**Beta3-Adrenoreceptor Stimulation Ameliorates Myocardial Ischemia-Reperfusion Injury Via Endothelial Nitric Oxide Synthase and Neuronal Nitric Oxide Synthase Activation**

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**Objectives**
This paper examined whether nebivolol protects the heart via nitric oxide (NO) synthase and NO-dependent signaling in an in vivo model of acute myocardial infarction.

**Background**
Beta3-adrenergic receptor (AR) activation promotes endothelial nitric oxide synthase (eNOS) activity and NO bioavailability. We hypothesized that specific beta3-AR agonists would attenuate myocardial ischemia-reperfusion (MI/R) injury via eNOS activation and increased NO bioavailability.

**Methods**
Mice were subjected to 45 min of myocardial ischemia in vivo followed by 24 h of reperfusion (R). Nebivolol (500 ng/kg), CL 316243 (1 μg/kg), BRL-37344 (1 μg/kg), or vehicle (VEH) was administered at the time of R. Myocardial area-at-risk (AAR) and infarct size (INF)/AAR was measured at 24 h of R. Cardiac tissue and plasma were collected to evaluate eNOS phosphorylation, neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase expression, and nitrite and nitrosothiol levels.

**Results**
Nebivolol (500 ng/kg) reduced INF/AAR by 37% (p < 0.001 vs. VEH) and serum troponin-I levels from 41 ± 4 ng/ml to 25 ± 4 ng/ml (p < 0.05 vs. VEH). CL 316243 and BRL-37344 reduced INF by 39% and 42%, respectively (p < 0.001 vs. VEH). Nebivolol and CL 316243 increased eNOS phosphorylation at Ser-1177 (p < 0.05 vs. VEH) and increased nitrite and total nitrosylated protein levels. Nebivolol and CL 316243 significantly increased myocardial nNOS expression. Nebivolol failed to reduce INF after MI/R in beta3-AR−/−, eNOS−/−, and in nNOS−/− mice.

**Conclusions**
Our results indicate that beta3-AR agonists protect against MI/R injury. Furthermore, the cardioprotective effects of beta3-AR agonists are mediated by rapid eNOS and nNOS activation and increased NO bioavailability.

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disease by improvement of coronary circulation, protection from cardiomyocyte apoptosis, and reduction of infarction (4–6). These beta-blockers target the beta1 and beta2 ARs as their primary mode of action (7,8). However, the beta3-AR has recently emerged as a potential target for the treatment of heart disease (9,10).

Nebivolol (alpha, alpha'-(imidomethylene)bis[6-fluoro-2-chromanethanol]) is a third-generation beta blocker approved by the U.S. Food and Drug Administration for the treatment of hypertension. The selectivity of nebivolol for the beta1-AR is considerably higher than that of bisoprolol, carvedilol, or bucindol, and it was shown to reduce mortality and morbidity in elderly patients with heart failure (11–13). Nebivolol also exhibits nitric oxide (NO)-mediated vasodilating properties thought to result from the stimulation of the endothelial beta3-AR (14,15). Stimulation of the beta3-AR and activation of endothelial nitric oxide synthase (eNOS) increases NO release after nebivolol treatment to cause peripheral vasodilatation and improved endothelial function (16,17). Nebivolol might also activate the beta3-AR (18–20).

NO exerts profound cardioprotection in animal models of MI/R injury (21–23). The overexpression of eNOS decreases MI/R injury, whereas eNOS deficiency exacerbates MI/R injury (24–26). Nitric oxide also inhibits mitochondrial respiration and prevents platelet aggregation and neutrophil adherence to vascular endothelium (27,28).

In the present study, we examined whether nebivolol or highly specific beta3-AR agonists protect the heart via eNOS and NO-dependent signaling in an in vivo murine model of acute myocardial infarction.

**Methods**

An expanded Methods section is available in the Online Appendix.

**Animals.** Male C57BL6/J mice, 10 to 12 weeks of age (Jackson Labs, Bar Harbor, Maine), eNOS deficient (eNOS−/−) mice (26) (10 to 12 weeks old), and beta3 adrenergic receptor deficient (beta3-AR−/−) mice (12 to 14 weeks) were used (29,30). All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85–23, Revised 1996). Procedures were approved by the Emory University Institutional Animal Care and Use Committee.

**Materials.** Nebivolol was provided by the Forest Research Institute (Jersey City, New Jersey). The beta3 receptor agonists CL 316243 and BRL-37344 were purchased from Sigma-Aldrich (St. Louis, Missouri). Nitro-L-arginine methyl ester (L-NAME) (hydrochloride) and the beta3 AR antagonist L-748,337 were purchased from Cayman Chemical (Ann Arbor, Michigan).

**MI/R protocol.** Surgical ligation of the left coronary artery (LCA), myocardial INF determination, and troponin I measurements were performed as previously described (25,31).

**Western blot analysis.** Western blot analysis was performed as previously described (25).

**NO metabolites.** NO and NO metabolite analysis was performed as previously described (32).

**Measurement of nebivolol.** Liquid chromatography tandem mass spectrometry was used for determination of d/l-nebivolol in mouse plasma. See the Online Appendix for detailed methods.

**Hemodynamic parameters.** Mice were anesthetized with isoflurane (1.0 to 2.0 l/min) in 100% oxygen. The right carotid artery was exposed for a length of approximately 5 mm. A 1.0-F Millar pressure catheter (Millar Instruments, Houston, Texas) connected to the computerized data-acquisition system (PowerLab 4/30, AD Instruments, Colorado Springs, Colorado) was advanced into the aorta through the right carotid artery to record heart rate (HR), systolic, diastolic, and mean arterial pressures. These values were recorded with LabChart 6 PRO (AD Instruments).

**Echocardiography.** Baseline echocardiography images were obtained 1 week before LCA ischemia to avoid cardioprotective effects of the isoflurane, as previously described with slight modification. Mice were anesthetized with isoflurane, and transthoracic echocardiography of the left ventricle (LV) was performed with a 30-MHz RMV scanhead interfaced with a Vevo 2100 (Visualsonics, Toronto, Ontario, Canada) to obtain high-resolution M mode images at the rate of 1,000 frames/s. These were used to measure LV end-diastolic diameter, LV end-systolic diameter, and ejection fraction. Echocardiography images were obtained again 1 week after the MI/R protocol.

**Statistical analysis.** All data in this study are expressed as the mean ± SEM. Differences in data between the groups were compared with Prism 4 (GraphPad Software, La Jolla, California) with Student unpaired 2-tailed t test when 2 groups were compared or a 1-way analysis of variance, when 3 or more groups were compared. If a significant difference was found with the analysis of variance test, a Tukey’s (Figs. 1 to 5, Online Fig. 2) or Dunnett’s (Online Fig. 3) multiple comparison test was used for post hoc analysis. A p value <0.05 was considered significant.
Results

Nebivolol limits myocardial injury. We subjected male C57BL/6J mice to 45 min of LCA ischemia followed by reperfusion. Nebivolol (250 ng/kg to 50 μg/kg) or vehicle (VEH) was administered into the LV lumen at reperfusion (Fig. 1A). We evaluated myocardial INF at 24 h of reperfusion. Representative mid-ventricular cross-sections of VEH- and nebivolol-treated (500 ng/kg) mice are shown in Figure 1B. The area-at-risk (AAR)/LV was similar (p < NS) in all groups (Fig. 1C). Evaluation of INF revealed a dose-response curve, with 250 and 500 ng/kg displaying the most cytoprotection, as assessed by 2,3,5-triphenyltetrazolium chloride staining (Fig. 1C). Mice receiving 250 ng/kg displayed an INF/AAR of (34.7 ± 5.5%) compared with VEH (55.3 ± 2.8%), a 37% reduction. Mice receiving 500 ng/kg displayed an INF/AAR of (35.2 ± 4.7%), a 36% reduction. The dose of 500 ng/kg was used in all subsequent in vivo studies.

We evaluated circulating plasma levels of the cardiac-specific isoform of troponin-I as a marker of myocardial injury (Fig. 1D). The cardiac-specific isoform of troponin-I levels were significantly reduced in nebivolol-treated animals, containing 25.5 ± 3.8 ng/ml versus the VEH level of 40.9 ± 4.6 ng/ml.

Effects of nebivolol on hemodynamic status. We measured mean arterial blood pressure (MABP) and HR after administration of nebivolol to determine the hemodynamic effects (Table 1). The MABP and HR were measured at baseline and at increasing doses to 500 ng/kg nebivolol. There was no significant change in MABP or HR at 500 ng/kg versus baseline. The dose was then increased to 1 mg/kg, which significantly decreased MABP and slightly decreased HR.

Nebivolol activates NO synthase. We investigated the effects of acute nebivolol on expression and phosphorylation of eNOS at serine residue-1177 (eNOS-P^S^er1177) to determine eNOS activation (Fig. 2). Total eNOS expression remained constant (Figs. 2A and 2B). However, nebivolol significantly increased eNOS-P^S^er1177 at 5 min after administration and returned to basal levels after 30 min (Figs. 2A and 2C).

We also evaluated neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) (Fig. 3). Interest-
ingly, we observed a 1.7-fold increase in the expression of nNOS in the heart 15 min after treatment, which returned to baseline levels by 30 min (Figs. 3A and 3B). Cardiac levels of iNOS remained unchanged (Fig. 3C).

Nebivolol increases NO. We next investigated plasma and cardiac NO levels (Fig. 4). Mice treated with nebivolol displayed a 2.5-fold increase in plasma nitrite (an NO storage molecule) 30 min after intracardiac injection (Fig. 4A). Additionally, plasma total nitrosylated protein (RXNO) levels were significantly increased (p < 0.01) at 30 min and 2 h after administration (2.6- and 2.5-fold, respectively) (Fig. 4B). Cardiac nitrite levels significantly increased at 2 h (2.1-fold) (Fig. 4C). Cardiac RXNO levels did not show a significant difference after nebivolol administration (Fig. 4D). In contrast, cardiac NO-Heme levels significantly increased (4.5-fold) after 30 min (Fig. 4E).

Nebivolol protects through beta3-AR and NO synthase. We investigated the effects of nebivolol therapy in eNOS−/− (Fig. 5A) and nNOS−/− mice (Fig. 5B). Myocardial INF was not reduced in eNOS−/− mice after administration of nebivolol. Similar results were found in nNOS−/− mice. We also investigated nebivolol treatment in beta3-AR−/− mice. Myocardial INF was not reduced in beta3-AR−/− mice after administration of nebivolol (Fig. 5C).

Inhibition of NO synthase and beta3-AR abolishes nebivolol-mediated cardioprotection. The L-NAME inhibits the 3 nitric oxide synthase (NOS) isofoms. Therefore, we evaluated myocardial INF in wild-type mice treated with both L-NAME (25 mg/ml) and nebivolol (Fig. 6A). When L-NAME was co-administered, the cardioprotective effects of nebivolol were lost. Subsequently, we studied nebivolol co-administered with a specific beta3-AR antagonist, L-748,337 (33). When L-748,337 (100 μg/kg) was co-administered, the cardioprotective effects of nebivolol were also lost (Fig. 6B).

Beta3-AR specific agonists limit injury after MI/R. We investigated 2 beta3-AR specific agonists, CL 316243 (34) and BRL-37344 (35), to confirm the effects of beta3-AR stimulation on MI/R injury (Fig. 5D). Mice receiving 1 g/kg of CL 316243 displayed an INF/AAR of (35.0 ± 3.3%) compared with VEH (59.0 ± 1.8%), a 39% reduction. Mice receiving 1 g/kg of BRL-37344 displayed an INF/AAR of (33.1 ± 4.7%), a 42% reduction.

CL 316243 mediates NOS activation. We also evaluated the effect of CL 316243 on the expression and phosphorylation levels of eNOS-PSer1177 in sham-operated mice. Total eNOS expression remained constant (Online Fig. 2A). However, 2 h after CL 316243 administration, eNOS-PSer1177 increased significantly (Online Fig. 2A), whereas eNOS-PThr495 remained constant (Online Fig. 2A). The expression of nNOS and iNOS were also measured. There was a significant increase in nNOS protein expression after 15

![Figure 2 Nebivolol Increases eNOS-PSer1177](image)
min of CL 316243 administration (Online Fig. 2B). The expression levels of iNOS were not significantly different (Online Fig. 2B). We also measured plasma and cardiac nitrite and RXNO levels and NO-Heme levels (Online Fig. 3). We observed an apparent increase in plasma nitrite levels and a significant increase in cardiac nitrite levels after CL 316243 injection that persisted to 2 h (1.8-fold in the heart) (Online Figs. 3A and 3C). The RXNO values also exhibited a similar trend with a 3.1-fold increase in plasma RXNO after 2 h, when compared with VEH (Online Fig. 3B). Heart RXNO and NO-Heme values also increased significantly after 30 min, showing a 1.9- and 3.6-fold increase, respectively.

Circulating levels of nebivolol. We measured the circulating levels of nebivolol at different time points (1, 5, and 15 min) after intracardiac injection, to evaluate the pharmacokinetics. Nebivolol was administered to 3 different groups of mice (n = 3), and blood samples were taken to isolate plasma. The concentration of nebivolol was 1.2 ± 0.29 ng/ml after 1 min, then 0.4 ± 0.1 ng/ml after 5 min, and below detection after 15 min.

Nebivolol and LV function. We measured LV function and cardiac dimensions of mice treated with nebivolol and VEH (Table 2). Wild-type mice were subjected to 45-min myocardial ischemia and received nebivolol or VEH at reperfusion. No significant difference was observed in the ejection fraction, LV end diastolic diameter, LV end systolic diameter, and fractional shortening.

Discussion

The effects of beta1- and beta2-ARs are well-established in mammals, because their stimulation mediates an increase in HR, myocardial contraction force, and acceleration of relaxation (36). The beta2-AR has been shown to produce a negative inotropic effect that antagonizes the activity of beta1- and beta2-ARs (37,38). The beta3-AR is activated at higher concentrations of catecholamines than required for beta1- and beta 2-ARs and is thought to act as a counter-mechanism during sympathetic overstimulation (39). The beta3-AR–mediated signaling also results in NO generation from eNOS (40).

Previous studies suggest that beta3-AR attenuates obesity (41). The beta3-AR agonists such as BRL 37344 (34) and CL 316243 (35) were developed as a treatment for obesity but failed in clinical trials. The involvement of the beta3-AR in cardiac function is primarily explained by the activation of eNOS, but the precise signaling mechanism has yet to be elucidated.

We investigated the cardioprotective actions of nebivolol and 2 other beta3-AR specific agonists (i.e., BRL 37344 and CL 316243) in the setting of a murine model of acute MI/R
injury. Here we show that nebivolol treatment results in significant cardioprotection in the setting of MI/R via the stimulation of the beta3-AR. Nebivolol stimulates NO production in endothelial cells by activation of the beta3-AR (17,33). Both exogenous NO therapy and actions that promote endogenous NO production from eNOS protect the ischemic myocardium (10,42). We have demonstrated that activation of the beta3-AR in the heart by nebivolol, CL 316243, and BRL-37344 significantly reduces INF/AAR through an NO-dependent mechanism.

Several reports show inconsistencies with potency and specificity of beta3-AR agonists (43–45); however, we provide evidence with multiple agonists that supports beta3-AR stimulation as a novel approach to address cardiovascular disease. We also show that L-NAME, a known NOS inhibitor, abolishes the cardioprotective actions of nebivolol treat-
ment in MI/R. Previous evidence suggests L-748,337, a specific beta 3-AR antagonist, is effective in reducing the effects of nebivolol (33). We also observed this in our MI/R studies.

Our study provides evidence that stimulation of the beta3-AR acts as a dual activator of eNOS and nNOS. Some studies have shown that the beta 3-AR modulates nNOS or iNOS (46,47), whereas the role of eNOS has been confirmed (40,48). We show a rapid increase in the activation of eNOS and the expression of nNOS after administration of nebivolol or CL 316243. We also report the absence of cardioprotection by nebivolol in eNOS knockout mice, nNOS knockout mice, and beta3-AR knockout mice.

Nebivolol has been shown to provide beneficial effects on LV dysfunction after myocardial infarction (49). In the present study we failed to demonstrate significant improvements in LV function at 7 days after reperfusion, which is in contrast to the Sorrentino et al. (49) study. There are, however, clear differences in methodology, because our experiments are based on a single dose of nebivolol (500 ng/kg) before reperfusion as opposed to treatment of 10 mg/kg/day for 30 days (49).

Nitric oxide is well-established as a trigger and mediator of cardioprotection and is involved in many protective signaling cascades (22,23). Activation of the beta3-AR likely stimulates many cytoprotective signaling cascades, which ultimately result in the cardioprotection we have observed. Our findings demonstrate that a single dose of nebivolol

![Figure 5](image-url)
confers significant cardioprotection against MI/R injury in mice when administered at the time of reperfusion. We show for the first time that a beta1-blocker/beta3-AR agonist and a specific beta3-AR agonist attenuates myocardial INF by an NO-mediated mechanism involving both eNOS and nNOS. We also demonstrate that the beta1-blocker properties of nebivolol do not seem to be part of this cardioprotection, because hemodynamic measurements at the doses investigated in this study did not affect MABP or HR. At higher doses (twice that used in our experiments) we did observe a significant decrease in MABP but did not observe a reflex increase in HR. This might be because NO blocker properties of nebivolol conefrom reperfusion fails to protect. These data suggest that nebivolol can attenuate the baroreflex HR increase (50,51). We also observe a reflex increase in HR. This might be because NO

TABLE 2

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<th>Baseline</th>
<th>7-Day Post-MI/R</th>
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<tbody>
<tr>
<td></td>
<td>VEH (n = 10)</td>
<td>Nebivolol (n = 9)</td>
</tr>
<tr>
<td>EF (%)</td>
<td>66.0 ± 1.87</td>
<td>64.4 ± 2.43</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.4 ± 0.04</td>
<td>3.5 ± 0.06</td>
</tr>
<tr>
<td>LVEDS (mm)</td>
<td>2.2 ± 0.06</td>
<td>2.3 ± 0.08</td>
</tr>
<tr>
<td>FS</td>
<td>35.6 ± 1.39</td>
<td>34.6 ± 1.92</td>
</tr>
</tbody>
</table>

Values are mean ± SE. p = NS (nebivolol vs. vehicle [VEH]).

EF = ejection fraction; FS = fractional shortening; LVEDD = left ventricular end-diastolic diameter; LVEDS = left ventricular end-systolic diameter; MI/R = myocardial ischemia-reperfusion.

use after myocardial ischemia. Future research will be aimed at further elucidation of the signaling mechanisms responsible for these cardioprotective actions. The involvement of nNOS as a redundant and additive signal for NO generation after acute administration of beta3-agonists also requires additional investigation.

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Key Words: beta 3-adrenergic receptor, cardiac ischemia, endothelial nitric oxide synthase, neuronal nitric oxide synthase, nitric oxide.

APPENDIX
For supplementary figures and text, please see the online version of this article.