or respiratory distress, cyanosis, lethargy) were euthanized earlier, in keeping with the protocol approved by the Institutional Animal Care and Use Committee. Manifest RHF was a survival end point and recorded as an event in the survival analyses.\(^\text{9}\)

### Hemodynamic Evaluation

Rats were evaluated by echocardiography 10 days after (monocrotaline) injection and at end of study (when manifest RHF developed, or 31 days after injection).\(^\text{9,19}\) At end of study, open-chest RV catheterization (Millar Instruments, Houston, TX) was performed under general anesthesia in all rats (isoflurane induction: 4.0% in 1:1 \(\text{O}_2/\text{air}\) mix; see the online-only supplement).\(^\text{9}\) Noninvasive estimations of disease progression and RV wall stress are described in the online-only supplement.

Using custom made algorithms (programmed in MATLAB 2007b, The MathWorks, Natick, MA) RV (peak-) systolic pressures and RV end-diastolic pressures were automatically determined from steady state measurements, as well as arterial elastance (\(E_a\)), a measure for RV afterload.\(^\text{13,20}\) From occlusion data, end-systolic elastance (\(E_{es}\); contractility) and end-diastolic elastance (\(E_{ed}\); filling) were determined.\(^\text{20,21}\) 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 (\(E_{es}\)) and filling (\(E_{ed}\)).\(^\text{22}\) In addition, we calculated the preload recruitable stroke work and the dP/dmax end-diastolic volume relation to assess RV contractile performance.\(^\text{23}\) The ratio \(E_{es}/E_a\) was calculated as an estimate for ventriculo-arterial coupling (cardiac adaptation in relation to its load).\(^\text{13,20}\)

### Histomorphology 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. Cardiomyocyte cross-sectional area, cardiac fibrosis, relative wall thickness of pulmonary arterioles, myocardial capillary density (using CD31-antibodies), and myocardial inflammation (using CD45-antibodies) were determined (see the online-only supplement).\(^\text{9,24}\)

### Protein Analyses \(\beta\)-Adrenergic Signaling

Phosphorylation of cardiac myosin binding protein C (cMyBPC) and cardioprotein one (cTnI) was determined as described before.\(^\text{25}\) All RV samples were treated with trichloroacetic acid, to preserve phosphorylation of cMyBPC and cTnI. Samples were separated on a gradient gel (Criteron Tric-HCL 4% to 15% gel, Bio-Rad Laboratories, Berkeley, CA), and proteins were stained for 1 hour with ProQ Diamond Phosphoprotein Stain (Molecular Probes, Eugene, OR). Fixation, washing, and destaining were performed according to manufacturer’s guidelines. Subsequently, gels were stained with SYPRO Ruby staining (Molecular Probes) for determination of total protein levels of cMyBPC and cTnI. The phosphorylation status of cMyBPC and cTnI was expressed relative to total protein levels to correct for differences in sample loading.

### Statistical Analysis

All analyses were performed in a blinded fashion. All data were verified for normal distribution. Data are presented as \(\text{mean} \pm \text{SEM. A probability value} <0.05\) was considered statistically significant. Comparison of telemetric registrations of PH rats before/after bisoprolol treatment was performed by 2-way ANOVA for repeated measurements, and the interaction between bisoprolol treatment and time was tested and reported. One-way ANOVA was used for the analyses of disease progression, pressure-volume relation, autopsy data, and protein analyses, with Bonferroni posthoc comparison between PH rats with/without bisoprolol treatment. Survival estimates were performed by Kaplan-Meier analysis, with posthoc comparison performed by log rank (Mantel-Cox) test between PH rats with/without bisoprolol treatment. Survival data, and protein analyses, with Bonferroni posthoc comparison between PH rats with/without bisoprolol treatment. Survival estimates were performed by Kaplan-Meier analysis, with posthoc comparison performed by log rank (Mantel-Cox) test between PH rats with/without bisoprolol treatment. Survival estimates were performed by Kaplan-Meier analysis, with posthoc comparison performed by log rank (Mantel-Cox) test between PH rats with/without bisoprolol treatment.

### 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 pulmonary artery ac-
Cardiac function was maintained partially by bisoprolol treatment (tricuspid annular plane systolic excursion: control: 4.0 ± 0.1; PH: 1.4 ± 0.1; PH + bisoprolol: 2.4 ± 0.2 mm, PH versus PH + bisoprolol: P < 0.001; cardiac output: control: 88 ± 2.1; PH: 17 ± 1.6; PH + bisoprolol: 34 ± 1.9 mL/min, PH versus PH + bisoprolol: P < 0.001; Figures 3D and E). No differences were observed in RV wall thickness and RV dilatation between bisoprolol- and vehicle-treated PH rats (Figures 3B and C). In addition, end-systolic wall stress was similar in bisoprolol- and vehicle-treated PH rats (RV end-systolic wall stress, control: 80 ± 5; PH: 336 ± 26; PH + bisoprolol: 308 ± 14 mm Hg; PH versus PH + bisoprolol: P = 0.26).

**Bisoprolol Improved Cardiac Function, Without Affecting RV Afterload**

Right ventricular pressure-volume measurements at end of study (Figures 4A–C) revealed that RV systolic pressures were significantly elevated in PH rats compared with control, but no difference was found between bisoprolol- and vehicle-treated PH rats (Figure 4D), which is in line with previous echo findings (Figures 3A and B). Ea (measure of vascular resistance) was elevated also 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. We also found an equal rise in (wet) lung mass observed at autopsy, and comparable remodeling of the pulmonary arteries during histological examination (Tables S4 and S5; see the online-only supplement).

On the other hand, bisoprolol treatment increased Ees (measure of contractility; Figure 4F), resulting in partial normalization of the ventriculo-arterial coupling (Ees/Ea; Figure 4G). Preload recruitable stroke work (control: 211 ± 35, PH: 1362 ± 156, PH + bisoprolol: 2161 ± 312 mm Hg; PH versus PH + bisoprolol: P = 0.04).

**Figure 2.** Bisoprolol treatment in PH significantly delayed time to manifest right heart failure. Log rank test: X² = 4.54, P < 0.05.

**Part II: Effects of 10 mg/kg Bisoprolol in Established PH**

**Bisoprolol Delayed the Progression Toward Right Heart Failure**

In a separate group of rats, we determined PH status 10 days after (monocrotaline) injection, by echocardiography, right heart catheterization, and histomorphology. Monocrotaline-treated rats (n = 5) revealed lower PAATT/cl, indicating higher RV systolic pressure⁶ and higher RV wall thickness, indicating (moderate) RV hypertrophy (Table S1; see the online-only supplement). In addition, increased RV systolic pressures, pulmonary vascular remodeling, and RV hypertrophy at day 10 were confirmed by RV catheterization and histomorphometric analyses (Tables S1 and S2; see the online-only supplement). The PH state before start of bisoprolol treatment, thereby, was confirmed in all monocrotaline-treated rats. Compared with vehicle-treated PH rats, bisoprolol delayed the time to manifest RHF, as defined in the Methods section (Figure 2). This finding was confirmed by serial echocardiography, demonstrating that bisoprolol significantly delayed the progression of RV dilatation and reduced the decline in cardiac function, whether measured by tricuspid annular plane systolic excursion or cardiac output (Figure 3; Table S3 [see the online-only supplement]; P < 0.05). Preservation of cardiac output was mainly the result of an increase in stroke volume rather than increased heart rate.

At end of study, cardiac function was maintained partially by bisoprolol treatment (tricuspid annular plane systolic excursion, control: 4.0 ± 0.1; PH: 1.4 ± 0.1; PH + bisoprolol 2.4 ± 0.2 mm, PH versus PH + bisoprolol: P < 0.001; cardiac output, control: 87 ± 2.1; PH: 17 ± 1.6; PH + bisoprolol: 34 ± 1.9 mL/min, PH versus PH + bisoprolol: P < 0.001; Figures 3D and E). No differences were observed in RV wall thickness and RV dilatation between bisoprolol- and vehicle-treated PH rats (Figures 3B and C). In addition, end-systolic wall stress was similar in bisoprolol- and vehicle-treated PH rats (RV end-systolic wall stress, control: 80 ± 5; PH: 336 ± 26; PH + bisoprolol: 308 ± 14 mm Hg; PH versus PH + bisoprolol: P = 0.26).

**Figure 1.** Averaged 24-hour registration of PH rats before and after treatment of 10 mg/kg bisoprolol. This dosage was able to completely blunt heart rate response during the whole active phase (between 18:00 and 06:00 hours) of the rats (A: gray area). In addition, only a moderate effect on systemic blood pressure was observed (B), without an (adverse) effect on daily physical activity (C). Data presented as mean ± SEM, n = 4 (PH/PH + bisoprolol). Probability values represent interactive term (time * treatment). PH (solid/red line) indicates vehicle-treated PH rats; PH + bisoprolol (dotted/blue line), bisoprolol-treated PH rats.
PH normalization of Ees for RV mass, no significant difference rats (Ees/RV volume, control: 2.14 improved toward normal value in the bisoprolol-treated PH tility was reduced significantly in vehicle-treated PH rats and sus PH). After normalization of Ees for RV volume, contrac-


duced RV end-diastolic pressures and Eed (measure of filling; Figures 4H and I).


Bisoprolol Reduced RV Fibrosis and RV Myocardial Inflammation

In line with previous echo findings, the right ventricles of PH rats at end of study were hypertrophied compared with controls (Figure 3B). No differences were observed between bisoprolol- and vehicle-treated PH rats, whether expressed as RV mass (irrespective of normalization), RV/(LV+S) ratio, or RV cardiomyocyte cross-sectional area (Tables S4 and S5; see the online-only supplement).

At start of treatment, no difference was observed between control and PH rats in RV capillary density or fibrosis, and there were no signs of cardiac inflammation (Table S2; see the online-only supplement). At end of study, the findings for RV capillary density were similar; compared with control, capillary density was reduced in PH, without a significant difference between the 2 PH groups (Figure 5A, D, and G). More RV interstitial fibrosis was observed between PH rats and controls; interestingly, bisoprolol treatment significantly reduced RV fibrosis (Figure 5B,E,and H). Furthermore, the presence of (CD45+) inflammatory cells in RV myocardium of bisoprolol-treated PH rats was significantly less, compared with vehicle-treated PH rats (Figure 5C, F, and I). Leukocyte infiltration in the left ventricle was increased in bisoprolol- and vehicle-treated PH rats; however, these values were low and comparable with control values of the right ventricle (Table S5; see the online-only supplement). Autopsy and assessment of LV histology revealed no effect of bisoprolol (Tables S4 and S5; see the online-only supplement), compatible with a RV-specific effect of bisoprolol.

Bisoprolol Restored RV β-Adrenergic Receptor Signaling Pathway

Phosphorylation of both cMyBPC and cTnI (protein kinase A-mediated downstream targets of the βAR) were significantly higher in bisoprolol-treated PH rats in comparison with vehicle-treated PH rats (Figure 6).

Discussion

This study investigated the effects of bisoprolol treatment in experimental PH, focusing on RV function and remodeling. Using a comprehensive set of physiological and pathological end points, we have demonstrated that:

1. Chronic low-dosed bisoprolol treatment was well tolerated, and delayed time to manifest RHF.
2. Bisoprolol treatment improved cardiac function by improving RV contractility (Ees), filling (Eed), and ventriculo-arterial coupling (Ees/Ea).

3. The cardiac-selective effects of bisoprolol can be attributed to restoration of RV β-adrenergic receptor signaling, 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 not recommended, because PH patients are believed not to tolerate the acute (but transient) negative inotropic and chronotropic effects. To address this legitimate argument, we used an approach that was inspired by successful β-blocker use in LHF.

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**Low Versus High Dose**

By definition, patients with LHF are hemodynamically compromised, and like in PH, their adrenergic system is overactivated as well. To some extent, these 2 patient groups are therefore comparable. Interestingly, most LHF 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 dose, followed by gradual 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 with other rat studies that used bisoprolol...
prolol (typically 60 mg/kg), the dose used in this study can be considered low.\textsuperscript{29}

Hemodynamic data at day 10 suggests limited functional hemodynamic compromise of the PH rats at start of treatment. Unfortunately, the progressiveness of the model did not permit starting treatment at a later time point. However, like in LHF,\textsuperscript{11} \&blocker use most likely will be limited to PH patients with some cardiac reserve, in order to cope with its acute negative inotropic effects.

**Selective Versus Unselective \&blocker**

Of all \&blockers, only bisoprolol, carvedilol, and metoprolol have been proven to reduce mortality in LHF.\textsuperscript{11} Of these 3, bisoprolol is the most \textsubscript{1}-cardioselective.\textsuperscript{10} We chose bisoprolol to avoid potential harmful effects of \textsubscript{2}-mediated blockade. The \textsubscript{2}-subtype is the predominant \textsubscript{AR} present in the pulmonary vasculature. Blockade of the \textsubscript{2}-receptors may lead to smooth muscle contraction, which could result in a further increase in pulmonary vascular resistance and RV pressures. Nevertheless, the clinical relevance of \textsubscript{1}-selectivity in PH is unknown.

**Beneficial Effects of Bisoprolol**

To ease the clinical interpretation of our findings, we used robust and clinically 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,\textsuperscript{20,26} and more specifically, to address the potential risk of ventriculo-arterial uncoupling after \&blocker use in PH, as raised by others.\textsuperscript{13} In contrast to what was feared, we observed partial normalization of the ventriculo-arterial coupling, which may be explained by chronic opposed to acute drug administration.

Measurements of RV wall thickness and diastolic diameter at end of study protocol failed to detect changes that could explain the beneficial effects of bisoprolol. However, it should be noted that these measurements were obtained at a stage of terminal right heart failure, which was reached at a later time point in the bisoprolol-treated PH group than the PH control group: eg, the progression of RV dilatation was significantly less in the bisoprolol-treated rats (Figure 3C: RV end-diastolic diameter; Table S3 [see the online-only supplement]). Hence, the beneficial effect of bisoprolol between
both groups are reflected more by the time elapsed to reach right heart failure than the hemodynamic findings at right heart failure. In addition, we observed less RV inflammation and fibrosis, together with an increase in RV contractility normalized for hypertrophy (Ees/RVmass), in the bisoprolol-treated PH group compared with the control PH group. This implies that bisoprolol treatment results in improved intrinsic properties of RV cardiomyocytes that cannot be detected by relatively crude measures such as RV wall thickness and diastolic diameters.

Only a few studies have evaluated the (chronic) effects of \( \beta \)-blockers in the context of PH.\(^{14} \) A recent paper by Bogaard et al\(^{15} \) provides some hemodynamic and molecular insights in the effects of carvedilol (a nonspecific \( \alpha_1/\beta_1/\beta_2 \)-adrenergic receptor blocker) on echocardiographic parameters and molecular analyses in PH rats. For the first time, we demonstrate that the positive effects of \( \beta \)-blocker treatment are RV-specific and could delay disease progression, improve RV function, and (partially) restore ventriculo-arterial coupling and \( \beta \)-AR-signaling. Bogaard et al\(^{16} \) also observed less RV fibrosis after \( \beta \)-blocker treatment. They found an increase in RV capillary density, which we could not confirm. We, on the other hand, observed a reduction in RV inflammation, which was not studied in detail by Bogaard et al.\(^{16} \) Differential effects of the \( \beta \)-blockers used might explain these subtle differences. An alternative explanation is related to differences in disease severity of the experimental models used; in our monocrotaline model, all rats developed right heart failure within 4 weeks, whereas all hypoxia/Sugen rats survived 8 weeks after induction of PH. As a consequence, treatment duration in our monocrotaline model was relatively short (median treatment was 2.5 weeks) compared with Bogaard’s Sugen/hypoxia model (4 weeks).\(^{16} \) It is possible that bisoprolol treatment reduced RV inflammation and RV fibrosis (early effects), but that its treatment duration was insufficient to enhance RV capillary density (late effect). Usui et al\(^{18} \) also investigated the effect of carvedilol in monocrotaline-treated rats. They too observed survival benefit with \( \beta \)-blocker, but unfortunately they did not report any measures on cardiac function, and focused mainly on LV rather than RV remodeling. Also, no information was provided on possible side effects and tolerability. In addition, Ishikawa et al\(^{31} \) reported beneficial effects of arottenol (a nonspecific \( \beta \)-blocker) using the same PH rat model. However, the clinical implications of this study are limited; arottenol was studied to prevent rather than to treat PH-associated RHF, and, unlike bisoprolol, arottenol is not clinically used or Food and Drug Administration approved.

**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 and protein analysis may provide some mechanistic insights, based on the experiences with \( \beta \)-blockers in LHF.

Although the \( \beta \)-adrenergic system in LHF is incompletely understood, it is generally believed to exert its beneficial effects by blunting of the sympathetic overactivity, resulting in reduced desensitization of the \( \beta \)AR and restoration of \( \beta \)AR-signaling.\(^{32} \) Protein kinase A is a central player in the \( \beta \)AR-signaling.\(^{33} \) Therefore, we investigated the effect of bisoprolol on phosphorylation of contractile proteins cMyBPC and cTnI, 2 main targets of protein kinase A. Interestingly, we observed that bisoprolol restored phosphorylation of both cMyBPC and cTnI, compared with vehicle treatment. This suggests that bisoprolol restored RV cardiomyocyte \( \beta \)AR-signaling.

An alternative explanation for the observed beneficial effects of bisoprolol therapy might be that a reduction in heart rate by \( \beta \)-blockers could have prevented sustained high levels of RV wall stress. We previously observed that exercise increased myocardial inflammation in experimental PH, and related this to increased RV wall stress during episodes of activity,\(^{9} \) comparable with what has been described in detail by Sun et al.\(^{34} \) This is in agreement with the observation of the present study that bisoprolol prevents rather than reverses RV inflammation, as no inflammation was observed at start of treatment, which also argues against direct cardiac inflammatory effects of monocrotaline.
Clinical Relevance
We demonstrated that β-blocker therapy is beneficial in experimental PH. However, the monocrotaline model exhibits alterations in the β-adrenergic system that resemble those in human PH; others have previously demonstrated that in monocrotaline-treated rats with RHF, 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.

Despite bisoprolol treatment all PH rats eventually developed right heart failure. However, we want to emphasize that the effects of bisoprolol were achieved in the absence of “traditional” vasodilating therapies (eg, bosentan) that modulate the progression of pulmonary vascular remodeling. The findings of this study therefore provide a rationale to investigate the role of (cardioselective) β-blockers as an add-on therapy in the management of clinical PH.

Conclusions
In our PH rat model, bisoprolol treatment was well tolerated and beneficial. It delayed the progression toward RHF, which was attributed to improved RV contractility and compliance, and was accompanied by restored β-adrenergic receptor signaling and reduced RV fibrosis and inflammation. Future studies are necessary to address the clinical implications of our findings.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

The use of beta-blockers is presently contraindicated in the clinical management of patients with pulmonary arterial hypertension (PH). Increased sympathetic activity is believed to be a compensatory mechanism to maintain cardiac output by increasing heart rate and right ventricular (RV) contractility. However, as has been shown for left heart failure, chronic adrenergic overstimulation might be detrimental in the long run. In the present study, we therefore studied the chronic effects of a cardio-selective β-blocker (bisoprolol) on disease progression, RV function, and β-adrenergic signaling. In our PH rat model, 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 was accompanied by restored β-adrenergic receptor signaling and reduced RV fibrosis and inflammation. These findings provide a rationale to investigate the clinical application of β-blockers in PAH. However, the acute negative inotropic effects of β-blockers on the pressure overloaded right ventricle necessitates a very careful approach. For that reason, we are currently conducting a prospective clinical study to evaluate whether a cautious introduction of β-blockers in New York Heart Association II/III PAH patients is tolerated and safe.
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SUPPLEMENTAL MATERIAL

Bisoprolol Delays Progression Towards Right Heart Failure in Experimental Pulmonary Hypertension

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Expanded Methods

Part I – Dose-finding by pressure-telemetry

A group of 8 pulmonary arterial hypertensive (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. 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.

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. Telemetry does not only allow continuous recordings of heart rate and systemic blood pressure, free of artefacts like stress or anaesthesia, 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.

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.

Hemodynamic evaluation

Echocardiography

Rats were evaluated by echocardiography 10 days after (monocrotaline-)injection and at end-of-study (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),¹³ which allows for serial assessment of cardiac dimensions and hemodynamics in rats with equal diagnostic accuracy as cardiac MRI, due to its high temporal resolution.⁵ Analyses were performed off-line (Image-Arena 2.9.1, TomTec Imaging Systems, Unterschleissheim / Munich, Germany). Measured parameters for right ventricular (RV) function were: cardiac output (Doppler-derived stroke volume, heart rate), and tricuspid annular plane systolic excursion (TAPSE). Parameters for RV remodelling were: RV end-diastolic diameter (RVEDD) 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: \[ \Delta \text{cardiac output} = \frac{[\text{cardiac output}_{\text{end}} - \text{cardiac output}_{\text{start}}]}{\text{cardiac output}_{\text{start}}} \times 100\% \]. Other parameters for disease progression were calculated similarly.

**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.
RV wall stress was estimated using LaPlace’s law: \( RV \) wall stress = \( \frac{RVSP \times RVEDD}{4 \times RV \text{ wall thickness}} \).

**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 \( \mu \)m) were used to determine left ventricular (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 \( \mu \)m) 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 \( \mu \)m) 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 \( \mu \)m, randomly distributed over the lungs, were measured. Relative wall thickness of pulmonary arterioles (PA) was calculated as:

\[ PA \text{ wall thickness} = 2 \times \text{medial wall thickness} / \text{outer diameter} \times 100\% . \]
Immunofluorescence microscopy

For the analyses of cardiac capillarisation 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.

Additional measurements at start-of-treatment

Ten days after sham- or monocrotaline-injection, pressure-volume measurements and histomorphological analyses were performed in a separate group of 10 rats (5 control; 5 MCT-treated), to evaluate disease severity and cardiac inflammation in more detail.
Supplemental References


Table S1: Hemodynamic assessment at start-of-treatment (day 10)

<table>
<thead>
<tr>
<th></th>
<th>Control (n=5)</th>
<th>PH (n=5)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV wall thickness (mm)</td>
<td>0.84 ±0.02</td>
<td>0.94 ±0.02</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TAPSE (mm)</td>
<td>3.6 ±0.1</td>
<td>3.5 ±0.1</td>
<td>0.50</td>
</tr>
<tr>
<td>RV end-diastolic diameter (mm)</td>
<td>3.6 ±0.1</td>
<td>3.6 ±0.1</td>
<td>0.68</td>
</tr>
<tr>
<td>PAAT/CL (%)</td>
<td>22.2 ±1.0</td>
<td>12.6 ±1.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stroke volume (ml)</td>
<td>0.25 ±0.01</td>
<td>0.23 ±0.01</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>Pressure-volume analyses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV systolic pressure (mmHg)</td>
<td>26.9 ±0.9</td>
<td>37.0 ±0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RV end-diastolic pressure (mmHg)</td>
<td>2.2 ±0.2</td>
<td>1.9 ±0.1</td>
<td>0.22</td>
</tr>
<tr>
<td>Ees (mmHg/ml)</td>
<td>135 ±43</td>
<td>143 ±35</td>
<td>0.89</td>
</tr>
<tr>
<td>Ea (mmHg/ml)</td>
<td>130 ±16</td>
<td>212 ±27</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ees/Ea</td>
<td>0.95 ±0.20</td>
<td>0.74 ±0.12</td>
<td>0.39</td>
</tr>
<tr>
<td>Eed (mmHg/ml)</td>
<td>6.8 ±0.7</td>
<td>7.6 ±2.3</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Data presented as mean ±SEM. TAPSE, tricuspid annular plane systolic excursion; PAAT/CL, pulmonary artery acceleration time / cyclus length; Ees, end-systolic elastance; Ea, arterial elastance; Ees/Ea, ventriculo-arterial coupling factor; Eed, end-diastolic elastance.
Table S2: Histomorphology at start-of-treatment (day 10)

<table>
<thead>
<tr>
<th></th>
<th>Control (n=5)</th>
<th>PH (n=5)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV cross-sectional area (µm²)</td>
<td>286 ±15</td>
<td>360 ±28</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LV cross-sectional area (µm²)</td>
<td>475 ±33</td>
<td>421 ±32</td>
<td>0.24</td>
</tr>
<tr>
<td>PA wall thickness (%)</td>
<td>6.5 ±0.4</td>
<td>20.0 ±1.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RV capillary density (/mm² *1000)</td>
<td>2.99 ±0.23</td>
<td>3.01 ±0.26</td>
<td>0.96</td>
</tr>
<tr>
<td>LV capillary density (/mm² *1000)</td>
<td>2.73 ±0.13</td>
<td>2.78 ±0.06</td>
<td>0.74</td>
</tr>
<tr>
<td>RV fibrosis (area%)</td>
<td>9.3 ±1.5</td>
<td>10.4 ±1.8</td>
<td>0.47</td>
</tr>
<tr>
<td>LV fibrosis (area%)</td>
<td>7.3 ±0.7</td>
<td>8.4 ±1.8</td>
<td>0.57</td>
</tr>
<tr>
<td>RV inflammation (CD45+ nuclei /mm²)</td>
<td>22.8 ±7.7</td>
<td>18.6 ±8.5</td>
<td>0.72</td>
</tr>
<tr>
<td>LV inflammation (CD45+ nuclei /mm²)</td>
<td>13.4 ±4.0</td>
<td>16.2 ±5.1</td>
<td>0.68</td>
</tr>
</tbody>
</table>

PA wall thickness, relative wall thickness of pulmonary arteries.
Table S3: 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>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta )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>
</tr>
<tr>
<td>( \Delta )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>
</tr>
<tr>
<td>( \Delta )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>
</tr>
<tr>
<td>( \Delta )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>
</tr>
<tr>
<td>( \Delta )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>
</tr>
<tr>
<td>( \Delta )Heart rate</td>
<td>-0.1 ±0.0</td>
<td>-3.2 ±0.5</td>
<td>-1.4 ±0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>( \Delta )Stroke volume</td>
<td>0.3 ±0.2</td>
<td>-6.8 ±0.6</td>
<td>-3.2 ±0.4</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\( \Delta \)PAAT/cl, daily percentage change in pulmonary artery acceleration time normalized for cardiac cycle length; \( \Delta \)RV wall thickness, daily percentage change in right ventricular wall thickness; \( \Delta \)RV end-diastolic diameter, daily percentage change in right ventricular end-diastolic diameter; \( \Delta \)TAPSE, daily percentage change in tricuspid annular plane systolic excursion; \( \Delta \)cardiac output, daily percentage change in cardiac output; \( \Delta \)Heart rate, daily percentage change in heart rate; \( \Delta \)Stroke volume, daily percentage change in stroke volume; PH, PH-rats (treated with vehicle); PH+biso: PH-rats treated with 10 mg/kg bisoprolol once daily from day 10.
<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>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body mass (g)</strong></td>
<td>337 ±6</td>
<td>234 ±4</td>
<td>245 ±5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>BMchange (%/2d)</strong></td>
<td>1.4 ±0.2</td>
<td>-6.1 ±0.9</td>
<td>-6.5 ±0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Tibia length (mm)</strong></td>
<td>36.5 ±0.4</td>
<td>32.1 ±0.4</td>
<td>32.7 ±0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Lungs/tl (g/mm*1000)</strong></td>
<td>33.6 ±1.1</td>
<td>66.8 ±3.0</td>
<td>68.3 ±5.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Lung wet/dry ratio</strong></td>
<td>4.7 ±0.1</td>
<td>4.6 ±0.1</td>
<td>4.4 ±0.1</td>
<td>0.84</td>
</tr>
<tr>
<td><strong>Heart/tl (g/mm*1000)</strong></td>
<td>34.6 ±0.8</td>
<td>39.0 ±1.4</td>
<td>37.7 ±1.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><strong>RV mass/tl (g/mm*1000)</strong></td>
<td>5.2 ±0.3</td>
<td>8.8 ±0.3</td>
<td>8.9 ±0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>LV mass/tl (g/mm*1000)</strong></td>
<td>21.9 ±0.7</td>
<td>16.9 ±0.6</td>
<td>16.0 ±0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>RV / (LV+S)</strong></td>
<td>0.24 ±0.02</td>
<td>0.53 ±0.03</td>
<td>0.56 ±0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Liver/tl (g/mm*1000)</strong></td>
<td>367 ±8</td>
<td>249 ±10</td>
<td>271±11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Spleen/tl (g/mm*1000)</strong></td>
<td>17.5 ±0.5</td>
<td>17.4 ±1.7</td>
<td>16.5 ±1.4</td>
<td>0.93</td>
</tr>
<tr>
<td><strong>Kidneys/tl (g/mm*1000)</strong></td>
<td>60.7 ±1.6</td>
<td>50.0 ±2.0</td>
<td>52.4 ±1.7</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

BMchange, percentage change in body mass of the last 2 days; …/tl, organ mass normalized for tibia length; RV / (LV+S), RV-to-LV (including septum) mass ratio.
Table S5: Histological 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>
</tr>
</thead>
<tbody>
<tr>
<td>PA wall thickness (%)</td>
<td>7.5 ±0.4</td>
<td>40.2 ±2.1</td>
<td>38.5 ±1.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RV cross-sectional area (μm²)</td>
<td>322 ±16</td>
<td>540 ±23</td>
<td>553 ±41</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LV cross-sectional area (μm²)</td>
<td>488 ±16</td>
<td>377 ±18</td>
<td>393 ±18</td>
<td>&lt;0.001</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>0.11</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>
</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>
</tr>
</tbody>
</table>

PA wall thickness, relative wall thickness of pulmonary arterioles.