Nebivolol Exerts Beneficial Effects on Endothelial Function, Early Endothelial Progenitor Cells, Myocardial Neovascularization, and Left Ventricular Dysfunction Early After Myocardial Infarction Beyond Conventional $\beta_1$-Blockade

Sajoscha A. Sorrentino, MD,*† Carola Doerries, MD,*‡ Costantina Manes, MD,*‡ Thimoteus Speer, MD,‡ Chantal Dessy, PhtD,§ Irina Lobysheva, PhtD,§ Wazma Mohmand, MD,* Razma Akbar, MD,* Ferdinand Bahmann, MD, PhtD,† Christian Besler, MD,*‡ Arnd Schaefer, MD,* Denise Hilfiker-Kleiner, PhtD,* Thomas F. Lüscher, MD,‡ Jean-Luc Balligand, MD, PhtD,§ Helmut Drexler, MD,* Ulf Landmesser, MD*‡ 
Hannover, Germany; Zurich, Switzerland; and Brussels, Belgium

Objectives
The aim of this study was to investigate whether nebivolol has added effects on left ventricular (LV) dysfunction and remodeling early after myocardial infarction (MI) beyond its $\beta_1$-receptor-blocking properties.

Background
Nebivolol is a third-generation selective $\beta_1$-adrenoreceptor antagonist that stimulates endothelial cell nitric oxide (NO) production and prevents vascular reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation. Both endothelial NO synthase–derived NO production and NADPH oxidase activation are critical modulators of LV dysfunction early after MI.

Methods
Mice with extensive anterior MI (n = 90) were randomized to treatment with nebivolol (10 mg/kg/day), metoprolol-succinate (20 mg/kg/day), or placebo for 30 days starting on day 1 after surgery.

Results
Infarct size was similar among the groups. Both $\beta_1$-adrenergic receptor antagonists caused a similar decrease in heart rate. Nebivolol therapy improved endothelium-dependent vasorelaxation and increased early endothelial progenitor cells 4 weeks after MI compared with metoprolol and placebo. Nebivolol, but not metoprolol, inhibited cardiac NADPH oxidase activation after MI, as detected by electron spin resonance spectroscopy analysis. Importantly, nebivolol, but not metoprolol, improved LV dysfunction 4 weeks after MI (LV ejection fraction: nebivolol vs. metoprolol vs. placebo: 32 ± 4% vs. 17 ± 6% vs. 19 ± 4%; nebivolol vs. metoprolol: p < 0.05) and was associated with improved survival 4 weeks post-MI compared with placebo. Nebivolol had a significantly more pronounced inhibitory effect on cardiomyocyte hypertrophy after MI compared with metoprolol.

Conclusions
Nebivolol improves LV dysfunction and survival early after MI likely beyond the effects provided by conventional $\beta_1$-receptor blockade. Nebivolol induced effects on NO-mediated endothelial function, early endothelial progenitor cells and inhibition of myocardial NADPH oxidase likely contribute to these beneficial effects of nebivolol early after MI. (J Am Coll Cardiol 2011;57:601–11) © 2011 by the American College of Cardiology Foundation

$\beta_1$-adrenergic receptor blockers have become a hallmark in the management of patients after acute myocardial infarction (MI) as well as in the treatment of chronic heart failure (1). In contrast to conventional $\beta_1$-selective adrenergic receptor antagonists such as metoprolol-succinate, the $\beta_1$-selective adrenergic receptor blocker nebivolol has been

From the *Division of Cardiology and Angiology, Medical School of Hannover, Hannover, Germany; †Division of Nephrology, Medical School of Hannover, Hannover, Germany; ‡Cardiovascular Center, University Hospital Zurich, Cardiovascular Research, Institute of Physiology, University of Zurich, Zurich, Switzerland; and the §Unit of Pharmacology and Therapeutics, University of Louvain Medical School, Brussels, Belgium. This study was supported in part by Deutsche Forschungsgemeinschaft (LA 1342/3-1), an unrestricted vascular biology grant from Berlin Chemie, Berlin, Germany, a Swiss National Research Grant (310030-122339), the Zurich Center for Integrative Human Physiology, and the Fondation Leducq grant support 05CVD "Adaptive and Maladaptive Signaling in Cardiac Growth and Regeneration." Dr. Landmesser receives speaker fees from Menatini. All other authors have reported that they have no relationships to disclose. Helmut Drexler is deceased. Drs. Sorrentino and Doerries contributed equally to this work.

Manuscript received October 20, 2009; revised manuscript received August 23, 2010, accepted September 2, 2010.
Examined the effect of nebivolol and metoprolol-succinate resonance spectroscopy (ESR) early after MI. Moreover, we mediated vasodilation, early EPC mobilization, and myocardial dysfunction early after MI (16,17). Dysfunction after MI, supporting the concept that NADPH oxidase activation after MI improved LV cells (EPCs) (14,19). Furthermore, endothelium- or cardiomyocyte-targeted effects on LV dysfunction and survival after MI (13,14,19). Increased eNOS-derived NO availability may exert beneficial effects on LV dysfunction and survival after MI (13–15). In this respect, we recently observed that statins improve endothelium-dependent vasodilation, LV dysfunction, and survival after experimental MI that was critically dependent on their effect on eNOS, suggesting that increased eNOS-derived NO production may exert beneficial effects on LV dysfunction and survival after MI (13,14,19). Furthermore, endothelium- or cardiomyocyte-targeted overexpression of eNOS resulted in improved LV function after MI (13,19). In contrast, suppression of eNOS-dependent NO production resulted in increased LV dysfunction (15), reduced myocardial neovascularization (20), and impaired mobilization of early endothelial progenitor cells (EPCs) (14,19).

In addition, we and others have observed that prevention of NADPH oxidase activation after MI improved LV dysfunction after MI, supporting the concept that NADPH oxidase activation contributes importantly to LV dysfunction early after MI (16,17).

The present study was therefore designed to examine the effect of nebulonol therapy compared with metoprolol-succinate or placebo on endothelium-dependent, NO-mediated vasodilation, early EPC mobilization, and myocardial NADPH oxidase activation as analyzed by electron resonance spectroscopy (ESR) early after MI. Moreover, we examined the effect of nebivolol and metoprolol-succinate therapy on LV dysfunction, survival and cardiomyocyte hypertrophy early after MI.

Methods

Animals, MI, and experimental protocol. The local committee on animal research approved all procedures involving experimental animals, and all procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health.

In male C57BL/6J mice, age 14 to 16 weeks, MI was induced by permanent ligation of the left anterior descending coronary artery as described previously (14,16). On the first day, 24 h after MI, these mice were randomized into 3 groups (30 in each group): 1) treatment with nebivolol (10 mg kg⁻¹ day⁻¹); 2) metoprolol-succinate (20 mg kg⁻¹ day⁻¹); or 3) inert vehicle given orally via gastric gavage for 30 days starting on day 1 after surgery. Sham-operated mice (n = 30) served as controls. The doses of nebivolol and metoprolol-succinate were used to achieve similar reductions in heart rate (as observed in preliminary experiments).

Furthermore, in additional experiments, MI was induced in eNOS⁺/⁻ mice that were randomized on day 1 to nebivolol or vehicle therapy (n = 10 in each group) using the previously mentioned dose. Sham-operated eNOS⁻/⁻ mice (n = 10) served as controls.

Studies of endothelium-dependent, NO-mediated vasorelaxation. Endothelium-dependent, NO-mediated vasorelaxation in response to acetylcholine and endothelium-independent relaxation in response to nitroglycerin were studied in ring segments of thoracic aortas, as described previously (14,21,22). Notably, it has been shown that acetylcholine responses are lacking in eNOS-deficient mice (21,23), which indicates that these responses are dependent on eNOS.

Early EPC culture assay. Early EPCs were cultured as described in detail previously (14,24–26). In brief, monocellular cells were isolated from 1 ml of peripheral blood by density gradient centrifugation with Histopaque (Sigma, Munich, Germany) (27), seeded on tissue culture coverslips (9 × 10⁵ cells) coated with rat vitronectin (Sigma) in endothelial basal medium (endothelial growth medium–2 MV, Clonetics, Lonza, Basel, Switzerland) and supplemented with endothelial growth medium–2 MV single quots (containing fetal bovine serum, human vascular endothelial growth factor A, human fibroblast growth factor B, human epidermal growth factor, insulin-like growth factor–1, and ascorbic acid in appropriate amounts). After 4 days in culture, nonadherent cells were removed by washing with phosphate-buffered saline. The cell culture was maintained through day 7, and fluorescence chemical detection was performed. To detect the uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethyldinocarbocyanine–labeled acetylated LDL (acLDL-DiI), Molecular Probes, Invitrogen, Carlsbad, California), cells were incubated with acLDL-DiI (6 μg/ml, 37°C, 2 h). Cells were then fixed with 1% parafo-
maldehyde for 10 min and incubated with fluorescein-labeled *Griffonia simplicifolia* lectin I (BS-1 lectin, Vector Laboratories, Burlingame, California) for 1 h. After being stained, samples were analyzed with an inverted fluorescence microscope (Leica, Wetzlar, Germany), and double-stained cells for both BS-1 lectin and acLDL-DiI were counted as early EPCs in at least 4 randomly selected high-power fields (28).

Histomorphometric analysis and immunohistochemistry. Tissue morphometry was performed in a blinded fashion with the Quantimet 500MC (Leica, Cambridge, England) digital image analyzer. After in situ fixation, LV tissue slices were embedded in paraffin and cut into 4-μm sections, as described previously (16,29). Mean cardiomyocyte cross-sectional area (CSA) or length and infarct size were determined in hematoxylin–eosin–stained sections or sections stained with antibodies recognizing wheat germ agglutinin to visualize myocyte boundaries (WGA) (Vector Laboratories) using the digital image analyzer as described previously (29–32). Nuclei were stained with 4,6-diamino-2-phenyindole. For measurements of the cardiomyocyte CSA or cardiomyocyte length, only cross-gated cardiomyocytes and respectively longitudinally sectioned cardiomyocytes were analyzed. Furthermore, the length of cardiomyocytes isolated from in situ formalin-fixed LV tissue was measured using the potassium hydroxide (KOH) method as described in detail previously (33). In brief, LV samples were cut into small pieces, placed overnight in a 12.5-mol/L KOH solution and transferred to phosphate buffer. Samples were then mixed using a vortexer, centrifuged, and resuspended in 10% formaldehyde–phosphate buffer. Nuclei were stained by Mayer’s hemalaum solution to differentiate between cell fragments and intact myocytes. Myocyte length (50 structurally intact myocytes per sample) was determined by using an Olympus BX51 microscope (Olympus, Volketswil, Switzerland) and the software program Analysis 5.0 (Soft Imaging System, Muenster, Germany).

For immunohistochemistry analysis of capillary density, mid-LV specimens were obtained and capillary density was determined, as described in detail previously (14). Sections were immunostained with the antiplatelet and endothelial cell adhesion molecule-1 (CD31) rabbit polyclonal antibody (H-300, Santa Cruz Biotechnology, Heidelberg, Germany) and the software program Analysis 5.0 (Soft Imaging System, Muenster, Germany). The avidin-biotin-peroxidase labeling system. Counterstain and detection of the primary antibody was performed using the H-300, Santa Cruz Biotechnology, Heidelberg, Germany) and the software program Analysis 5.0 (Soft Imaging System, Muenster, Germany).

Echocardiographic measurements. Echocardiographic analysis was performed with the mice under light anesthesia (ketamine 100 mg/kg, xylazine 1.25 mg/kg, and atropine 0.6 mg/kg intraperitoneally), and spontaneous respiration was provided with a commercially available ultrasound system (ATL5000 CV, Philips Medical System, Bothell, Washington) with a linear 15-MHz high-frequency transducer, as described previously (14,16). The investigator (A.S.) was blinded to the experimental group.

Measurement of myocardial NADPH oxidase activity by ESR spectroscopy analysis. Activity of NADPH oxidase was determined in remote LV myocardium (50 μg protein) by ESR spectroscopy, as described previously by using the spin probe 1-hydroxy-3-carboxy-2-pyrrolidine (CP-H, Axxora, Gruenberg, Germany) and a MiniScope ESR spectrometer (Magnettech, Berlin, Germany) (16,34). The ESR settings were the following: field center, 3,367.67 G; field sweep width, 108.92 G; microwave frequency, 9.82 GHz; microwave power, 20 mW; magnetic field modulation frequency, 100 kHz; modulation amplitude, 2 G. The intensity of ESR spectra was quantified after subtraction of the ESR signal of samples without NADPH (obtained for each sample).

Measurement of superoxide production in human aortic endothelial cells (HAECs) by ESR spectroscopy analysis. HAECs purchased from Lonza were grown according to standard procedures. Serum-starved HAECs were exposed to metoprolol (10 μmol/l), nebivolol (10 μmol/l), or the solvent after 60-min pre-incubation with various pharmacological modulators (e.g., brupanolol 10 μmol/l or nadolol 10 μmol/l), as described in detail previously (6).

Superoxide production in HAECs in response to 24-h angiotensin II stimulation was measured by ESR spectroscopy and the spin trap CP-H, as described in detail previously (16,34).

Blood pressure measurements. Systolic blood pressure was measured by a computerized noninvasive tail cuff system (Blood Pressure Analysis System BP-200, Visitech Systems, Apex, North Carolina), as described previously (14,22).

Statistical analysis. All data are expressed as mean ± SEM. Statistical analysis was performed using analysis of variance followed by the Newman-Keuls multiple comparison test. For the comparison of 2 groups (eNOS-deficient mice), the 2-tailed, unpaired Student *t* test was used. Comparison of survival was performed using Kaplan-Meier analysis and the log–rank test. A *p* value <0.05 was considered statistically significant. Data were analyzed by using GraphPad Prism 4.03 (GraphPad Software, San Diego, California).

Results

Effect of nebivolol and metoprolol-succinate treatment on endothelium-dependent, NO-mediated vasodilation after MI. In sham-operated mice, acetylcholine produced endothelium-dependent relaxations of 93 ± 10%. These responses were substantially impaired in aortas of wild-type mice 4 weeks after MI (21 ± 4%: *p* < 0.05) (Fig. 1A). Nebivolol treatment markedly improved endothelium-dependent vasodilation in response to acetylcholine in mice after MI (55 ± 7%: *p* < 0.05 vs. placebo), whereas metoprolol had no effect (21 ± 3%, *p* = NS vs. vehicle) (Fig. 1A). In contrast, endothelium-independent vasodilation in response to nitroglycerin was not impaired in mice after MI and was not changed by nebivolol or metoprolol.
In eNOS/−/− mice, no endothelium-dependent vasodilation in response to acetylcholine was observed, indicating that these responses are dependent on eNOS (data not shown).

Effect of nebivolol and metoprolol-succinate treatment on early EPCs after MI. Increasing evidence suggests that eNOS activation plays a critical role in the mobilization of early EPCs (14,35). However, it is not known whether the number of early EPCs is altered by β-blocker treatment in the presence of an ischemic stimulus. Notably, nebivolol therapy markedly increased early EPCs compared with placebo (Figs. 2A and 2B). In contrast, metoprolol-succinate treatment had no significant effect on early EPC numbers compared with vehicle (Figs. 2A and 2B). As shown in Figure 2D, nebivolol therapy did not change early EPC numbers in eNOS/−/− mice, suggesting that this response is dependent on eNOS.

Effect of nebivolol and metoprolol-succinate treatment on myocardial capillary density after MI. The effect of nebivolol and metoprolol-succinate therapy on capillary density after MI was examined in at least 6 representative high-power fields of the infarct border zone. Nebivolol, but not metoprolol-succinate, therapy resulted in a significant increase in capillary density in the infarct border zone in wild-type mice after MI (Fig. 2C). In contrast, nebivolol therapy had no effect on capillary density in eNOS/−/− mice after MI (Fig. 2E).

ESR spectroscopic analysis of myocardial NADPH oxidase activity after MI. NADPH oxidase activation has been shown to play a pivotal role in cardiomyocyte hypertrophy and LV dysfunction after MI (16,17). Furthermore, nebivolol has been shown to inhibit vascular NADPH oxidase activation in response to hyperlipidemia (9). We therefore examined the effect of nebivolol and metoprolol-succinate therapy on myocardial NADPH oxidase activity by using ESR spectroscopy. NADPH oxidase activity in LV remote myocardium was markedly increased after MI (Figs. 3A and 3B). After 30 days of treatment with nebivolol, but not with metoprolol, myocardial NADPH oxidase activity was substantially reduced as assessed by ESR spectroscopy, indicating a suppression of myocardial NADPH oxidase activation by nebivolol after MI (Figs. 3A and 3B).

Effect of nebivolol and metoprolol on cardiomyocyte hypertrophy and cardiomyocyte length after MI. After MI, we observed cardiomyocyte hypertrophy as indicated by an increased LV weight/body weight ratio and increased cardiomyocyte length and CSA in the remote myocardium (Fig. 4, Table 1). Although both β-blocker therapies decreased cardiomyocyte hypertrophy after MI, treatment with nebivolol had a significantly more pronounced inhibitory effect on LV weight/body weight and cardiomyocyte CSA compared with metoprolol (p < 0.05 vs. metoprolol in Fig. 4A, p < 0.001 vs. metoprolol in Table 1). In eNOS/−/− mice, the response of nebivolol therapy on LV mass and hypertrophy was attenuated compared with wild-type mice (Table 2), suggesting that this effect is at least partly dependent on eNOS.

Our data suggest that cardiomyocyte lengthening contributed to the observed LV weight/body mass differences after MI (i.e., measurements of cardiomyocyte length of isolated cardiomyocytes by using the KOH method indicated an increase in cardiomyocyte length after MI that was significantly attenuated by both β-blockers after MI (Fig. 4B).
The measurements of cardiomyocyte length of in situ fixed cardiac samples indicated a cardiomyocyte lengthening after MI that was reduced to a significantly greater extent by nebivolol compared with metoprolol-succinate therapy (Figs. 4C and 4D). A similar trend for a more pronounced effect of nebivolol therapy on cardiomyocyte length was observed by measurements of isolated cardiomyocytes using the KOH method that, however, did not reach statistical significance (Fig. 4B).

**Effect of nebivolol and metoprolol on LV dysfunction after MI.** LV ejection fraction was substantially decreased 4 weeks after MI compared with sham-operated animals. Notably, nebivolol, but not metoprolol, therapy significantly improved fractional shortening and LV ejection fraction 4 weeks after MI (Figs. 5A to 5C, Table 1), suggesting that nebivolol therapy is associated with an early beneficial effect on LV function after MI. This effect of nebivolol therapy was not observed in eNOS−/− mice after MI, suggesting that the eNOS is involved in this effect (Figs. 5D and 5E, Table 2).

**Survival post-MI.** Nebivolol therapy was associated with a significantly improved survival at 4 weeks after MI compared with vehicle therapy (p < 0.05 vs. vehicle). The impact of metoprolol was not statistically significant (Fig. 6).

**ESR spectroscopic analysis of superoxide production in HAECs.** The effect of blockade of specific β-adrenoreceptors was tested on endothelial superoxide production in response to angiotensin II, known to be dependent on NADPH oxidase activation (36). As shown in Figure 7, pre-treatment of HAECs with the β1-blocker nadolol (10 μmol/l) had no effect on nebivolol-induced reduction of endothelial superoxide production, whereas the complete β1-2-3-blocker bupranolol prevented nebivolol’s effect on endothelial superoxide production in response to angiotensin II, suggesting that the antioxidant effect of nebivolol was mediated via the β3-receptor.

**Infarct size, heart rate, and blood pressure.** Infarct size did not differ between the treatment groups (Tables 1 and 2). Heart rate and blood pressure were similarly decreased by either β-blocker treatment (Table 1).

**Discussion**

The present study demonstrates that nebivolol, a β1-selective adrenoreceptor antagonist with eNOS-stimulating
properties, attenuates LV dysfunction and cardiomyocyte hypertrophy early after MI, and is associated with improved survival, which likely goes beyond the effects of conventional β-blockade. These effects of nebivolol therapy on LV dysfunction and cardiomyocyte hypertrophy early after MI were largely blunted in eNOS-deficient mice, supporting a critical role of eNOS in this respect. Thus, the present study supports the notion that beneficial effects of conventional β-blockade can be increased by cardiac NO-dependent actions, which may be related, at least in part, to activation of β₂-receptors by nebivolol that may increase eNOS-dependent NO availability both, by preventing NADPH oxidase activation and stimulation of eNOS.

Our present observations provide novel mechanistic insights concerning the early beneficial effects of nebivolol on LV function post-MI. Nebivolol therapy exerted a beneficial effect on endothelium-dependent, NO-mediated vasodilation, early EPC mobilization, and myocardial neovascularization after MI, likely independent of its β₁-receptor-blocking effects because it was not observed with the β₁-selective adrenoreceptor antagonist metoprolol. Furthermore, in contrast to metoprolol-succinate, nebivolol inhibited myocardial NADPH oxidase activation after MI. Moreover, our data suggest that the ability of nebivolol to decrease angiotensin II–induced NADPH oxidase–dependent superoxide production is dependent on β₂-receptor activation. In contrast to the neutral effect of β₁-β₂-blockade by nadolol, the β₁-β₂-β₃-blocker bupranolol significantly inhibited nebivolol’s effect on superoxide production.

Several studies have firmly established the beneficial effects of conventional β-blockers on LV remodeling processes post-MI (37). However, although prolonged β-blocker therapy has been shown to improve LV function in patients with chronic heart failure and LV systolic dysfunction, the present study suggests that nebivolol exerts a beneficial effect on LV function and survival early after MI likely beyond β₁-blockade that may be mediated by prevention of NADPH oxidase activation and enhanced eNOS-dependent NO availability.

Nebivolol is a third-generation highly selective β₁-adrenoreceptor blocker. There is evidence that nebivolol, in addition to its β₁-adrenoreceptor blocking effects, can stimulate endothelial NO production, which has been suggested to be mediated, at least in part, by a β₃-agonistic effect (5,38). Furthermore, nebivolol has been suggested to exert antioxidant effects that have been attributed, at least in part, to prevent NADPH oxidase activation in response to hyperlipidemia or angiotensin II (9,12). Our data suggest that the β₃-agonistic effect of nebivolol is involved in the inhibition of endothelial NADPH oxidase activation.

In the present study, nebivolol improved LV dysfunction and survival early after MI, which was not observed with metoprolol-succinate therapy. In this respect, we and others recently showed that both eNOS-dependent NO production and NADPH oxidase activation play a pivotal role in LV dysfunction and survival early after MI (13–17,19). In fact,
Figure 4  LV Weight and Cardiomyocyte Length

(A) Left ventricular (LV) weight/body weight in sham-operated and vehicle-, metoprolol-, and nebivolol-treated mice 4 weeks after surgery. (B) Cardiomyocyte length as determined from isolated cardiomyocytes (potassium hydroxide method) in sham-operated and vehicle-, metoprolol-, and nebivolol-treated mice 4 weeks after surgery. (C) Cardiomyocyte length as determined by wheat germ agglutinin staining in sham-operated and vehicle-, metoprolol-, and nebivolol-treated mice 4 weeks after surgery (representative photographs are shown in D) (n = 7).

Table 1  Wild-Type Mice: Echocardiographic, Hemodynamic, and Morphometric Analyses

<table>
<thead>
<tr>
<th></th>
<th>Sham WT</th>
<th>Vehicle WT</th>
<th>Metoprolol WT</th>
<th>Nebivolol WT</th>
<th>p Value (Metoprolol vs. Nebivolol)</th>
<th>p Value (Vehicle vs. Metoprolol)</th>
<th>p Value (Vehicle vs. Nebivolol)</th>
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<tr>
<td>Echocardiography</td>
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<tr>
<td>EDD, mm</td>
<td>3.8 ± 0.1</td>
<td>6.3 ± 0.3</td>
<td>5.3 ± 0.3</td>
<td>5.2 ± 0.2</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ESD, mm</td>
<td>2.4 ± 0.1</td>
<td>5.7 ± 0.3</td>
<td>4.9 ± 0.4</td>
<td>4.3 ± 0.2</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FS, %</td>
<td>36.7 ± 1.1</td>
<td>9.6 ± 1.7</td>
<td>7.6 ± 3.3</td>
<td>16.6 ± 2.6</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>EF, %</td>
<td>55.9 ± 3.1</td>
<td>18.6 ± 3.7</td>
<td>17.1 ± 5.5</td>
<td>31.7 ± 3.5</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.05</td>
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<tr>
<td>Weight and morphometric analysis</td>
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<tr>
<td>LV, mg/g body weight</td>
<td>3.6 ± 0.2</td>
<td>6.2 ± 0.5</td>
<td>4.8 ± 0.3</td>
<td>4.0 ± 0.1</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>29 ± 1.6</td>
<td>33 ± 1.4</td>
<td>26 ± 0.3</td>
<td>29 ± 0.5</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
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<tr>
<td>LV, mg</td>
<td>105 ± 6</td>
<td>205 ± 21</td>
<td>124 ± 9</td>
<td>114 ± 4</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Cardiomyocyte cross-sectional area, μm²</td>
<td>259 ± 12</td>
<td>553 ± 70</td>
<td>451 ± 8</td>
<td>362 ± 10</td>
<td>&lt;0.001</td>
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<td>Infarct size, %</td>
<td>NA</td>
<td>42 ± 3</td>
<td>41 ± 14</td>
<td>41 ± 12</td>
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<td>NS</td>
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<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>110 ± 3</td>
<td>112 ± 2</td>
<td>106 ± 2</td>
<td>104 ± 3</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>Heart frequency, min⁻¹</td>
<td>598 ± 20</td>
<td>674 ± 15</td>
<td>547 ± 12</td>
<td>553 ± 23</td>
<td>NS</td>
<td>&lt;0.01</td>
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EDD = end-diastolic diameter; EF = ejection fraction; ESD = end-systolic diameter; FS = fractional shortening; LV = left ventricular; NA = not available; WT = wild-type mice.
statin-induced improvement of LV function and survival early after MI were critically dependent on eNOS because they were not observed in eNOS-deficient mice (14). Moreover, endothelial or cardiomyocyte-targeted overexpression of eNOS resulted in improved LV function after MI, further suggesting an important role for eNOS-derived NO production for LV dysfunction early after MI (13,19). Similarly, drugs that act as NO enhancers have been recently shown to exert beneficial effects post-MI (39), however, in the absence of concomitant β-blocker therapy. Based on the present observations, it is conceivable that enhancement of NO activity in addition to β-blockade provides additive effects early post-MI.

### Table 2 eNOS-Deficient Mice: Echocardiographic, Hemodynamic, and Morphometric Analyses

<table>
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<tr>
<th></th>
<th>Sham</th>
<th>Vehicle</th>
<th>Nebivolol</th>
<th>p Value (Vehicle eNOS−/− vs. Nebivolol WT)</th>
<th>p Value (Nebivolol eNOS−/− vs. Nebivolol WT)</th>
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<td><strong>Echocardiography</strong></td>
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<tr>
<td>EDD, mm</td>
<td>3.8 ± 0.1</td>
<td>5.9 ± 0.5</td>
<td>5.9 ± 0.2</td>
<td>5.2 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>ESD, mm</td>
<td>2.3 ± 0.1</td>
<td>5.3 ± 0.7</td>
<td>5.3 ± 0.2</td>
<td>4.3 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>FS, %</td>
<td>39.3 ± 2.6</td>
<td>9.6 ± 1.9</td>
<td>9.6 ± 1.8</td>
<td>16.6 ± 2.6</td>
<td>NS</td>
</tr>
<tr>
<td>EF, %</td>
<td>60.5 ± 4.3</td>
<td>19.7 ± 3.4</td>
<td>19.6 ± 3.2</td>
<td>31.7 ± 3.5</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Weights and morphometric analysis</strong></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>LV, mg/g body weight</td>
<td>3.8 ± 0.1</td>
<td>6.1 ± 0.3</td>
<td>5.9 ± 0.4</td>
<td>4.0 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>22 ± 0.8</td>
<td>27 ± 0.5</td>
<td>28 ± 0.3</td>
<td>29 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>LV, mg</td>
<td>84 ± 4</td>
<td>162 ± 12</td>
<td>167 ± 10</td>
<td>114 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td>Cardiomyocyte cross-sectional area, μm²</td>
<td>337 ± 26</td>
<td>582 ± 16</td>
<td>443 ± 11</td>
<td>362 ± 10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Infarct size, %</td>
<td>NA</td>
<td>49 ± 2</td>
<td>49 ± 7</td>
<td>41 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td>Heart frequency, min⁻¹</td>
<td>549 ± 19</td>
<td>562 ± 27</td>
<td>473 ± 11</td>
<td>553 ± 23</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

eNOS = endothelial nitric oxide synthase; other abbreviations as in Table 1.

(A and B) LV fractional shortening and LV ejection fraction of sham-treated and post-myocardial infarction mice treated with vehicle, metoprolol, or nebivolol as assessed by echocardiography. (C) Representative photographs of M-mode echocardiography. (D and E) LV fractional shortening and LV ejection fraction of eNOS-deficient mice after myocardial infarction treated with vehicle or nebivolol. Abbreviations as in Figures 2 and 4.
Moreover, in the present study, we observed that nebivolol therapy increased early EPCs and myocardial neovascularization post-MI in an eNOS-dependent manner, which was not observed in eNOS-deficient mice. The observed association of an increase in early EPCs, improved myocardial neovascularization, and improved LV function after nebivolol therapy that were dependent on eNOS does not prove a cause-and-effect relationship. However, there is recent evidence to support the concept that bone marrow–derived progenitor cells may promote cardiac neovascularization after MI and may contribute to an improved LV function after MI. Several recent studies showed that administration of ex vivo expanded early EPCs increased myocardial neovascularization at the infarct border and improved LV function (40–43). Cho et al. (43) reported that cardiac transplantation of early EPCs stimulated the host production of several angiogenic growth factors in the peri-infarct myocardium. Furthermore, Fazel et al. (44) observed that in mice with a mutation of the c-kit receptor, the mobilization of early EPCs after MI was impaired and was associated with decreased cardiac neovascularization and increased LV dysfunction early after MI. Furthermore, eNOS-derived NO production has been shown to be critically important for the mobilization and angiogenic and endothelial repair capacity of mobilized early EPCs (14,24,35). More recently, eNOS was observed to be essential for the effects of bone marrow–derived mononuclear cells on LV dysfunction after MI, indicating an important role of eNOS-containing cells in the effects on cardiac function after MI (45). However, several potential local mechanisms have also been suggested whereby increased eNOS-dependent NO availability may improve myocardial neovascularization including decreased expression of growth inhibitors (i.e., angiostatin [20]), and improved local vascular endothelial growth factor expression and activity that have been observed after overexpression of eNOS (46). Therefore, it is conceivable that both the eNOS-dependent

Figure 6  Survival
Kaplan-Meier survival curves for mice after myocardial infarction (MI) treated with vehicle, metoprolol, or nebivolol. Percentages of surviving mice are plotted.

Figure 7  Superoxide Production
Electron spin resonance spectroscopy analysis of superoxide production in angiotensin II–stimulated human aortic endothelial cells (HAECs). Effect of treatment with metoprolol (β₁), nebivolol (β₂), nadolol (β₁,₂), and bupranolol (β₁,₂,₃), 12 mice in each experiment.
effects of nebivolol therapy on bone marrow–derived progenitor cells and stimulation of local eNOS-dependent mechanisms may have contributed to increased myocardial neovascularization and the observed beneficial effects on LV dysfunction early after MI.

In the present study, we observed an inhibition of cardiac NADPH oxidase activation early after MI in response to nebivolol, but not metoprolol, therapy. Notably, we and others recently demonstrated that prevention of NADPH oxidase activation early after MI by using p47phox- or Nox2-deficient mice attenuated cardiac hypertrophy and improved LV function and survival (16,17). These studies provide further evidence to suggest that the ancillary properties of nebivolol after MI observed in the present study may be useful in the early post-MI period and may provide potentially important beneficial cardiac effects beyond conventional β-blockade. Furthermore, our studies on endothelial cells suggest that nebivolol inhibits endothelial NADPH oxidase activation via its β3-receptor–stimulating effect. Although prolonged β1-receptor blockade has been shown to exert beneficial effects on LV function in patients with chronic heart failure and decreased systolic function, the present study indicates that nebivolol possesses important additional beneficial effects likely independent of its β1-receptor blocking action and likely related to its effects on eNOS and the NADPH oxidase system in the early post-MI period.

Study limitations. In the present study, we compared the effects of nebivolol therapy with those of metoprolol-succinate treatment, a selective β1-adrenoreceptor antagonist without eNOS-stimulating or NADPH oxidase–inhibiting properties, on LV dysfunction in mice early after MI, and observed that nebivolol therapy exerts additional effects on LV dysfunction early after MI compared with metoprolol-succinate. As described here, our findings suggest that the effects of nebivolol treatment on LV dysfunction early after MI were dependent on eNOS because there was no response in eNOS-deficient mice. However, this does not exclude that another third-generation β-blocker, in particular carvedilol, may also have ancillary effects, including effects on eNOS-derived NO availability that are relevant for LV dysfunction early after MI. In particular, we and others have observed that NADPH oxidase inhibition has the potential to increase eNOS-dependent NO availability (22,24). In 2 recent studies, both nebivolol and carvedilol (at higher concentrations) were observed to inhibit NADPH oxidase–dependent superoxide production in isolated neutrophils (9) and in heart membranes from angiotensin II–infused rats (12) that was not detected after metoprolol or atenolol treatment. Furthermore, carvedilol, but not metoprolol, therapy has been observed to improve endothelial function in patients with type 2 diabetes (47). Our findings therefore do not exclude that another third-generation β-blocker, in particular carvedilol, may also have ancillary effects on eNOS-dependent NO availability that are relevant for LV dysfunction and remodeling after MI that remains to be determined in future studies and was beyond the scope of the present study.

In the present study, we performed several measurements of LV hypertrophy post-MI, including determination of the LV weight/body weight ratio, cardiomyocyte length, and CSA because cardiomyocyte hypertrophy post-MI likely involves both, cardiomyocyte lengthening and an increase in cardiomyocyte width that can be stimulated by increased mechanical stretch and neurohumoral activation (48). The measurements of cardiomyocyte CSA likely need to be interpreted with caution due to potential inherent limitations including analysis from selected representative cross sections of cardiomyocytes. Although we cannot exclude that the cardiomyocyte CSA measurements may have overestimated the magnitude of differences between the groups, overall, the different measurements performed to examine LV hypertrophy in the present study support the notion that both β-blockers decreased LV hypertrophy post-MI and that this effect was more pronounced after nebivolol compared with metoprolol-succinate treatment.

Conclusions

The present study provides novel evidence that nebivolol treatment is associated with beneficial effects on LV dysfunction, cardiomyocyte hypertrophy, and survival early after MI, likely independent of β1-receptor blocking effects because it was not observed with metoprolol therapy. We speculate that improved NO-dependent vasodilation, mobilization of early EPCs, and inhibition of myocardial NADPH oxidase activation, as observed after nebivolol therapy, but not after metoprolol therapy, are contributing underlying mechanisms that are involved in these beneficial ancillary properties of nebivolol in the early post-MI period. This notion is supported by the observation that nebivolol therapy did not improve LV dysfunction early after MI in eNOS-deficient mice.

Acknowledgment

The authors thank Eva Niemczyk at Phenos GmbH for her excellent technical assistance.

Reprint requests and correspondence: Dr. Ulf Landmesser, Cardiovascular Center, University Hospital Zürich, Rämistr 100 (C-Hof 111), 8091 Zürich, Switzerland. E-mail: Ulf.Landmesser@usz.ch.

REFERENCES


Key Words: beta-adrenoceptor blocker • early endothelial progenitor cells • endothelial function • left ventricular remodeling • myocardial infarction.