Nebivolol improves diastolic dysfunction and myocardial remodeling through reductions in oxidative stress in the transgenic (mRen2) rat

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Submitted 5 December 2011; accepted in final form 14 March 2012

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ELEVATIONS IN SYSTOLIC BLOOD PRESSURE (SBP) over time contribute to pressure-dependent left ventricular (LV) hypertrophy (LVH) and maladaptive tissue remodeling, which leads to heart failure (19, 21, 34, 53). The initial manifestations of heart failure are characterized by increases in interstitial fibrosis and diastolic dysfunction (34, 53). The reductions in initial filling rate (IFR) and increases in relaxation time that characterize diastolic dysfunction occur due to remodeling of the LV and are associated with alterations in the elastic properties driven by the development of interstitial fibrosis (38, 44). Inappropriate activation of the renin-angiotensin-aldosterone system (RAAS) plays a central role in blood pressure regulation as well as contributes to myocardial fibrosis through the generation of ROS (35, 37, 38, 43). These RAAS-induced increases in ROS are due to mitochondrial uncoupling and increased activation of NADPH oxidase (22, 40). Excess ROS contribute to reductions in bioavailable nitric oxide (NO) by the conversion of locally released NO to peroxynitrite (ONOO−), a highly reactive species that contributes to lipid peroxidation (28, 45). NO is an essential component of endothelial function as well as several metabolic pathways that modulate growth and proliferation, such as 5′-AMP-activated protein kinase (AMPK) signaling pathways (15). Targeting NO to reduce oxidative stress and improve metabolic signaling has the potential to reduce myocardial fibrosis and to correct diastolic dysfunction (6).

β1-Adrenergic receptor blockade has been shown to improve contractile function, and recent data have suggested that nebivolol targets improvements in diastolic function in human and rodent models of heart failure (26, 49, 55). Nebivolol is a highly cardiac-selective β1-adrenergic receptor blocker that contributes to vasodilation by increasing bioavailable NO through its coupling to the β3-receptor subtype (13). It has been reported that nebivolol treatment leads to reductions in NADPH oxidase activity in the heart and vascular tissue (13, 32, 41, 43), and recent work has highlighted improvements in insulin metabolic signaling and enhancement in bioavailable NO in skeletal muscle (25) and kidney tissue (51) in a transgenic (TG) (mRen2)27 (Ren2) rat model. This Ren2 rat manifests tissue overexpression of the mouse renin gene and tissue ANG II with elevations in SBP, insulin resistance, and increases in myocardial oxidative stress and diastolic dysfunction (4, 50). The Ren2 rat provides a unique model to investigate the effects of nebivolol on hypertension/renin-angiotensin system (RAS)-induced diastolic dysfunction as a result of oxidative stress and impaired metabolic signaling. Thereby, we hypothesized that targeting reductions in myocardial interstitial fibrosis with nebivolol would correct myocardial diastolic dysfunction through improvements in metabolic signaling pathways by reducing NADPH oxidase activity and stimulation of AMPK and bioavailable NO.

METHODS

Animals and Treatments

Animal procedures were approved by the Animal Care Committees of the University of Missouri and housed according to National
Institutes of Health guidelines. Male transgenic heterozygous (+/−) Ren2 and Sprague-Dawley (SD) rats were received at 5–6 wk of age from the Wake Forest University School of Medicine and Vascular Research Center (Winston-Salem, NC). Rats were randomly assigned to placebo (SD-C or Ren2-C) or nebivolol treatment (SD-N and Ren2-N) groups. Ren2-N and SD-N rats received 10 mg·kg⁻¹·day⁻¹ nebivolol released via an implanted osmotic minipump for 21 days (51). Insulin stimulation was performed by an intravenous injection of 2 units of insulin 5 min before euthanization to evaluate insulin-induced Akt activation.

SBP and LV Weight

Restraint conditioning was initiated on the day of initial blood pressure measurements. SBP was measured in triplicate on separate occasions throughout the day using the tail-cuff method (Student Oscillometric Recorder, Harvard Systems) before the initiation of treatment and on days 19 or 20 before euthanization. At autopsy, the LV plus septum was dissected free of the right ventricle, atria, and great vessels. The LV plus septum normalized to body weight is a commonly used index of LVH in nonobese rodent models.

In Vivo Cine-MRI

Noninvasive cine-MRI scans were performed on rats after treatment with nebivolol or vehicle using a 7-T/210-mm horizontal-bore MRI (Agilent, Palo Alto, CA) equipped with a 63-mm quad rupture great vessels. The LV plus septum normalized to body weight is a commonly used index of LVH in nonobese rodent models.

LV systolic function. LV stroke volume (SV) was calculated as follows: SV = EDV – ESV, where EDV is end-diastolic volume and ESV is end-systolic volume. LV ejection fraction (EF) was measured as follows: EF = SV/EDV × 100%. LV cardiac output (CO) was assessed as follows: CO = SV × HR, where HR is heart rate.

LV diastolic function. First derivatives of LVV against time were calculated to extract the diastolic filling rates and relaxation time. Diastolic IFR was defined as the slope of the first four time points on the early diastolic curve (Fig. 1C). Diastolic peak filling rate (PFR) was defined as the maximum derivative of the LVV curve. Diastolic relaxation time (DRT) was defined as the time duration from the end of systolic phase to the peak filling phase. Normalized DRT, which is the ratio of DRT to the R-R interval, was used to compare LV diastolic relaxation among groups, where normalized DRT = [DRT × (HR/6,000)].

Myocardial Interstitial Fibrosis

Fixed paraffin sections of the LV were evaluated with Verhoef-van Gieson (VVG) stain, which stains elastin (black), nuclei (blue-black), collagen (pink), and connective tissue (yellow), as previously described (45, 55). Briefly, 4-μm longitudinal and transvers sections of the LV were stained with VVG. Slides were blindly analyzed by one or two observers with a Nikon50i (Nikon, Tokyo, Japan) microscope. To keep uniformity and avoid error, each section was thoroughly checked. Five representative areas were captured with ×40 images from each section with a CoolSNAP cf camera (Roper Scientific Germany, Trenton, NJ). The areas and intensities of pink regions, which are indicative of interstitial fibrosis, were quantified on both transverse and longitudinal sections of the LV using MetaVue software (Molecular Devices, Sunnyvale, CA). The average grayscale intensity due to collagen was recorded. An average value of these intensities was determined for each animal.

Markers of Oxidative Stress

ROS formation. Myocardial ROS was measured by chemiluminescence as previously described (55). Briefly, LV tissue sections were homogenized and centrifuged, and supernatants (whole homogenate) were then removed and placed on ice. Whole homogenate (100 μl) was added to 1.4 ml of 50 mM phosphate (KH2PO4) buffer (150 mM NaCl, 1 mM EGTA, 5 μM lucigenin, and 100 μM NADPH; pH 7.0) in dark-adapt counting vials. After dark adaptation, samples were counted on a luminoimeter, and all counts were averaged. Samples were then normalized to total protein in the whole homogenate.

NADPH oxidase activity. NADPH oxidase activity was determined in LV plasma membrane fractions as previously described (14, 50, 55). Briefly, plasma membrane fractions were incubated with NADPH (100 μmol/l) at 37°C. Total enzyme oxidase activity was determined by measuring the conversion of Radical Detector (Cayman Chemical, Ann Arbor, MI) in the absence and presence of the NADPH inhibitor diphenylepinoiodonium sulfate (500 μmol/l) using spectrophotometric (450 nm) techniques.

Immunostaining. LV sections were immunostained for Rac1, p47phox, phosphorylated (p)-Akt (Thr308), p-endothelial NO synthase (eNOS; Ser1177), and heme oxygenase (HO) and quantitated as previously described (14, 50, 55). Briefly, 4-μm paraffin-embedded transverse sections of the LV from different treatments were dewaxed in citriSolv, rehydrated in ethanol series, and antigen retrieved in sodium citrate buffer at 95°C. Sections were then incubated with mouse anti-Rac1 antibody (1:200), mouse anti-p47phox (1:100), mouse anti-p-eNOS (Ser1177; 1:50), rabbit anti-HO (1:75) antibodies overnight at room temperature. Sections were washed, incubated with 1:300 of secondary antibodies, and developed with a DAB chromogen. Images were acquired and signal intensities were measured by MetaVue software as average grayscale intensities.

Quantification of AMPK and p-AMPK by Western blot analysis. Total AMPK and p-AMPK were analyzed by Western blot analysis. Protein concentrations of tissue homogenates were measured as previously described (55). Samples (40 μg/lane) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). Blots were incubated overnight at 4°C with.
primary antibodies against AMPK and p-AMPK (1:1,000 dilution, Cell Signaling Technology). After a rinse, blots were incubated with secondary antibodies (1:5,000 dilution of each antibody) for 1 h at room temperature. Bands were visualized by chemiluminescence, and images were recorded using a Bio-Rad ChemiDoc XRS image-analysis system. Quantitation of phosphorylated protein band density, normalized to the density of total protein for each sample, was performed using Image Lab (Bio-Rad).

Statistical Analysis

All values are expressed as means ± SE. Statistical analyses were performed with Sigma Stat (Aspire Software, Ashburn, VA) using Student’s t-tests or ANOVA with Fisher’s least-significant-difference test for post hoc comparisons. Significance was accepted at $P < 0.05$.

RESULTS

Experimental Parameters

As previously described (25, 51), SBP was elevated in the Ren2-C group compared with the SD-C group (207 ± 8 vs. 144 ± 8 mmHg, $P < 0.05$). Moreover, there was increase in the insulin resistance index in the vehicle-treated Ren2 model compared with the SD-C group at the end of the treatment period. Treatment with nebivolol in the Ren2 model led to reductions in SBP (183 ± 4 mmHg, $P < 0.05$) and the insulin resistance index compared with age-matched controls over 3 wk of treatment (51) but had no impact on total body weight.

Nebivolol Improves Diastolic Relaxation But Not Hypertrophy in the Ren2 Heart

LV systolic and diastolic functions were determined using in vivo cardiac-MRI after 2 wk of treatment with placebo or nebivolol (Table 1). There were increases in DRT along with reductions in IFR as indexes of diastolic function in the Ren2-C group compared with the SD-C group ($P < 0.01$; Fig. 1, A–C). Treatment with nebivolol over 2 wk led to reductions in DRT as well as increases in IFR ($P < 0.05$). There were no observable changes in systolic function and body weight in the
Nebivolol Reduces NADPH Oxidase in the Ren2 Heart

NADPH oxidase is a major source of ROS in the myocardium, and it generates ROS through the assembly of a multisubunit protein complex including the cytoplasmic subunit p47^{phox} and the GTP-binding protein Rac1. There were increases in total NADPH oxidase activity in the Ren2-C myocardium compared with the SD-C myocardium (P < 0.05; Fig. 4A), and this increased activity was attenuated by nebivolol treatment for 3 wk (P < 0.05). Commensurate with total enzyme activity, there were increases in NADPH oxidase subunits p47^{phox} and Rac1 in Ren2 rats, which were similarly improved with nebivolol treatment (Fig. 4B).

Nebivolol Increases p-eNOS in the Ren2 Heart

Nebivolol treatment resulted in a substantial reduction in 3-NT content, a marker for ONOO− formation. The ONOO− radical is a highly ROS that can be formed endogenously by the interaction of NO and O_2. Thus, to ascertain whether nebivolol induces eNOS activation in the Ren2 heart, Ser^{177} phosphorylation of eNOS (activation) was measured. p-eNOS (Ser^{177}) was diminished in the Ren2-C group compared with the SD-C group (P < 0.05) and was significantly increased with nebivolol treatment in the Ren2 myocardium (P < 0.05; Fig. 5A).

Nebivolol Improves Insulin-Induced Akt Activation in the Ren2 Heart

There were decreases in p-Akt (Thr^{308}) in the Ren2-C heart compared with the SD-C heart (P < 0.05), and Akt activation was restored by nebivolol treatment in the Ren2 heart (P < 0.05; Fig. 5B).

Nebivolol Increases p-AMPK in the Ren2 Heart

Next, we sought to determine whether nebivolol treatment could increase AMPK activation. While there were no differences in p-AMPK (Thr^{172}) over total AMPK in the Ren2-C group compared with the SD-C group, there was a substantial increase in p-AMPK after treatment with nebivolol for 3 wk (P < 0.05; Fig. 5C).

**DISCUSSION**

Herein, we demonstrated that nebivolol treatment reduced myocardial indexes of oxidative stress associated with increased tissue RAS in the Ren2 rat. Indeed, 3 wk of treatment with a cardioselective β-blocker substantially reduced NADPH oxidase activity and improved myocardial oxidative stress in the Ren2 heart.

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**Table 1. Effects of nebivolol on in vivo cardiac function in Ren2 and SD rats evaluated by cine-MRI**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SD-C Group</th>
<th>SD-N Group</th>
<th>Ren2-C Group</th>
<th>Ren2-N Group</th>
</tr>
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<tbody>
<tr>
<td>Sample size</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>310 ± 14</td>
<td>322 ± 23</td>
<td>273 ± 16</td>
<td>266 ± 16</td>
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<tr>
<td>Heart rate and left ventricular morphology</td>
<td>328 ± 14</td>
<td>342 ± 18</td>
<td>386 ± 14*</td>
<td>352 ± 9</td>
</tr>
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<td>Septal wall thickness, mm</td>
<td>1.49 ± 0.09</td>
<td>1.46 ± 0.03</td>
<td>1.86 ± 0.05</td>
<td>1.98 ± 0.05</td>
</tr>
<tr>
<td>End-diastolic volume, μl</td>
<td>570 ± 24</td>
<td>592 ± 34</td>
<td>544 ± 52</td>
<td>507 ± 47</td>
</tr>
<tr>
<td>End-systolic volume, μl</td>
<td>123 ± 17</td>
<td>124 ± 16</td>
<td>132 ± 25</td>
<td>152 ± 25</td>
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<tr>
<td>Systolic indexes</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Stroke volume, μl</td>
<td>448 ± 15</td>
<td>469 ± 23</td>
<td>412 ± 30</td>
<td>354 ± 33</td>
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<tr>
<td>Cardiac output, ml/min</td>
<td>147 ± 5</td>
<td>160 ± 9</td>
<td>158 ± 7</td>
<td>125 ± 12</td>
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<td>Ejection fraction, %</td>
<td>79 ± 2</td>
<td>79 ± 2</td>
<td>77 ± 3</td>
<td>71 ± 4</td>
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<td>Diastolic indexes</td>
<td></td>
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<td>Initial filling rate, μl/ms</td>
<td>7.69 ± 0.86</td>
<td>7.12 ± 0.54</td>
<td>1.88 ± 0.6*</td>
<td>5.22 ± 1.06*</td>
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<td>Peak filling rate, μl/ms</td>
<td>9.90 ± 1.17</td>
<td>11.40 ± 1.16</td>
<td>12.05 ± 0.64</td>
<td>9.89 ± 1.06</td>
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<tr>
<td>Diastolic relaxation time, ms</td>
<td>28.0 ± 3.7</td>
<td>32.9 ± 1.7</td>
<td>40.3 ± 3.1*</td>
<td>31.8 ± 3.4</td>
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<td>Normalized diastolic relaxation time (to R-R interval)</td>
<td>0.15 ± 0.02</td>
<td>0.19 ± 0.00</td>
<td>0.26 ± 0.02*</td>
<td>0.19 ± 0.02*</td>
</tr>
</tbody>
</table>

Data are means ± SE. Sprague-Dawley (SD) and mRen2 rats were randomly assigned to placebo (SD-C or Ren2-C) or nebivolol treatment (SD-N and Ren2-N) groups. *P < 0.05 vs. the SD-C group; †P < 0.05 vs. the Ren2-C group.

Ren2-C group compared with the SD-C group, nor was there a treatment effect.

Commensurate with the observed changes in diastolic function, there were increases in measures of LV mass. There were increases in interventricular septal wall thickness on MRI as well as LV weight normalized to total body weight in Ren2-C rats compared with SD-C rats (P < 0.01; Fig. 2, A and B). Despite reducing SBP, nebivolol treatment did not improve septal wall thickness or LV weight in the Ren2-N group.

**Nebivolol Improves Interstitial Fibrosis in the Ren2 Heart**

To determine whether the improvement in diastolic relaxation in nebivolol-treated Ren2 was attributable to fibrosis rather than hypertrophy, we evaluated myocardial fibrosis by VVG staining (for collagen). There were increases in interstitial fibrosis in the Ren2-C group compared with the SD-C group (P < 0.05). This increase in collagen content and interstitial fibrosis was reduced with nebivolol treatment in the Ren2-N group (Fig. 2C).

**Nebivolol Reduces Oxidative Stress in the Ren2 Heart**

An imbalance in redox status and “oxidant stress” contributes to the development of fibrosis. In this context, ROS are important signaling molecules that activate many redox signaling pathways, and nitrosylation of tyrosine residues (3-NT) can alter protein functions. There were increases in total ROS formation as well as 3-NT content in the Ren2-C group compared with the SD-C group (P < 0.05; Fig. 3, A and B). Treatment with nebivolol led to improvements in 3-NT content to a greater extent than total ROS formation. HO catalyzes a multistep reaction to release Fe(III) and the antioxidant biliverdin (5). There was a trend of reductions in HO compared with the SD-C group, which was significantly increased with nebivolol treatment in the Ren2-N group (P < 0.05; Fig. 3C).

**Nebivolol Reduces NADPH Oxidase in the Ren2 Heart**

Nebivolol Improves Insulin-Induced Akt Activation in the Ren2 Heart

Next, we sought to determine whether nebivolol treatment could increase AMPK activation. While there were no differences in p-AMPK (Thr^{172}) over total AMPK in the Ren2-C group compared with the SD-C group, there was a substantial increase in p-AMPK after treatment with nebivolol for 3 wk (P < 0.05; Fig. 5C).
oxidase activity, as previously reported in skeletal muscle (25) and kidney tissue (51). Nebivolol treatment also increased myocardial HO levels. Since HO catalyzes a multistep reaction to release Fe(III) and the antioxidant biliverdin (5), this may have contributed to the reduced myocardial ROS and 3-NT after treatment with the β-blocker. Ren2 heart tissue displayed reduced AMPK and eNOS activity as well as reduced insulin-mediated Akt activation. Significant increases in the levels of p-AMPK (Thr172), p-eNOS (Ser1177), and p-Akt (Thr308) (Fig. 5), indicative of stimulatory phosphorylation and activation of these proteins in the Ren2 myocardium after nebivolol treatment are consistent with our observations of improved insulin...
metabolic signaling, increased bioavailable NO, and reduced interstitial fibrosis in the Ren2 myocardium. Moreover, these effects of nebivolol treatment are consistent with the protective effects of nebivolol observed in the heart, skeletal muscle (25), and kidneys of Zucker obese rats (51). Our findings extend previous work from preclinical and clinical work suggesting that nebivolol improves heart failure associated with preserved EF, an important finding given the paucity of effectiveness of current treatment approaches for the correction of diastolic dysfunction (8, 13, 26, 41, 49, 55).

The finding in the present investigation of reduced filling rate and increases in diastolic relaxation of LV on cine-MRI are consistent with observations of diastolic dysfunction in this TG model (14, 50). Noninvasive cardiac MRI has superior spatial and temporal resolution that permits the visualization of the entire heart, enabling an accurate estimation of cardiac dimensions and volumes over time (1, 42, 48, 54, 55). Volume-time curves on cine-MRI provide a determination of filling rates derived from changes of LVV during early diastole and have demonstrated great accuracy compared with echocardi-
ography in preclinical and clinical models (1, 17, 42). Although a faster frame rate is preferred to increase the accuracy of measurements, the present frame rate of 8–12 ms/frame is sufficient for the analysis of the heart in the rat, as shown in Fig. 1, B and C. In the present study, the diastolic function parameters were derived based on the estimation of LVV and HR. Therefore, we presented normalized DRT (to R-R interval; Table 1 and Fig. 1A) to eliminate effects on this parameter from variations of HR. We made the same treatment for IFR and PFR; the HRs did not show any effects on these results. Diastolic dysfunction is associated with deceased filling rates and increased diastolic relaxation. The presence of diastolic dysfunction is a critical prognosticator for the future loss of systolic function. That our findings occurred in a model of

Fig. 4. Nebivolol reduces NADPH oxidase activity and NADPH oxidase subunit protein expression in the Ren2 heart. A: bar graph demonstrating increased NADPH oxidase activity in the myocardium of Ren2-C rats relative to SD-C rats (P < 0.05). Nebivolol reduced NADPH oxidase activity in the Ren2-N myocardium compared with the Ren2-C myocardium (P < 0.05). OD, optical density units. B, top: representative confocal images showing immunofluorescence for the NADPH oxidase subunits Rac1 (left) and p47phox (right). Bottom, bar graphs showing average grayscale intensities for the NADPH oxidase subunits Rac1 (left) and p47phox (right). *P < 0.05 vs. the SD-C group; †P < 0.05 vs. the Ren2-C group.
tissue RAS overactivation extends previous work on the impact of metabolic heart disease on the initiation and progression of heart failure (14, 50). Thereby, the finding that the RAS, when inappropriately activated, initiates changes in myocardial tissue relaxation complements previous work done in obese rodent models such as the Zucker obese rat and \( \text{db/db} \) mouse (54, 55). Furthermore, our observation that nebivolol improves diastolic function in this RAS-dependent model is novel.

Myocardial interstitial deposition of the extracellular matrix (ECM) is a critical remodeling event associated with functional alterations in diastolic relaxation. Our observation of interstitial fibrosis in the Ren2 heart with VVG staining, which demonstrates excess interstitial collagen deposition, further supports the relationship between interstitial fibrosis and diastolic dysfunction in this model. ANG II has been shown to enhance collagen deposition and contributes to stiffening of the myocardium, reductions in elasticity, and associated impairments in relaxation and filling (3, 19, 21, 27). This enhanced deposition of ECM proteins has also been observed in a variety of rodent models in which cardiac hypertrophy develops as a result of hypertension associated with the activation of the RAAS (34). The observation that hypertrophy occurred in parallel with interstitial fibrosis in the Ren2 heart further refines this relationship. However, our finding that nebivolol improved interstitial fibrosis but not hypertrophy is novel and suggests that this compound has a specific impact on metabolic signaling pathways that influence collagen turnover mechanisms.

Recent work has suggested that oxidative stress is associated with interstitial fibrosis in the progression of heart failure (44) through the generation of the superoxide anion by the enzyme complex NADPH oxidase (38). The balance between ROS production and elimination plays a key role in preserving cardiac function. In the present study, transgenic Ren2 rats exhibited increases in NADPH oxidase enzyme activity and expression of the subunits \( p47^{\text{phox}} \) and \( \text{Rac1} \) in the myocardium associated with reductions in the antioxidant HO and increases in ROS production, as determined by total ROS production and ONOO\(^{-} \) formation. Our finding that nebivolol improved HO suggests that targeting bioavailable NO leads to improvements in antioxidant mechanisms. In this regard, the data suggest that NO stabilizes and increases HO expression in vascular smooth
muscle cells and in the myocardium (7, 9). Moreover, an increase in ONOO\textsuperscript{−} formation was observed in the Ren2 myocardium, which was significantly reduced by nebivolol treatment. Reduction in ONOO\textsuperscript{−} formation in the Ren2 myocardium by nebivolol treatment appears to be particularly important in that superoxide may react with NO released by eNOS to generate ONOO\textsuperscript{−}, further supporting a direct role for nebivolol in improving bioavailable NO, antioxidant mechanisms, and diastolic function. ONOO\textsuperscript{−} is a potent oxidizing agent implicated in several pathologies. ONOO\textsuperscript{−} can modify proteins by nitration of tyrosine residues, which modulate the catalytic activity of the enzyme or prevent protein phosphorylation of the OH moiety on the tyrosine residue. These modifications result in alterations of function and promote a biological effect (36). Recently, a proteomic approach identified a total of 48 putative cardiac proteins containing nitrotyrosine that undergo age-dependent protein tyrosine nitrification (20). However, investigators recently have demonstrated that among these proteins, MnSOD, a key mitochondrial antioxidant enzyme, and sarco (endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase type 2 are nitrated at one or more tyrosine residues and implicated in different pathological condition, including diabetes, atherosclerosis, and ANG II-induced hypertension (52).

Recent work has suggested that hearts with hypertrophy due to pressure overload are characterized by alterations in AMPK activity (46). AMPK is a stress-activated protein kinase that works as a metabolic sensor of cellular ATP levels and is a critical intermediary in myocardial energetics. AMPK activation plays a cardioprotective role by improving LV function and survival in heart failure (2, 12). Alternatively, deficiency in AMPK has been shown to exacerbate cardiac hypertrophy and contractile dysfunction (47). There is increasing interest in the cross talk between NO and AMPK (23), a kinase that generally downregulates oxidant and fibrotic pathways. Previous studies have demonstrated that AMPK stimulates the production of endothelium-derived NO, and targeting activation of AMPK by pharmacological intervention reduces agonist-stimulated protein synthesis in cultured cardiac myocytes via an Akt-dependent pathway (16, 18, 33, 46), thereby suggesting that AMPK may be a critical energy modulator of interstitial fibrosis and LV function. Further work supports that AMPK enhances eNOS activity by direct Ser\textsuperscript{1177} phosphorylation and increases NO bioavailability (45). Our finding that nebivolol restored p-AMPK in the Ren2 rat support that nebivolol actions on NO may occur in an AMPK-dependent mechanism. Furthermore, our observation that insulin-stimulated p-Akt was improved with nebivolol suggests the cross talk with AMPK and Akt. AMPK is known to regulate insulin-dependent pathways in skeletal muscle and heart tissue (24). AMPK activation has also been shown to regulate insulin-dependent activation of PBK/Akt in cardiac tissue glucose uptake as well as maladaptive tissue remodeling (4, 11, 16, 29). In this context, recent reports have suggested that the activation of AMPK is dependent, in part, on ANG II activation of H\textsubscript{2}O\textsubscript{2} production, suggesting that AMPK is a redox-sensitive kinase (4, 29).

The Ren2 model develops cardiac hypertrophy that leads to adaptive alterations in the level of the contractile filaments (56). The increase in β-myosin heavy chain expression (56) may partially be responsible for the observed LV diastolic dysfunction in the Ren2 heart. A small shift from the α- to β-myosin heavy chain isomer could cause functional dysfunctions or heart failure in the human heart (30) and animal models (48). However, the unique feature of this β-blocker, and what we have observed, is that nebivolol does not impact hypertrophy but rather corrects fibrotic abnormalities. The present work supports that nebivolol improves diastolic dysfunction by targeting NO and reducing myocardial oxidative stress, which decrease interstitial fibrosis. Future work is needed on exploring the other potential mechanisms of action of nebivolol, such as effects on sarcomere proteins, in improving heart function.

**Limitations**

Our data indicate that targeting increases in bioavailable NO using nebivolol may play a role in improving diastolic dysfunction in Ren-2 rats. It should be noted that while there is no direct evidence linking bioavailable NO to diastolic dysfunction, there are correlative data in humans suggesting that nebivolol improves diastolic function and reduces mortality in elderly patients with heart failure (10, 31, 49). However, further studies are required to elucidate this mechanism. Moreover, the observation that nebivolol led to modest reductions in weight deserves mention as traditional β-blockers promote modest weight gain. It is possible that putative β\textsubscript{3}-agonist properties of nebivolol mediate modest weight loss by inducing the transdifferentiation of white adipose tissue into brown adipose tissue. Indeed, in rodents and humans, β\textsubscript{3}-receptor agonists stimulate the oxidation of fats, reduce fat weight, improve insulin sensitivity, and spare lean body mass (39). However, we did not include a comparator to confirm that all the beneficial effects of nebivolol are through its ability to increase bioavailable NO and not via its β-blocking effect. Our data on weight loss in Ren-2 rats, although interesting, should also be explored further to determine weight loss may be mitigating a portion of this response. It is possible the modest weight loss contributed to the improvements in diastolic function.

**Summary**

In summary, our observations support that targeting NO improves diastolic function through AMPK/Akt and reduced NADPH oxidase generation of ROS and improved HO. Biochemical alterations are associated with interstitial fibrosis in this model of RAAS overactivation.

**ACKNOWLEDGMENTS**

Exceptional support was provided by the Veterans Affairs Biomolecular Imaging Center of the Harry S. Truman Veterans Affairs Hospital. The authors acknowledge the technical contributions of Rebecca Schneider, Nathan Rehmer, and Mona Garro as well as students Safwan Hyder and Bennett Krueger. The authors thank Brenda Hunter for assistance in preparing the manuscript.

**GRANTS**

This work was supported by National Institutes of Health Grants R01-HL-73101 and R01-HL-107910 (to J. R. Sowers), R03-AG-040638 (to A. Whaley-Connell), and HL-051952 (to C. M. Ferrari). There was also support from Veterans Affairs Merit System Grant 0018 (to J. R. Sowers) as well as CDA-2 (to A. Whaley-Connell) and the ASN-ASP Junior Development Grant in Geriatric Nephrology (to A. Whaley-Connell) supported by a T. Franklin Williams Scholarship Award. Funding was also provided by Atlantic Philanthropies, Incorporated, the John A. Hartford Foundation, the Association of Specialty Professors, and the American Society of Nephrology.

AJP-Heart Circ Physiol • doi:10.1152/ajpheart.01126.2011 • www.ajpheart.org
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