NEBIVOLOL HAS A BENEFICIAL EFFECT IN MONOCROTALINE-INDUCED PULMONARY HYPERTENSION

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Complete List of Authors:	Pankey, Edward; Tulane University School of Medicine, Pharmacology Edward, Justin; Tulane University School of Medicine, Pharmacology Swan, Kevin; Tulane University School of Medicine, Pharmacology Bourgeois, Camille; Tulane University School of Medicine, Physiology Bartow, Matthew; Tulane University School of Medicine, Pharmacology Yoo, Daniel; Tulane University School of Medicine, Pharmacology Peak, Taylor; Tulane University School of Medicine, Pharmacology Song, Bryant; Tulane University School of Medicine, Pharmacology Chan, Ryan; Tulane University School of Medicine, Pharmacology Murthy, Subramanyam; Tulane University School of Medicine, Pharmacology Prieto, Minolfa; Tulane University School of Medicine, Physiology Giles, Thomas; Tulane University School of Medicine, Internal Medicine / Cardiology Kadowitz, Philip; Tulane University School of Medicine, Pharmacology
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HYPERTENSION

Edward A. Pankey, M.D.[£], Justin A. Edward M.S.[£], Kevin W. Swan, M.S.[£], Camille R. T. Bourgeois M.S.[§], Matthew J. Bartow M.S.[£], Daniel Yoo, M.S.[£], Taylor A. Peak, B.S.[£], Bryant M. Song, B.A.[£], Ryan A. Chan, M.S.[£], Subramanyam N. Murthy, Ph.D.[£], Minolfa C. Prieto M.D. Ph.D.^{§£}, Thomas D. Giles M.D.[¥], and Philip J. Kadowitz, Ph.D.[£]

£Department of Pharmacology, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, Louisiana 70112-2699 USA

§Department of Physiology, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, Louisiana 70112-2699 USA

¥ Department of Internal Medicine, Division of Cardiology, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, Louisiana 70112-2699 USA

Corresponding Author: Philip J. Kadowitz, Ph.D.
Department of Pharmacology, SL83
Tulane University School of Medicine
1430 Tulane Avenue
New Orleans, Louisiana 70112-2699 USA
Phone: 504 988-2634
Fax: 504 988-5283
Email: pkadowi@tulane.edu

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Abstract

Pulmonary hypertension is a rare disorder that without treatment is progressive and fatal within 3-4 years. Current treatment involves a diverse group of drugs that target the pulmonary vascular bed. In addition strategies that increase nitric oxide (NO) formation have a beneficial effect in rodents and patients. Nebivolol, a selective β_1 adrenergic receptor-blocking agent reported to increase NO production and stimulate β_3 receptors has vasodilator properties suggesting that it may be beneficial in the treatment of pulmonary hypertension. The present study was undertaken to determine if nebivolol has a beneficial effect in monocrotaline-induced (60mg/kg) pulmonary hypertension in the rat. These results show that nebivolol treatment (10 mg/kg qd/bid) attenuates pulmonary hypertension, reduces right ventricular hypertrophy and improves pulmonary artery remodeling in monocrotaline-induced pulmonary hypertension. This study demonstrates the presence of β_3 adrenergic receptor immunoreactivity in pulmonary arteries and airways and that nebivolol has pulmonary vasodilator activity. Studies with β_3 receptor agonists (mirabegron, BRL 37344) and antagonists suggest that β_3 receptor-mediated decreases in systemic arterial pressure occur independent of NO release. Our results suggest that nebivolol a selective vasodilating β_1 receptor antagonist stimulates β_3 adrenergic receptors and induces vasodilation by increasing NO production may be beneficial in treating pulmonary hypertensive disorders.

Key words: nebivolol, beta receptors, pulmonary hypertension, nitric oxide, monocrotaline, pulmonary vascular bed, vasodilator response, cardiac output.

Introduction

Pulmonary hypertension is a rare, progressive, and ultimately fatal disorder with 5-year survival rates of approximately 60% (Farber et al. 2015; McLaughlin et al. 2009). However, patients with early diagnosis and adequate treatment have survived for periods up to 18 years. A sustained increase in pulmonary arterial pressure regardless of cause is associated with increased morbidity and mortality (McLaughlin et al. 2009).

The treatment of pulmonary hypertension involves the use of a diverse group of drugs that target the pulmonary vascular bed to decrease pulmonary arterial pressure. These agents include prostacyclin and analogs, calcium channel and endothelin receptor antagonists, PDE5 inhibitors, anticoagulants, and riociguat, a recently approved soluble guanylate cyclase stimulator (Ghofrani et al. 2013; Hyman and Kadowitz 1979; McLaughlin et al. 2009; Sitbon et al. 2002). The effects of NO have been demonstrated to be beneficial in experimental models of pulmonary hypertension mostly in rodent models and in human subjects (Ardehali et al. 2001a,b; Botha et al. 2007; Griffiths and Evans 2005; Ichinose et al. 2004; Pankey et al. 2012). It has also been reported that β receptor antagonists have a favorable effect in the treatment of left ventricular failure and reverse right ventricular remodeling and dysfunction in experimental animal models of pulmonary hypertension (Bogaard et al. 2010; de Man et al. 2012; Ishikawa et al. 2009). However, β receptor blocking agents are not used for the treatment of pulmonary hypertension for fear of worsening right ventricular failure because first generation β receptor antagonists such as propranolol have vasoconstrictor properties and may increase right ventricular afterload (Powell 1980). The mortality of pulmonary hypertension is related to the extent of right ventricular

dysfunction, and it has been suggested and that the β_1 and β_3 adrenergic receptor may be novel targets for treatment of pulmonary hypertension (Rubin 2015). Carvedilol, a third generation β receptor antagonist with vasodilator properties, which is related to alpha-1 receptor blocking effects has been shown to be safe in human subjects with pulmonary hypertension (Rubin 2015). However, less is known about the effects of nebivolol on the pulmonary vascular bed. The present study was undertaken to investigate the effects of nebivolol on the pulmonary vascular bed and on monocrotaline-induced pulmonary hypertension in the rat. The results of these studies show that nebivolol has vasodilatory effects in the pulmonary vascular bed and that chronic treatment with nebivolol has a beneficial effect in monocrotaline-induced pulmonary hypertension in the rat.

Methods

Design of experiments: monocrotaline-induced pulmonary hypertension

The Institutional Animal Care and Use Committee of the Tulane University School of Medicine approved the experimental protocol employed in these studies and all procedures were conducted in accordance with institutional guidelines. All experiments were carried out in Sprague-Dawley rats weighing 300-350g for studies on monocrotaline-induced pulmonary hypertension. The animals were injected with monocrotaline 60 mg/kg into the tail vein and housed in the vivarium. Beginning on day 14, monocrotaline-treated rats received once or twice daily oral gavage of nebivolol (10 mg/kg) for 21 consecutive days. Hemodynamic values were assessed on day 28 in the monocrotaline-treated control rats and day 35 in the nebivolol-treated monocrotaline

rats. The rationale for carrying out hemodynamic assessment on day 35 in nebivolol treated monocrotaline injected rats and on day 28 in control monocrotaline injected rats was to evaluate the hemodynamic and survival benefits of nebivolol treatment since the control monocrotaline treated rats experience mortality after day 28. Long term studies with nebivolol treatment in monocrotaline injected rats should be carried out in the future. The dose of nebivolol used in acute and chronic studies were determined from pilot studies and studies in the literature (Vanhoutte and Gao 2013). The heart and lungs were removed for histological analysis, and all animals were euthanized. Pulmonary arterial pressures and right ventricular hypertrophy are increased on day 28 in monocrotaline rats and compared to vehicle control rats (Tanaka et al. 1996). The mortality rate of monocrotaline-treated control rats markedly increases with an approximate survival rate of 50% by day 35 both in studies in the literature and in previous studies in our laboratory (Abe et al. 2004; Fontoura et al. 2014; Okumura et al. 2015; Pankey et al. 2012). By extending the treatment duration to 35 days, the survival benefit of therapeutic agents can be studied (Cowan et al. 2000; Pankey et al. 2012). In addition, long term studies with nebivolol should be carried out in the future in monocrotaline treated rats.

Measurement of hemodynamic values

For the measurement of hemodynamic variables the animals were anesthetized with thiobutabarbital (Inactin® hydrate) (100 mg/kg ip) (Sigma-Aldrich, St. Louis, MO) and placed in the supine position on an operating table. Supplemental doses of thiobutabarbital were administered ip in order to maintain an adequate level of

anesthesia. The level of anesthesia was assessed by monitoring changes in respiratory activity, heart rate and systemic arterial pressure. Body temperature was maintained with a heating lamp. The trachea was cannulated with a short segment of PE-240 tubing to maintain a patent airway. The animals spontaneously breathed room air. A femoral artery was catheterized with PE-50 tubing for measurement of systemic arterial pressure. The left jugular and femoral veins were catheterized with PE-50 tubing for iv injections and infusions of agents. For pulmonary arterial pressure measurement, a specially designed 3F single lumen catheter with a curved tip and with radio-opaque marker was passed from the right jugular vein and into the main pulmonary artery under fluoroscopic guidance (Picker-Surveyor Fluoroscope, Cleveland, OH) as previously described (Badejo et al. 2010; Pankey et al. 2012). Pulmonary and systemic arterial pressures were measured with Namic Perceptor DT transducers (Boston Scientific, Marlborough, MA), digitized by a Biopac MP100 data acquisition system (Biopac Systems, Goleta, CA) and stored on a Dell PC. Cardiac output was measured by the thermodilution technique with a Cardiomax II computer (Columbus Instruments, Columbus, OH). A known volume (0.2 ml) of room temperature 0.9% NaCl solution was injected into the jugular vein catheter with the tip near the right atrium and changes in blood temperature were detected by a 1.5F thermistor microprobe catheter (Columbus Instruments, Columbus, OH) positioned in the aortic arch from the left carotid artery. The indicator dilution data were stored on the PC. Pulmonary and systemic vascular resistances were calculated by dividing pulmonary arterial and systemic arterial pressure by the cardiac output and are usually expressed as mmHg/ml/min.

Right ventricular hypertrophy assessment

To measure ventricular weight, the hearts were removed following sternotomy and the atria and major vessels were carefully dissected free from the ventricles. The right ventricular free wall (RV) was carefully dissected and separated from the left ventricle and septum (LV+S) and weighed. Fulton's index was calculated as the ratio of RV weight to LV+S weight (RV/LV+S) and used as a measurement of RV hypertrophy (Mam et al. 2010; Pankey et al. 2012).

Histological analysis of small pulmonary arteries

Following euthanasia, the chest was opened, the pulmonary artery was cannulated and the lungs were perfused with 0.9% heparinized saline, infused with 10% paraformaldehyde solution and harvested. The tissues were fixed in 10% paraformaldehyde for 24 hours, paraffin embedded, and 5 μ m sections were obtained for hematoxylin and eosin staining and immunohistochemistry. Digital images of the specimens were photographed using a Nikon Eclipse 50i fluorescence microscope and digital camera (Nikon Instruments Inc., Melville, NY). Lung tissue was analyzed from several animals in each group. This was not intended to be a morphometric study and only qualitative changes in vessel morphology are reported in lung sections from some of the rats.

Immunohistochemistry

Immunohistochemical studies were performed as previously described (Feng et al. 2012). Paraffin-embedded tissue sections were treated in xylene (3X), hydrated in

alcohol baths (100, 95, and 75%) and processed using sequential antibody incubations. For β_3 adrenergic receptor detection a monoclonal rabbit anti- β_3 -adrenergic receptor antibody (1:200 dilution, cat. no. orb15066; Biorbyt, Cambridge, UK) followed by an anti-rabbit secondary antibody, detection using a Vectastain ABC kit (PK-4001, Vector Laboratories, Burlingame, CA), NovaRed (chromogen, SK-4800, Vector Laboratories, Burlingame, CA) and methyl green to counterstain (cat. no. H-3402; Vector Laboratories, Burlingame, CA). The specificity of immunostaining was determined by the omission of the primary antibody and substitution with normal horse serum (data not shown). For immunofluorescence, β_3 -adrenergic receptor was detected in similar tissue sections using a secondary donkey-anti rabbit (Alexa Fluor 568; 1,8000, Life Technology Corp., Grand Island, NY) antibody followed by co-staining of smooth muscle actin (SMA) detected with a donkey anti-mouse antibody (Alexa Fluor 488, 1:8,000 dilution; Life Technology, Corp., Grand Island, NY).

Drugs

Nebivolol (Forest Research Institute, Jersey City, NJ) and BRL 37344 (Tocris Biosciences, Minneapolis, MN) were dissolved in a 15% cyclodextran (Sigma-Aldrich, St. Louis, MO) solution in 0.9% saline. Dobutamine hydrochloride (Cayman Chemical, Ann Arbor, MI), N-w-Nitro-L-Arginine Methyl Ester hydrochloride (L-NAME), and SR59230A (Sigma-Aldrich, St. Louis, MO) were dissolved in 0.9% NaCl. Monocrotaline hydrochloride (Sigma Aldrich, St. Louis, MO) was dissolved in 1 N HCl, neutralized with 0.5N NaOH, and diluted with PBS. The filtered solution was then injected into the tail vein in a dose of 60 mg/kg. U46619 (Cayman Chemical, Ann Arbor,

MI) was dissolved in 95% ethyl alcohol and diluted in 0.9% NaCl solution. Mirabegron (Astellas Pharm US, Northbrook, IL) was dissolved in 70% 0.9% NaCl, 15% Cremophor EL (Sigma Aldrich, St. Louis, MO), and 15% N,N-dimethylformamide (Sigma Aldrich, St. Louis, MO).

Statistics

The hemodynamic data and heart weight data are expressed as mean $\pm$ SE and were analyzed using ANOVA with a post hoc test for repeated measures and with unpaired *t*-tests where indicated. The criteria used for statistical significance was $p < 0.05$.

Results

Response to monocrotaline

The effects of monocrotaline treatment on hemodynamic values in the rat are summarized in figure 1. Monocrotaline when administered in a dose of 60 mg/kg iv resulted in a significant increase in pulmonary arterial systolic, diastolic, and mean pressures with no significant change in systemic arterial pressure or cardiac output when values were measured on day 28 when compared with values in normal control rats (Fig. 1). Monocrotaline treatment increased pulmonary arterial pressure, whereas systemic arterial pressure and cardiac output were unchanged, indicating that pulmonary vascular resistance was increased whereas systemic vascular resistance was unchanged. The 28-day time period was chosen because significant mortality was observed with the monocrotaline treatment after day 28 and this procedure and the 35

day time of hemodynamic measurement have been used in previous studies (Abe et al. 2004; Fontoura et al. 2014; Pankey et al. 2012).

Effect of chronic Nebivolol treatment

The effect of chronic nebivolol treatment on monocrotaline-induced pulmonary hypertension was investigated and these data are summarized in figure 1. Since cardiac output and systemic arterial pressure were unchanged calculated systemic vascular resistance was unchanged. Once a day oral gavage with nebivolol (10 mg/kg) starting on day 14 and continuing for 21 days resulted in a significantly lower pulmonary arterial pressure without a significant change in systemic arterial pressure or cardiac output when values were measured on day 35 when compared to hemodynamic values in untreated animals with monocrotaline-induced pulmonary hypertension (Fig. 1). Twice daily oral gavage with nebivolol (10 mg/kg) resulted in significantly lower pulmonary arterial pressure when compared to pulmonary arterial pressure in the once daily nebivolol treatment group (Fig. 1A). Both groups of animals treated with nebivolol exhibited no mortality and longer term survival studies should be carried out in the future. Previous experiments in our laboratory and studies in the literature indicate that monocrotaline-treated control animals experience an increase in mortality after day 28 (Abe et al. 2004; Okumura et al. 2015; Pankey et al. 2012; Stenmark et al. 2009).

Effect of Nebivolol treatment on right ventricular hypertrophy and small vessel histology

In order to investigate the effect of nebivolol treatment on right ventricular mass

and the histology of small pulmonary arteries, the heart and lungs were removed from normal control rats, from rats with monocrotaline-induced pulmonary hypertension and from rats with monocrotaline-induced pulmonary hypertension treated with nebivolol 10 mg/kg once per day and twice per day. The rats with monocrotaline-induced pulmonary hypertension had significantly higher right ventricular mass (RV/LV + S) than healthy control rats (Fig. 1D). The rats with monocrotaline-induced pulmonary hypertension that were treated with nebivolol had significantly lower RV/LV+S weights when compared to values in untreated rats with monocrotaline-induced pulmonary hypertension (Fig. 1D).

The histologic sections from the left lower lobe lung from three rats show the development of medial hypertrophy in the small pulmonary arteries from animals with monocrotaline-induced pulmonary hypertension. Treatment with nebivolol (10 mg/kg once per day) resulted in a reduction in the extent of medial hypertrophy in small pulmonary arteries when compared to the degree of medial hypertrophy in pulmonary vessels from untreated animals with monocrotaline-induced pulmonary hypertension when evaluated in the lung from the rats and photomicrographs from typical experiments are shown in figure 2. This study was not designed to be a morphometric study and lung sections were not obtained in all experiments.

Acute pulmonary vascular responses to nebivolol

Acute pulmonary vascular responses to iv injections of nebivolol were investigated in the anesthetized rat and these data are summarized in figure 3A. The iv injection of nebivolol in doses of 0.1, 0.3, and 1 mg/kg produced dose-related decreases in pulmonary arterial pressure that were significant at the 0.3 and 1 mg/kg iv doses with

no significant change in cardiac output (Fig. 3A). The decrease in pulmonary arterial pressure under baseline tone conditions was significant at the 1 mg/kg dose of nebivolol and the pulmonary vasodilator response was significantly attenuated after administration of the NOS inhibitor L-NAME in a dose of 50 mg/kg iv (Fig. 3B). The decreases in pulmonary arterial pressure in response to iv injection of nebivolol was increased significantly when baseline pulmonary arterial pressure was increased to ~30 mmHg with an iv infusion of the thromboxane receptor agonist U46619 which was used to increase vasoconstrictor tone in the pulmonary vascular bed (Fig. 3B) (Pankey et al. 2012; Valentin et al. 1996).

The decreases in pulmonary artery pressure in response to the 1mg/kg iv injection of nebivolol in the U46619 infused animals occurred in the absence of a significant change in cardiac output. These results provide support for the hypothesis that the magnitude of pulmonary vasodilator responses to a vasodilator agent are dependent on the level of vasoconstrictor tone in the pulmonary vascular bed. The iv infusion of U46619 did not significantly increase systemic arterial pressure.

The effect of nebivolol on the increase in heart rate in response to the iv injection of the β_1 receptor agonist dobutamine is shown in figure 3C. After administration of nebivolol in a dose of 1mg/kg iv, the increase in heart rate in response to iv injection of dobutamine 3 μ g/kg was significantly attenuated (Fig. 3C). The iv injection of nebivolol, 1 mg/kg significantly reduced heart rate from 368 ± 9 to 325 ± 8 beats/min.

Analysis of systemic vascular responses to the β_3 -adrenergic receptor agonists BRL 37344, and mirabegron

The role of NOS and β_3 adrenergic receptors in mediating responses to nebivolol and the selective β_3 adrenergic receptor agonists BRL 37344 and mirabegron were investigated in the systemic vascular bed and these data are summarized in figure 4. After administration of the selective β_3 adrenergic receptor antagonist SR59230A in a dose of 2mg/kg iv, the decreases in systemic arterial pressure in response to iv injection of nebivolol, the β_3 receptor agonist BRL 37344, and mirabegron were significantly decreased (Fig. 4). In another set of experiments the decrease in systemic arterial pressure in response to iv injection of nebivolol was reduced significantly after administration of the NOS inhibitor L-NAME in a dose of 50 mg/kg iv (Fig. 4A). In contrast, after administration of L-NAME the decrease in systemic arterial pressure in response to iv injection of mirabegron was not changed and the response to BRL 37344 was enhanced significantly (Fig. 4). These data suggest that decreases in systemic arterial pressure in response to nebivolol are mediated by the activation of β_3 adrenergic receptors and NO, however the role of NOS and NO in modulating vasodepressor responses to BRL 37344 and mirabegron is different and the decrease in systemic arterial pressure in response to BRL 37344 was increased whereas the response to mirabegron was unchanged by L-NAME (Fig. 4).

Immunohistochemical localization of β_3 adrenergic receptor in the rat lung

The results of β_3 adrenergic receptor localization studies in the rat are illustrated in figures 5 and 6. Immunofluorescent evaluation of rat lung sections showed positive

immunoreactivity for the β_3 adrenergic receptor (red) on lung airway epithelial cells (white arrows) and on the endothelial cells of pulmonary vessels (white arrowhead) (Fig. 5A). Smooth muscle actin (SMA) was stained green and nuclei were stained blue with DAPI. The results of immunohistochemical studies using a peroxidase technique in the rat lung are illustrated in figure 6. Evaluation of rat lung sections showed positive and specific immunoreactivity for β_3 adrenergic receptors (brown) and nuclei counterstained with light green. There was strong positive immunoreactivity for the β_3 adrenergic receptor in the luminal aspect of airway epithelial cells and endothelial cells and smooth muscle cells of the associated pulmonary artery (Fig. 6). These studies indicate the presence of β_3 adrenergic receptors in the rat lung. Given that there is β_3 receptor expression in the airways and vessel wall, there is a possibility that β_3 receptor activation could alter the distribution of pulmonary blood flow and change V/Q matching.

DISCUSSION

The results of the present study show that chronic treatment with nebivolol in a dose of 10mg/kg by gavage once or twice daily starting on day 14 and continuing for 21 days significantly attenuated the pathologic changes associated with monocrotaline-induced pulmonary hypertension in the rat. In the present study, monocrotaline in a dose of 60mg/kg iv produced a large increase in pulmonary arterial pressure without significantly changing cardiac output or systemic arterial pressure when values were measured. The pulmonary hypertension develops over a 2-3 week period, and mortality begins to occur after day 28 (Gomez-Arroyo et al. 2012; Pankey et al. 2012; Stenmark et al. 2009). The increase in pulmonary arterial pressure in monocrotaline-treated rats was associated with an increase in right ventricular mass and remodeling in the

pulmonary vascular bed that involves medial hypertrophy in small pulmonary arteries (Pankey et al. 2012; Stenmark et al. 2009).

The present results show that treatment with nebivolol in a dose of 10 mg/kg once or twice per day by oral gavage starting on day 14 and continuing for 21 days resulted in a significant decrease in pulmonary arterial pressure when compared to values in untreated rats with monocrotaline-induced pulmonary hypertension when values were measured by right heart catheterization. The reduction in pulmonary arterial pressure with chronic oral nebivolol treatment was associated with a reduction in right ventricular mass and an improvement in the morphology of small intrapulmonary arteries. These beneficial effects of nebivolol treatment on the pulmonary vascular bed and the right ventricle in monocrotaline-induced pulmonary hypertension occurred without a significant change in systemic arterial pressure or cardiac output. The results of the present study indicate that chronic nebivolol therapy can have a beneficial effect on monocrotaline-induced pulmonary hypertension and that it does not cause systemic hypotension or a reduction in cardiac output. These results may suggest that selective β_1 receptor blockade along with β_3 receptor agonism and NO release may provide a beneficial effect in the treatment of pulmonary hypertensive disorders and are consistent with results showing that nebivolol has beneficial effects on pulmonary vascular remodeling not seen with metoprolol (Perros et al. 2015; Rubin 2015).

β adrenergic receptor blocking agents are used in the treatment of congestive heart failure and most patients with pulmonary hypertension succumb as a consequence of right heart failure (Bogaard et al. 2009; Voelkel et al. 2013). Treatment with β receptor blocking agents reduces mortality by about 30% in patients with left-

sided heart failure, but β blocking agents are not used in the treatment of pulmonary hypertension and cor pulmonale (Doughty et al. 1997; McLaughlin et al. 2009). Moreover, first generation β blocking agents can increase right ventricular afterload, which would have a deleterious effect in patients with pulmonary hypertension (Galderisi and D'Errico 2008; Powell 1980). Third generation β receptor blocking agents such as nebivolol and carvedilol have important vasodilator properties that may be useful in the treatment of pulmonary hypertension (Grinnan et al. 2014; Vanhoutte and Gao 2013). Nebivolol is a highly selective β_1 adrenergic receptor blocking agent with β_3 stimulating properties and the ability to release NO (Bowman et al. 1994). Nebivolol has been reported to induce vasodilation by releasing NO from the endothelium and also possesses important antioxidant properties (Broeders et al. 2000). The ability of nebivolol to increase NO formation and its reported antioxidant properties would be expected to enhance NO bioavailability and produce a beneficial effect in pulmonary hypertensive disorders (Vanhoutte and Gao 2013). In the present study in the anesthetized rat, the iv injection of nebivolol produced dose-related decreases in pulmonary arterial pressure which were enhanced when vasoconstrictor tone in the pulmonary vascular bed was increased to a high steady value with a U46619 infusion. Inasmuch as decreases in pulmonary arterial pressure could be elicited without a significant change in cardiac output, these data show that nebivolol has vasodilator activity in the pulmonary vascular bed. The results from histochemical studies indicate the presence of β_3 receptor immunoreactivity in the endothelium and smooth muscle cells in small pulmonary arteries from the rat and also show that β_3 receptors are highly expressed in the airways of the rat. The results of the present study show that nebivolol

has vasodilator properties in the pulmonary vascular bed, and suggest that the vasodilator response correlates with the expression of β_3 adrenergic receptors in pulmonary arteries in the rat. The results of hemodynamic studies and β_3 receptor localization studies are novel. The ability of nebivolol to reduce baseline heart rate and to attenuate the positive chronotropic effect of dobutamine confirms its β_1 receptor blocking properties.

The present data indicate that nebivolol induces vasodilation in the pulmonary vascular bed by releasing NO. It is postulated that impaired NO formation may play a role in monocrotaline-induced pulmonary hypertension and in a number of cardiovascular disorders (Napoli and Ignarro 2009; Stenmark et al. 2009). It has also been reported that interventions that increase NO concentration in the lung including studies with NO donors, nitrite injection, and gene and cell based therapies which increase NOS expression have a beneficial effect in monocrotaline-induced pulmonary hypertension (Baber et al. 2007; Casey et al. 2010; Champion et al. 2002; Pankey et al. 2012; Pankey et al. 2011; Somanna et al. 2014). It is therefore possible that enhanced NO formation may play an important role in the beneficial effect of nebivolol in the present study along with the β_1 blocking effects and the β_3 stimulating properties.

Patients with pulmonary hypertension usually die from right heart failure (Badesch et al. 2012; Borrie et al. 2011; McLaughlin et al. 2009). The major drugs for the treatment of pulmonary hypertension target the pulmonary vascular bed and decrease pulmonary arterial pressure and right ventricular afterload. Although it is known that β receptor blocking agents improve right ventricular function in heart failure, the first generation agents like propranolol have vasoconstrictor effects and are not

used to treat pulmonary hypertension (Galderisi and D'Errico 2008; Powell 1980).

Nebivolol is a selective β_1 receptor antagonist that stimulates β_3 receptors and releases NO from the endothelium. It is our hypothesis that by blocking β_1 receptors in the heart, stimulating β_3 receptors, and releasing NO in the lung that nebivolol would be effective in the treatment of pulmonary hypertensive disorder. In this regard, the present results indicate that nebivolol has a beneficial effect in monocrotaline-induced pulmonary hypertension and show that nebivolol has pulmonary vasodilator activity mediated by NO release and receptor stimulation. However, studies comparing responses to nebivolol and the newly developed, highly selective β_3 receptor agonist mirabegron suggest that the release of NO by nebivolol may not involve β_3 receptor activation in that systemic hypotensive responses to mirabegron and to BRL 37344 are not attenuated by the NOS inhibitor L-NAME whereas the β_3 receptor antagonist SR59230A attenuated the vasodepressor responses to nebivolol, BRL 37344, and mirabegron. The results of the present study may be interpreted to suggest that the effects of nebivolol on NO release and β_3 receptor activation may not be related but may be beneficial in the treatment of pulmonary hypertension.

In summary, the results of the present study show that chronic treatment with nebivolol has a beneficial effect on monocrotaline-induced pulmonary hypertension in the rat. These results indicate that β_3 receptors are located on the endothelium and smooth muscle cells in small pulmonary arteries and that nebivolol has vasodilator activity in the pulmonary vascular bed. The present study shows that nebivolol treatment decreases pulmonary arterial pressure without changing systemic arterial pressure or cardiac output in the rat with monocrotaline-induced pulmonary

hypertension. The reduction in pulmonary arterial pressure was associated with a decrease in right ventricular mass and improvement in the morphology of small pulmonary arteries. In these studies, decreases in systemic arterial pressure in response to nebivolol and the β_3 agonists BRL 37344 and mirabegron were attenuated by the β_3 receptor antagonist SR59230A, whereas only responses to nebivolol were attenuated by the NOS inhibitor L-NAME. The present results suggest that the beneficial effects of nebivolol may result from β_1 receptor blockade, β_3 receptor stimulation and the release of NO, which may not be related to β_3 receptor agonism. It is also possible that the reported antioxidant properties and inhibitory effect on asymmetrical dimethylarginine induced by nebivolol may increase NO bioavailability and may play a role in the beneficial effect of nebivolol in pulmonary hypertension.

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Figure Legend

Figure 1: Bar graphs comparing mean pulmonary arterial pressure (A), mean systemic arterial pressure (B), cardiac output (C), and RV/(LV-S) ratios (D) in control rats at 28 days, monocrotaline plus placebo treated rats at 28 days, monocrotaline plus nebivolol (10 mg/kg for 21 days) treated rats at 35 days, and monocrotaline plus nebivolol (10 mg/kg twice daily for 21 days) treated rats at 35 days. n = number of rats and * indicates $p < 0.05$ ANOVA with repeated measures, group comparison. These data show that increases in pulmonary arterial pressure and right ventricular mass in response to monocrotaline are significantly attenuated by treatment with nebivolol. Cardiac output and mean systemic arterial pressure are unchanged in these experiments so that systemic vascular resistance is not changed. In these experiments nebivolol has a beneficial effect in monocrotaline-induced pulmonary hypertension.

Figure 2: Photomicrographs of hematoxylin and eosin stained sections of the left lower lung lobe from a healthy control rat (A), from a monocrotaline plus placebo treated rat (B), and from a monocrotaline plus nebivolol 10 mg/kg treated rat (C). The small artery in B panel shows marked medial thickening when compared to the small artery in the control group in panel A. The section in panel C shows less medial thickening than the section in panel B. These data indicate that nebivolol treatment improves the morphology of small pulmonary arteries in monocrotaline-treated rats.

Figure 3: A) Bar graphs comparing changes in mean pulmonary arterial pressure and cardiac output in response to iv injection of nebivolol in doses of 0.1, 0.3, and 1 mg/kg. B) Bar graphs comparing responses to iv injection of nebivolol (1 mg/kg) under baseline tone conditions after treatment with L-NAME and when pulmonary arterial pressure was increased to ~ 30 mmHg with an iv infusion of U46619. C) Bar graph showing the effect of nebivolol in a dose of 1 mg/kg iv on the increase in heart rate in response to the β_1 receptor agonist dobutamine. n = number of rats. * indicates $p < 0.05$, group comparison. These data indicate that nebivolol has NO dependent pulmonary vasodilator activity.

Figure 4: Bar graphs showing the effect of the selective β_3 receptor antagonist SR52390A in a dose of 2 mg/kg iv and the NOS inhibitor L-NAME in a dose of 50 mg/kg iv the on changes in mean systemic arterial pressure in response to iv injections of A) nebivolol 0.3 mg/kg, B) BRL 37344 30 μ g/kg and C) mirabegron 0.3 mg/kg in the rat. n = number of rats and * indicates $p < 0.05$ when compared to control, group comparison. These data indicate that hypotensive responses to nebivolol, BRL 37344 and mirabegron are mediated by β_3 adrenergic receptors and that the response to nebivolol is mediated by activation of β_3 adrenergic receptors and the release of NO.

Figure 5: A) Enlarged image of a fluorescent photomicrograph showing the presence of β_3 adrenergic receptors (red) in the pulmonary airway epithelium (white arrows) and the endothelium of the associated vessel (white arrowhead). Nuclei are stained blue (DAPI) and smooth muscle actin is stained green. B) Immunofluorescence of β_3 adrenergic receptor was observed after using a secondary donkey-anti rabbit antibody (Alexa Fluor 568, Red). C) Immunofluorescence of SMA was observed after using a donkey anti-mouse antibody (Alexa Fluor 488, Green). This figure shows the presence of β_3 adrenergic receptors in the rat lung. Images were captured at 400x magnification.

Figure 6: Oil immersion photomicrograph of a rat airway and associated pulmonary artery stained for β_3 adrenergic receptors (brown) and a light green nuclear counterstain. The luminal cells of the airway showed positive and specific immunoreactivity on the apical aspect of the cells for β_3 adrenergic receptors. The associated pulmonary vessel showed positive and specific immunoreactivity in the endothelial cell layer and in the underlying vascular smooth muscle cells for β_3 adrenergic receptors. The observation that β_3 receptors are expressed in the airways and pulmonary arteries suggests that β_3 receptor activation could alter the V/Q relationship in the lung.

Figure 1

n = 6-10

Control Nebivolol 10 mg/kg qd
MCT Nebivolol 10mg/kg bid

A

B

C

D

Figure 2

Figure 3

A

B

C

Figure 4

Figure 5

Figure 6