Nebivolol Improves Diastolic Dysfunction and Myocardial Remodeling Through Reductions in Oxidative Stress in the Zucker Obese Rat

Xinli Zhou, Lixin Ma, Javad Habibi, Adam Whaley-Connell, Melvin R. Hayden, Roger D. Tilmon, Ashley N. Brown, Jeong-a Kim, Vincent G. DeMarco, James R. Sowers

Abstract—Insulin resistance is associated with obesity and may be accompanied by left ventricular diastolic dysfunction and myocardial remodeling. Decreased insulin metabolic signaling and increased oxidative stress may promote these maladaptive changes. In this context, the β-blocker nebivolol has been reported to improve insulin sensitivity, increase endothelial NO synthase activity, and reduce NADPH oxidase–induced superoxide generation. We hypothesized that nebivolol would attenuate diastolic dysfunction and myocardial remodeling by blunting myocardial oxidant stress and promoting insulin metabolic signaling in a rodent model of obesity, insulin resistance, and hypertension. Six-week-old male Zucker obese and age-matched Zucker lean rats were treated with nebivolol (10 mg · kg⁻¹ · day⁻¹) for 21 days, and myocardial function was assessed by cine MRI. Compared with untreated Zucker lean rats, untreated Zucker obese rats exhibited prolonged diastolic relaxation time (27.7 ± 2.5 versus 40.9 ± 2.0 ms; P < 0.05) and reduced initial diastolic filling rate (6.2 ± 0.5 versus 2.8 ± 0.6 μL/ms; P < 0.05) in conjunction with increased homeostatic model assessment of insulin resistance (7 ± 2 versus 95 ± 21; P < 0.05), interstitial and pericapillary fibrosis, abnormal cardiomyocyte histoarchitecture, 3-nitrotyrosine, and NADPH oxidase–dependent superoxide. Nebivolol improved diastolic relaxation (32.8 ± 0.7 ms; P < 0.05 versus untreated Zucker obese), reduced fibrosis, and remodeling in Zucker obese rats, in concert with reductions in nitrotyrosine, NADPH oxidase–dependent superoxide, and improvements in the insulin metabolic signaling, endothelial NO synthase activation, and weight gain (381 ± 7 versus 338 ± 14 g; P < 0.05). Results support the hypothesis that nebivolol reduces myocardial structural maladaptive changes and improves diastolic relaxation in concert with improvements in insulin sensitivity and endothelial NO synthase activation, concomitantly with reductions in oxidative stress. (Hypertension. 2010;55:880-888.)

Key Words: nebivolol ■ oxidative stress ■ insulin resistance ■ diastolic relaxation ■ MRI

Obesity-induced cardiomyopathy is characterized by impaired left ventricular (LV) relaxation in association with insulin resistance (IR).1,2 This cardiomyopathy develops independent of blood pressure, ischemia, impaired systolic function, and age.1,3 Impaired insulin metabolic signaling and increased generation of reactive oxygen species (ROS) play important roles in maladaptive myocardial remodeling3–5 and impaired diastolic relaxation.6,7 Excessive ROS in the heart and vasculature, derived from several enzymatic sources, including NADPH oxidase,8 can lead to decreased bioavailable NO and reduced delivery of glucose and insulin to myocardial tissue.4,9

β-Adrenergic receptor blockers have clinical use in treating heart failure, but traditional β-blockers have been associated with weight gain and worsening of IR. Nebivolol, a third-generation β₁-receptor blocker improves diastolic dysfunction10 and reduces mortality in elderly heart failure patients.10–12 Nebivolol does not have adverse effects on IR, nor does it cause weight gain.11–15 Potential beneficial effects of nebivolol on diastolic dysfunction associated with IR may be mediated through improvements in bioavailable NO, reductions in ROS, and improvements in insulin metabolic signaling.

We hypothesized that nebivolol would improve diastolic dysfunction in an IR rat through reductions in NADPH oxidase activity and improved insulin metabolic signaling. To address our hypothesis, we used young (6- to 7-week-old) Zucker obese (ZO) rats, an IR model that manifests increased myocardial oxidative stress and diastolic dysfunction.16–18 Thus, the ZO rat provides a unique model to investigate the effect of nebivolol on impaired myocardial diastolic relaxation as a result of oxidative stress, reduced bioavailable NO, and impaired insulin metabolic signaling.16
Methods

Animals
Animal procedures were approved by the University of Missouri animal care committees and housed according to National Institutes of Health guidelines.

Drug Preparation
Nebivolol was dissolved in 50% dimethyl sulfoxide/50% propylene glycol to a final concentration of 70 mg/mL and filter sterilized. The solution (or its vehicle) was loaded into a model 2004 Alzet pump and inserted subcutaneously behind the shoulder blades under brief isoflurane anesthesia.

Systolic Blood Pressure and Total Body Weight
Within a day or 2 of termination of the experiment, systolic blood pressures (SBPs) were measured in triplicate using the tail-cuff method as described previously.19

Homeostatic Model Assessment of IR
A venous blood sample was collected from a subset of fasting rats in each treatment group at the end of the study, and plasma was stored at −80°C. Glucose and insulin were measured by an automated hexokinase G-6-PDH assay and an ELISA kit specific for rat insulin, respectively. Homeostatic model assessment was calculated by taking the product of the glucose (in millimoles per liter) and insulin (in microunits per milliliter) values and dividing by 22.

Cine-MRI
MRI scans were performed on rats after 2 weeks of treatment with nebivolol or vehicle. For details describing procedures to determine LV functional parameters, please see the online Data Supplement at http://hyper.ahajournals.org.

Light Microscopic Analysis for Myocardial Interstitial Fibrosis
Fixed paraffin sections of the left ventricle were evaluated with Verhoeff-van Gieson stain, which stains elastin (black), nuclei (blue black), collagen (pink), and connective tissue (yellow), as described previously.19 For details of the morphometric analysis please see the online Data Supplement.

Ultrastructural Analysis With Transmission Electron Microscopy
Details of fixation, embedding, sectioning, and staining procedures have been described previously.19 A JOEL 1400-EX transmission electron microscope was used to view all of the samples.

Quantitative Analysis of Mitochondrial Number, Enzyme Level, and Activity

Mitochondrial Quantification
Fixed samples were immunolabeled with an antibody to complex IV-1 and viewed under a laser confocal microscope (Bio-Rad) and a multiphoton confocal system. Images were captured with Laser-sharp software and the immunofluorescence quantified using Meta-Morph software. For details please see the online Data Supplement.

Citrates Synthase Activity
Citrates synthase activity in mitochondrial fractions of LV tissue was determined as described previously.20 For details please see the online Data Supplement.

β-Hydroxyacyl-Coenzyme A Dehydrogenase Activity
β-Hydroxyacyl-coenzyme A dehydrogenase (β-HAD) activity was measured as described previously with modifications.21 For details please see the online Data Supplement.

Markers of Oxidative Stress

3-Nitrotyrosine Immunostaining
3-Nitrotyrosine (3-NT) was quantified as described previously.19,22

Superoxide Formation
Superoxide was determined by chemiluminescence, as described previously.19,22 Superoxide values were normalized to total protein in the whole homogenate and expressed as relative light units per second per milligram of protein.

NADPH Oxidase Activity
Activity was determined in plasma membrane fractions as described previously.19,22

Nox2, Nox4, Rac1, p47phox, Endothelial NO Synthase, and Ser1177 Endothelial NO Synthase Immunostaining
LV sections were immunostained and quantified as described previously.19,22

Quantification of IRS-1, Ser473 Akt, and Ser1177 eNOS by Western Blot Analysis
Protein concentrations of tissue homogenates were measured as described previously.19 Briefly, samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Reactive bands were detected by chemiluminescence, and images were recorded using a Bio-Rad ChemiDoc XRS image analysis system. Quantitation of protein band density, normalized to β-actin band density, was performed using Quantity One software (Bio-Rad). Data are reported as the normalized protein band density in arbitrary units.

Statistical Analysis
All of the values are expressed as mean±SE. Statistical analyses were performed with Sigma Stat (Aspire Software Intl) using Student t tests or ANOVA with the Fisher least significant difference test for post hoc comparisons. Significance was accepted as P<0.05.

Results

Nebivolol Effects on Body Weight and SBP
Both the ZO-control (C) and ZO-nebivolol (N) groups had higher body weights compared with the Zucker lean group (ZL-C; P<0.05), although there was a modest reduction in weight gain in the ZO-N rats compared with ZO-C rats (P<0.05; Table 1). Although SBP tended to be higher in ZO-C and normalized in ZO-N compared with ZL-C rats, the ANOVA main effect was not significant (P>0.05). A t test indicated a significant decrease in SBP in ZO-N compared with ZO-C rats (128±5 versus 156±12 mm Hg; P<0.05).

Nebivolol Effects on IR
ZO-C rats had elevated fasting plasma insulin levels (Table 1) compared with ZL-C (177±32 versus 21±5 μU/mL; P<0.05); nebivolol treatment tended to blunt this hyperinsulinemia, but the trend was not significant (134±29 μU/mL; P>0.05 versus ZO-C). ZO-C rats were slightly hyperglycemic compared with ZL-C rats (11.4±2 versus 7.3±0.5 mmol/L; P<0.05), although glucose levels were normalized in ZO-N rats (7.1±0.4 μM; P<0.05 versus ZO-C). Homeostatic model assessment-IR indicated a significant decrease in SBP in ZO-N compared with ZO-C rats (128±5 versus 156±12 mm Hg; P<0.05).
Nebivolol Improves Diastolic Relaxation
LV diastolic relaxation time, diastolic peak filling rate, and initial filling rate, as well as septal wall thickness, ejection fraction, and stroke volume were determined via cine MRI (Figure 1A and 1B; Table 2). There were increases in septal wall thickness in ZO-C relative to ZL-C rats (P<0.05), and nebivolol tended to blunt the increased septal wall thickness in the ZO strain (P<0.05). LV diastolic relaxation time was prolonged in ZO-C compared with ZL-C rats (40.88±1.94 versus 27.68±2.50 ms; P<0.05). Nebivolol decreased diastolic relaxation time in the ZO rats (ZO-N: 32.77±0.73 ms; P<0.05 versus ZO-C). Diastolic initial filling rate was reduced in ZO-C versus ZL-C rats (P<0.05). Heart rate, diastolic peak filling rate, systolic ejection fraction, and stroke volume were similar among all of the groups (Table 2).

Table 1. Effects of Nebivolol on Body Weight, Blood Pressure, Fasting Plasma Glucose and Insulin, and Homeostatic Model Assessment-IR of 9-Week–Old ZL and ZO Rats

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>ZL-N</th>
<th>ZO-C</th>
<th>ZO-N</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>245±7 (6)</td>
<td>240±7 (6)</td>
<td>381±7 (6)*</td>
<td>338±14 (6)*†</td>
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<td>SBP, mm Hg</td>
<td>136±11 (5)</td>
<td>133±10 (6)</td>
<td>156±12 (6)</td>
<td>128±5 (7)</td>
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<td>Glucose, mmol/L</td>
<td>7.3±0.5 (6)</td>
<td>6.6±0.6 (2)</td>
<td>11.4±1.2 (8)*</td>
<td>7.1±0.4 (6)†</td>
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<tr>
<td>Insulin, μU/mL</td>
<td>21±5 (6)</td>
<td>18±12 (2)</td>
<td>177±32 (8)*</td>
<td>134±29 (6)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>7.2±1.9 (6)</td>
<td>5.6±4.1 (2)</td>
<td>95±21 (8)*</td>
<td>41±8 (6)*†</td>
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</table>

Numbers in parentheses are sample sizes. HOMA indicates homeostatic model assessment. Data represent mean±SE. *P<0.05 vs ZL-C. †P<0.05 vs ZO-C.

Nebivolol Reduces Myocardial Interstitial Fibrosis
Interstitial fibrosis, as represented by the average grayscale intensity of Verhoeff-van Gieson staining (for collagen), was increased in ZO-C compared with ZL-C rats (P<0.001) and stolic relaxation time in the ZO rats (ZO-N: 32.77±0.73 ms; P<0.05 versus ZO-C). Diastolic initial filling rate was reduced in ZO-C versus ZL-C rats (P<0.05). Heart rate, diastolic peak filling rate, systolic ejection fraction, and stroke volume were similar among all of the groups (Table 2).

Figure 1. Nebivolol improves diastolic relaxation, reduces myocardial fibrosis, and improves ultrastructural remodeling of myocardial capillaries. A, Representative cine-MRIs illustrate early diastole phases (frame 7 to 12 of 16 captured) in a cardiac cycle. The top row demonstrates prolonged diastolic relaxation time in ZO-C compared with reduced diastolic relaxation time and increased initial filling rate in ZO-N rats shown in the bottom row. B, Bar graph shows diastolic relaxation times for experimental groups. C, Light micrographs show representative LV sections stained with Verhoeff-van Gieson stain, which stains collagen pink. The bar graph below shows that nebivolol attenuates the increased interstitial fibrosis in the ZO myocardium. Scale bar: 50 μm. *P<0.001 vs ZL-C; †P<0.01 vs ZO-C. D, Representative transmission electron microscopy micrographs at ×400 demonstrate constricted capillaries in ZO-C rats that improved with nebivolol treatment in ZO-N (top). Small dark arrows point to a capillary, which is shown at higher magnification in the white boxes. Scale bar: 2 μm. The X in the center of the bottom left panel shows pericapillary fibrosis in the ZO-C heart, and the area inside the dark box is shown at higher magnification in the white box (scale bar; 0.1 μm). From bottom to top, each image shows the capillary lumen, a single endothelial cell layer composing the capillary wall, a prominent layer of pericapillary collagen, and cardiomyocytes. A pericapillary collagen layer was not observed in the ZO-N (bottom right). White arrows indicate an area of abundant endothelial cell transcytotic vesicles in the ZO-N.
improved in ZO-N compared with ZO-C rats (P<0.01; Figure 1C). Coronary arterioles of ZO rats also exhibited perivascular fibrosis, which was attenuated by nebivolol (data not shown).

Nebivolol Improves Myocardial Capillary and Tissue Remodeling

On ultrastructural analysis, there were constricted capillaries with pericapillary fibrosis in the ZO-C myocardium, which improved with nebivolol treatment (Figure 1D). There was also an increase in endothelial cell transcytotic vesicles in ZO-N rats compared with ZO-C rats (Figure 1D).

Nebivolol Improves Mitochondrial Function and Structure

Mitochondrial complex IV subunit 1, the last enzyme in the respiratory electron transport chain and an index of mitochondrial number, was quantitated by protein immunofluorescence. Citrate synthase, a Kreb cycle enzyme used to assess aerobic capacity of mitochondria, and β-HAD, an enzyme involved in mitochondrial fatty acid β-oxidation, were also quantitated. Control ZO myocardium exhibited increased (P<0.05) mitochondrial numbers (increase in complex IV-1) without differences in citrate synthase and β-HAD (Figure 2A through C, respectively). Complex IV-1 signal in the ZO myocardium was reduced with nebivolol treatment (P<0.05). Normal levels of citrate synthase and β-HAD activities, coupled with the increased numbers of mitochondria in the ZO myocardium, suggest that it takes more mitochondria in the ZO myocardium to provide normal levels of ATP. Nebivolol treatment restored the enzyme activities and mitochondrial number in ZO rats to levels similar to those in the ZL-C and ZL-N myocardium. Compared with ZL-C, ZL-N, and ZO-N, ZO-C rats demonstrated increases in intermyofibrillar mitochondria and disorganized sarcomere structure (Figure 2D). ZO-C mitochondria exhibited swollen and disrupted cristae (Figure 2E). Nebivolol abrogated the increased mitochondrial biogenesis and improved mitochondrial sarcomere organization (Figure 2D and 2E).

<table>
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<tr>
<th>Parameter, Cine MRI Data</th>
<th>ZL-C</th>
<th>ZL-N</th>
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<td>Sample size, n</td>
<td>10</td>
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<td>10</td>
<td>6</td>
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<tr>
<td>Age, wk</td>
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<td>8.14±0.2</td>
<td>8.76±0.09</td>
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<td>Heart rate, bpm</td>
<td>368±7</td>
<td>383±6</td>
<td>376±10</td>
<td>363±8</td>
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<td>Stroke volume, μL</td>
<td>372.5±20.1</td>
<td>314.8±28</td>
<td>350.4±19.2</td>
<td>303.7±23.8</td>
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<tr>
<td>Ejection fraction, %</td>
<td>80.9±1.5</td>
<td>75.6±2.4</td>
<td>82.6±1.9</td>
<td>80.2±1.0</td>
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<td>End-diastolic septal wall thickness, mm</td>
<td>1.41±0.04</td>
<td>1.37±0.03</td>
<td>1.78±0.09*</td>
<td>1.61±0.04</td>
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<td>Initial filling rate, μL/ms</td>
<td>6.16±0.49</td>
<td>5.67±0.82</td>
<td>2.84±0.58*</td>
<td>3.88±0.35</td>
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<tr>
<td>Peak filling rate, μL/ms</td>
<td>8.07±0.61</td>
<td>7.80±0.91</td>
<td>8.61±0.98</td>
<td>7.06±0.51</td>
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<td>Diastolic relaxation, ms</td>
<td>27.68±2.50</td>
<td>27.50±3.33</td>
<td>40.88±1.95*</td>
<td>32.77±0.73†</td>
</tr>
</tbody>
</table>

Data represent mean±SE. *P<0.05 vs ZL-C. †P<0.05 vs ZO-C.

Nebivolol Reduces Myocardial NADPH Oxidase Activity

NADPH oxidase activity was elevated in ZO-C compared with that of ZL-C rats (P<0.05; Figure 3A). ZO-N rats had lower NADPH oxidase activity versus ZO-C rats (P<0.05). There were also increases in NADPH oxidase subunit immunostaining of Nox2, Nox4, Rac1, and p47phox in ZO-C rats compared with ZL-C rats (P<0.05 for each; Figure 3C). Expression levels for all of these proteins were normalized after nebivolol therapy (P<0.05 versus ZO-C rats for each protein).

Nebivolol Reduces Myocardial Oxidative Stress

There were increases in superoxide levels in ZO myocardium compared with ZL rats (P<0.05; Figure 3B). ZO-N rats had lower superoxide levels compared with ZO-C rats (P<0.05). There were increases in myocardial 3-NT content in the ZO-C (32.8±2.1 grayscale intensities) compared with ZL-C rats (19.2±2.1 grayscale intensities; P<0.05), and 3-NT was reduced in nebivolol-treated ZO rats (22.1±2.1 grayscale intensities; P<0.05; Figure 3D).

Nebivolol Modulates the IRS-1/Akt/eNOS Signaling Pathway

To explore whether nebivolol modulates myocardial insulin signaling, we examined the IRS-1/Akt/eNOS cascade by immunoblotting (Figure 4A through 4C). Although the protein levels of IRS-1 or the ratio of phosphorylated Akt at Ser473 to total Akt were similar in the ZL-C, ZL-N, and ZO-C groups, there was a 2.26-fold increase in IRS-1 content and a 2.05-fold increase in activated Akt, that is, the ratio of phosphorylated Akt at Ser473 to total Akt, in ZO rats treated with nebivolol compared with ZO controls (each P<0.05; Figure 4A and 4B).

To ascertain whether nebivolol induced eNOS activation in ZO heart tissue, Ser1177 phosphorylation of eNOS (activation) and total eNOS protein were measured using Western blots (Figure 4C). There were increases in Ser1177 eNOS (1.79-fold) and eNOS (1.59-fold) in ZO rats with nebivolol treatment compared with ZO controls (each P<0.05). The ratio of
Ser\textsuperscript{1177} eNOS to total eNOS in the ZO-C rat was not different from ZL-C rats (P>0.05); however, the ratio of Ser\textsuperscript{1177} eNOS to total eNOS was increased in ZO-N compared with ZO-C rats (P<0.05).

To more specifically evaluate myocardial or coronary arteriolar changes in total eNOS and phosphorylated eNOS at Ser\textsuperscript{1177}, we performed semiquantitative immunofluorescence analyses. Both total eNOS and Ser\textsuperscript{1177} eNOS immunofluorescent signals were detected in the myocardium and in the vascular wall of coronary arterioles (Figure 4D). Ser\textsuperscript{1177} eNOS was increased in ZO rats treated with nebivolol compared with ZO controls (Figure 4D; P<0.05).

**Discussion**

This investigation demonstrates that nebivolol improves LV diastolic function and insulin sensitivity; reduces myocardial NADPH oxidase activity, oxidative stress, and interstitial fibrosis; reduces capillary and mitochondria ultrastructural abnormalities; and enhances the IRS-1/Akt/eNOS signaling pathway. Finally, nebivolol, unlike traditional \( \beta \)-blockers, which promote weight gain,\textsuperscript{23} reduced weight gain in the ZO rat.

In metabolic heart disease, relaxation abnormalities often appear before the onset of contractile dysfunction. Diastolic dysfunction is characterized by a decrease in the ability of the left ventricle to fill with blood during the early diastolic filling.\textsuperscript{24} Using high-resolution cine-MRI, we observed delayed LV diastolic relaxation and decreased early diastolic filling in the ZO rat in the absence of measurable contractile dysfunction. Nebivolol treatment improved LV diastolic relaxation in concert with reductions in interstitial fibrosis.

There were substantive ultrastructural abnormalities of the coronary microvasculature and intermyofibrillar mitochondria in ZO hearts, which were reversed with nebivolol treatment. Capillaries were constricted, exhibited diffuse pericapillary fibrosis, and contained fewer transcytotic vesicles in endothelial cells. ZO hearts had marked increases in intermyofibrillar mitochondria, which resulted in disorganized sarcomere structure as observed previously in other rodent models of IR.\textsuperscript{19,25–27} The increase in complex IV-1 in ZO-C rats, whereas indicative of an increase in number of mitochondria, may also reflect a compensatory response, which limits oxidative stress by diverting molecular oxygen toward the terminal steps in aerobic metabolism leading to ATP synthesis. Indeed, there is recent evidence that mitochondria in the untreated diabetic ZO heart produce excessive superoxide.\textsuperscript{28} There was a marked reduction in mitochondria...
in nebivolol-treated ZO hearts, yet they exhibited improved cristae structure and sarcomere organization.

The balance between ROS production and elimination plays a key role in preserving cardiac function; excessive myocardial ROS precipitates impairment of myocardial function and abnormalities in cardiac structure. The NADPH oxidase complex serves as a major source for the generation of superoxide in the cardiovascular system. ZO myocardium had increases in NADPH oxidase activity, superoxide, and 3-NT; these increases were blunted with nebivolol therapy. There were increases in NADPH subunits Nox2, Nox4, p47phox, and Rac1 in ZO-C hearts; however, the expression of all of these proteins was significantly reduced by nebivolol treatment. These data suggest that NADPH oxidase activation may be the primary mediator of increased superoxide production in the heart of the ZO rat. In diabetic fatty rats, the mitochondria may also be a significant source of ROS in the myocardium. Interestingly, these young ZO rats did not exhibit increases in mitochondrial citrate synthase activity or β-HAD, a marker of fatty acid β-oxidation. Excessive fatty acid oxidation can lead to increased mitochondrial superoxide synthesis. Others have shown increased fatty acid uptake in ZO hearts in the absence of increased fatty acid oxidation.

IR is associated with endothelial dysfunction and impaired vasodilation, which may be partially dependent on excess generation of ROS. Superoxide may react with NO released by eNOS to generate peroxynitrite. The increased 3-NT staining in the ZO myocardium observed in this study is indirect evidence of formation of peroxynitrite. Peroxynitrite can contribute to endothelial dysfunction by reducing the bioavailability of NO in part by promoting uncoupling of eNOS, which results in eNOS-derived increases in superoxide synthesis.

eNOS activity is regulated by posttranslational modifications, including phosphorylation of specific sites and protein-
activation of the IRS-1/Akt pathway plays a central role in the regulation of myocardial glucose metabolism, cell survival, and cardiac function. In particular, the IRS-1/Akt pathway activates eNOS by phosphorylation at the Serine\(^{1177}\) residue, which promotes NO production. Our laboratory has observed previously that there was a relationship between increased NADPH oxidase activity and diminished Akt activation in vivo. In this study, there were significant increases in phosphorylated Ser\(^{1177}\) Akt, as well as total IRS-1 protein, in ZO rats with nebivolol treatment in the absence of significant differences in the expression of IRS-1, Akt phosphorylation, and eNOS, as well as eNOS phosphorylation between control ZO and ZL rats at 9 weeks of age. This somewhat surprising result suggests that there could be a compensatory mechanism under hyperinsulinemia in the young ZO rats. Despite the apparent normal state of eNOS activation in the untreated ZO myocardium observed in this study, it is likely that nebivolol acts to enhance the bioavailability of NO by reducing levels of ROS and promoting activation of the IRS-1/Akt/eNOS pathway. Indeed, nebivolol dilates the coronary arterial microvasculature via an agonist effect on endothelial \(\beta\)\(_3\)-adrenoreceptors that promotes the release of NO, in part by dephosphorylating eNOS on Thr\(^{495}\). Such a scenario predicts improvements in cardiac function and structure.

**Figure 4. Nebivolol improves insulin metabolic signaling and enhances coronary arteriolar eNOS activation.** A. The bar graph shows a quantitative densitometric analysis for IRS-1 protein (normalized to \(\beta\)-actin) as a percentage of ZL-C (ie, fold increase). Representative protein bands for IRS-1 and \(\beta\)-actin are shown above the bar graph. Nebivolol increased IRS-1 protein level in the ZO myocardium compared with ZO-C. B. Representative Western blots show phosphorylated Ser\(^{473}\) Akt and total Akt, as well as their corresponding \(\beta\)-actin bands. The bar graph shows the ratio of phospho-Akt/total Akt expressed as a percentage of ZL-C control. C, Representative Western blots show phosphorylated eNOS at Ser\(^{1177}\) and total eNOS, as well as their corresponding \(\beta\)-actin bands. The bar graph displays the ratio of phospho-eNOS Ser\(^{1177}\)/total eNOS expressed as a percentage of ZL-C control. D, Representative confocal micrographs show total eNOS (top row) and phospho-eNOS Ser\(^{1177}\) (bottom row) immunofluorescence in the myocardium and coronary arterioles of ZL and ZO rats. Scale bar: 50 \(\mu\)m. The bar graphs to the right show average grayscale intensity measures of total eNOS and phospho-eNOS Ser\(^{1177}\) immunofluorescence in the endothelium of coronary arterioles of ZL and ZO rats and indicate that nebivolol enhances eNOS phosphorylation at Ser\(^{1177}\).* "P<0.05 vs ZL-C; †P<0.05 vs ZO-C.

Phosphorylation of eNOS at Ser\(^{1177}\) is associated with increased enzyme activity. The ZO rat exhibits impairments in endothelial function and endothelium-dependent vasodilation. In this study, both eNOS expression and eNOS phosphorylation at Ser\(^{1177}\) were increased in coronary arterioles of ZO rats with nebivolol treatment. Commensurate with these changes in eNOS, transmission electron microscopy measurements demonstrated constricted capillaries with decreased endothelial transcytotic vesicles in ZO rats that improved with nebivolol treatment. Our data suggest that nebivolol improves capillary endothelial function and remodeling in the ZO rat and that this may be because of a reduction in oxidative stress, promotion of NO bioavailability, and an increase in NO biosynthesis. This compliments a previous observation that nebivolol inhibits endothelial dysfunction via diminishing superoxide formation by NADPH oxidase in the heart of angiotensin II–treated rats. In the heart, among the 3 isoforms of NO synthase, eNOS is constitutively expressed in both endothelial cells and cardiomyocytes. NO produced by eNOS is not only a primary determinant of blood vessel tone but also a regulator of cardiac function. In this regard, eNOS-derived NO facilitates increased myocardial diastolic relaxation and decreased O\(_2\) consumption.
The notion that traditional \(\beta\)-blockers promote modest weight gain may be attributed to several factors, including reductions in the metabolic rate and insulin sensitivity.\(^{23,41}\) However, our data suggest that nebivolol-treated ZO rats, on average, weighed less by 11% \((P<0.05)\), were more insulin sensitive, and were normotensive compared with control ZO rats. This is consistent with observations in human trials wherein nebivolol has largely been found to be weight and metabolically neutral compared with more traditional agents.\(^{14,15}\) However, it is possible that this modest weight loss contributes to the overall improvements in insulin sensitivity, SBP, and diastolic function. In obese humans, modest weight loss can lead to improvements in insulin sensitivity, decreased blood pressure, and improved endothelial function. It is also possible that the \(\beta_3\) agonist properties of nebivolol mediate modest weight loss by inducing transdifferentiation of white adipose tissue into brown adipose tissue.\(^{42}\) Indeed, in rodents and humans, \(\beta_3\) receptor agonists stimulate oxidation of fats, reduce fat weight, improve insulin sensitivity, and spare lean body mass.\(^{43}\)

**Perspectives**

This investigation indicates that nebivolol, a highly cardioselective \(\beta_1\)-receptor blocker, improves myocardial remodeling and diastolic dysfunction, as well as IRS-1/Akt/eNOS signaling pathways, by inhibiting myocardial NADPH oxidae–mediated superoxide formation in an obese insulin-resistant rodent model. These findings suggest that nebivolol prevents or at least delays the development of cardiomyopathy associated with IR. Finally, nebivolol, unlike traditional \(\beta\)-blockers, which promote weight gain, reduced weight gain in the ZO rat. These data are highly clinically relevant as a first report using an animal model of obesity that ascribes potential mechanisms to explain observed human improvements in diastolic function and insulin sensitivity in patients on nebivolol.

**Acknowledgments**

Exceptional support was provided by the Veterans’ Affairs Biomedical Imaging Center at the Harry S. Truman Veterans’ Affairs Hospital, as well as the Electron Microscope Core Center at the University of Missouri-Columbia for their help with tissue preparation of animal specimens.

**Sources of Funding**

This research was supported by the National Institutes of Health (RO1 HL73101-01A1; to J.R.S.), the Veterans’ Affairs Merit System grants 0018 (to J.R.S.) and CDA-2 and VISN15 (to A.W.C.), and the Forest Research Institute.

**Disclosures**

J.R.S. received investigator-initiated support from the Forest Research Institute.

**References**


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Hypertension. 2010;55:880-888; originally published online February 22, 2010;
doi: 10.1161/HYPERTENSIONAHA.109.145136

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Nebivolol Improves Diastolic Dysfunction and Myocardial Remodeling through Reductions in Oxidative Stress in the Zucker Obese Rat

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Running Title: Nebivolol Improves Diastolic Dysfunction

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Total Word Count: 5836

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Supplemental Methods

Cine-Magnetic Resonance Imaging
Noninvasive magnetic resonance imaging (MRI) scans were performed on age-matched Zucker obese and lean rats after 2 weeks treatment with nebivolol or vehicle using a Varian 7T horizontal bore MRI (Varian Inc., Palo Alto, CA) equipped with a 60 mm birdcage radiofrequency coil. Animals were weighed and anesthetized using 1.8–2.7% isoflurane on a nose-cone nonrebreathing system supplying continuous oxygen. Electrocardiogram (ECG) and respiratory monitoring and gating were performed with a small animal monitoring system (SA Instruments, Stony Brook, NY). Warm air was circulated through the MRI bore to maintain the body temperature. ECG/respiratory gated gradient echo sequences were acquired with 1 mm slice thickness and 65×45 mm² and 45×45 mm² field of view for the left ventricle (LV) in long- and short-axis images, respectively. Septum wall thickness measurements were determined on the midventricular axial image immediately after the R wave and with averaging of 5 measurements per heart. The LV functional parameters were determined using a series of cine images of the LV in long-axis view acquired at 16 equally spaced time points throughout the entire cardiac cycle with a frame rate of 8 – 12 ms/frame. At each time point, the endocardial borders were traced to measure the LV chamber area using VnmrJ software (Varian Inc., Palo Alto, CA) by two experienced MRI readers. The LV volumes at each phase were calculated with a modified ellipsoid equation, $8A^2/(3\pi L)$, where A is the endocardial area and L is the length of the LV long-axis chamber.¹ Left ventricular volume (LVV) curve was plotted as LV volumes versus time in 1 cardiac cycle. LV ejection fraction (EF) was measured as: $EF = (\text{end diastolic volume [EDV]} – \text{end systolic volume [ESV]})/\text{EDV} \times 100\%$. The first derivatives of the LVV against time were calculated to extract the diastolic filling rates and relaxation time. Diastolic initial filling rate (IFR) is defined as the slope of the first 4 time points at the early diastolic curve. Diastolic peak filling rate (PFR) is defined as the maximum derivative of the LVV curve. Diastolic relaxation time is defined as the time duration from the end of systolic phase to the peak filling phase.

Light Microscopic Analysis for Myocardial Interstitial Fibrosis
Fixed paraffin sections of LV were evaluated with Verhoeff-van Gieson stain, which stains elastin (black), nuclei (blue black), collagen (pink), and connective tissue (yellow), as previously described.² Slides were analyzed with a Nikon50i microscope and x40 images were captured with a Cool Snapcf camera. Morphometric analysis was performed with MetaVue software. For each animal a region of interest rectangle was placed at 9-10 randomly chosen sites that did not contain a blood vessel. For each of the 9-10 replicate images from a single rat heart, the average gray scale intensity due to collagen was recorded. An average value of these intensities was determined for each animal.

Quantitative Analysis of Mitochondrial number using Complex IV-1 Immunolabeling
Left ventricle sections were fixed in 3% fresh paraformaldehyde and infiltrated and embedded in paraplast. Four-micrometer sections were deparaffinized in CitriSolv and rehydrated in ethanol series and HEPES wash buffer. The epitopes were retrieved in citrate buffer at 95°C for 25 min. Nonspecific binding sites were blocked with goat blocker for 4 h. Sections were then incubated overnight with mouse anti-Complex IV subunit 1 monoclonal antibody at a concentration of 3 μg/ml in 10-fold diluted blocker (Mitosciences, Eugene, OR). After being washed with HEPES wash buffer, the sections were incubated with 1:300 goat-anti mouse Alexa fluor 647. Four hours later the slides were washed and incubated with 1:2,000 4,6-diamidino-2-phenylindole to counterstain nuclei. After 10 min, the slides were washed and mounted with Mowiol. Slides were checked under a laser confocal microscope (Bio-Rad) and a multiphoton confocal system. Images were captured with Laser-sharp software and the immunofluorescence quantified using MetaMorph software (Boyce Scientific, St. Louis, MO).

Citrate Synthase Activity

Hearts from animals were removed and homogenized as described above. Mitochondrial fractions were isolated from whole tissue homogenates after centrifugation at 33,000g for 60 min. Pellets were resuspended in sucrose homogenization buffer comprised of sucrose, HEPES, EDTA, complete EDTA-free protease inhibitor cocktail tablet (Roche, Germany), sodium pyrophosphate, and sodium orthovanadate. The protein content was determined with BCA protein assay kit (Thermo Scientific, Rockford, IL). Citrate synthase activity was determined as previously described. Briefly, 10 microgram of mitochondrial protein was incubated with the buffer containing 66.5 mM Tris (pH 8.3), 0.5 mM oxaloacetate, 0.43 mM acetyl-CoA, and 95 mM 5,5'-dithio-bis(2-nitrobenzoic acid (DTNB) at 37 °C. Spectrophotometric detection of reduced DTNB at the wavelength of 412 nm was measured every 20 second for 10 min. Amount of DTNB converted per minute was determined with the molar extinction coefficient for DTNB. The maximum slope was calculated and used as an indication of citrate synthase activity.

β-hydroxyacyl-CoA dehydrogenase (β-HAD) Activity

(β-HAD) activity was measured as previously described with modifications. Ten micrograms of mitochondrial protein was incubated in the assay buffer containing 0.1 M triethanolamine-HCl, 5 mM EDTA, and 0.45 mM NADH (pH 7.0) at 37 °C. After an initial 2-min absorbance reading at 340 nm, the reaction was initiated by adding 0.1 mM acetoacetyl-CoA, and the rates of disappearance of NADH was measured by change in absorbance 340 nm every 10 second for 10 min. The maximum slope was calculated and used as the indication of β-HAD activity. Enzyme activity was expressed as nanomoles per gram protein per minute.

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