Nebivolol Exerts Beneficial Effects on Endothelial Function, Early Endothelial Progenitor Cells, Myocardial Neovascularisation, and Left Ventricular Dysfunction Early After Myocardial Infarction

Beyond Conventional β_1-Blockade

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Abstract

Objectives - The aim was to investigate whether nebivolol has added effects on left ventricular (LV) dysfunction and remodelling early after MI beyond its β_1-receptor blocking properties.

Background - Nebivolol is a third-generation selective β_1-adrenoreceptor antagonist that stimulates endothelial cell nitric oxide (NO) production and prevents vascular NAD(P)H oxidase activation. Both, endothelial NO synthase-derived NO production and NAD(P)H oxidase activation are critical modulators of LV dysfunction early after myocardial infarction (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 β_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 as compared to metoprolol and placebo. Nebivolol, but not metoprolol inhibited cardiac NAD(P)H oxidase activation after MI as detected by electron spin resonance spectroscopy (ESR) 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 as compared to placebo. Nebivolol had a significantly more pronounced inhibitory effect on cardiomyocyte hypertrophy after MI as compared to metoprolol.

Conclusions - Nebivolol improves LV dysfunction and survival early after MI likely beyond the effects provided by conventional β_1-receptor blockade. Nebivolol induced effects on NO mediated, endothelial function, early endothelial progenitor cells and inhibition of myocardial NAD(P)H oxidase likely contribute to these beneficial effects of nebivolol early after MI.

Keywords: Myocardial Infarction, β-Adrenoreceptor Blocker, Early Endothelial Progenitor Cells, Left Ventricular Remodelling, Endothelial Function

Abbreviation list

CSA	Cross sectional area
EDD	End-diastolic diameter
EF	Ejection fraction
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cells
ESD	End-systolic diameter
ESR	Electron spin resonance spectroscopy
FS	Fractional shortening
HPF	High power field
LV	Left ventricular, left ventricle
MI	Myocardial infarction
NO	Nitric oxide
RR	Blood pressure

Introduction

β_1-Adrenergic receptor blockers have become a hallmark in the management of patients after acute myocardial infarction as well as in the treatment of chronic heart failure (1). In contrast to conventional β_1-selective adrenergic receptor antagonists such as metoprolol-succinate the β_1–selective adrenergic receptor blocker nebivolol has been shown to possess additional actions, in particular, to stimulate endothelial cell NO production (2-4), which is thought to be mediated by β_3-receptor activation (5-7) and by interaction with the estrogen receptor (8). The release of the endothelium-derived relaxing factor NO has been suggested to mediate vasodilatory properties of nebivolol, since nebivolol induced vasodilation is almost completely blocked by inhibitors of the NO synthase (3). Moreover, administration of nebivolol has been shown to prevent hypercholesterolemia induced uncoupling of endothelial NO synthase (9) and to restore NO availability in endothelial cells obtained from black Americans (10). Furthermore, nebivolol has been suggested to exert systemic antioxidant effects (11) and to inhibit inflammatory cell NAD(P)H oxidase activation (9) and vascular NAD(P)H oxidase activation in response to angiotensin-II (12).

Importantly, accumulating evidence suggests a critical role of both, endothelial nitric oxide synthase-derived nitric oxide availability (13-15) and NAD(P)H oxidase activation for left ventricular (LV) dysfunction and cardiomyocyte hypertrophy early after myocardial infarction (MI) (16-18). In this respect, we have recently observed that statins improve endothelium-dependent vasodilation, left ventricular dysfunction and survival after experimental myocardial infarction that was critically dependent on their effect on endothelial NO synthase (eNOS), suggesting that increased eNOS-derived NO production may exert beneficial effects on LV dysfunction and survival after MI (13,14,19). Furthermore, endothelial or cardiomyocyte-targeted overexpression of eNOS resulted in improved LV function after MI (13,19). Vice versa, suppression of eNOS-dependent NO production resulted in an augmented left ventricular dysfunction (15), reduced myocardial neovascularisation (20), and impaired mobilization of early endothelial progenitor cells (EPCs) (14,19).

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

The present study was therefore designed to examine the effect of nebivolol therapy as compared to metoprolol-succinate or placebo on endothelium-dependent, NO mediated vasodilatation, early endothelial progenitor cell (EPC) mobilization, as well as myocardial NAD(P)H oxidase activation as

analysed by electron resonance spectroscopy (ESR) early after myocardial infarction. Moreover, we examined the effect of nebivolol and metoprolol-succinate therapy on LV dysfunction, survival and cardiomyocyte hypertrophy early after myocardial infarction.

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 (NIH).

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

Furthermore, in additional experiments myocardial infarction was induced in $eNOS^{-/-}$ mice that were randomized on day 1 to nebivolol or vehicle therapy (n=10 each) using the above 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 endothelial nitric oxide synthase.

Early EPC Culture Assay

Early EPCs were cultured as described in detail previously (14,24-26). In brief, mononuclear cells were isolated from 1 mL of peripheral blood by density gradient centrifugation with Histopaque (Sigma) (27), seeded on tissue culture coverslips ($9x10^5$ cells) coated with rat vitronectin (Sigma) in endothelial basal medium (endothelial growth medium-2 MV, Clonetics), and supplemented with EGM-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'-tetramethylindocarbocyanine–labeled acetylated LDL (acLDL-DiI; Molecular Probes), cells were incubated with acLDL-DiI (6 µg/mL, 37°C, 2 hours). Cells were then fixed with 1% paraformaldehyde for 10 minutes and incubated with fluorescein-labeled *Griffonia simplicifolia* lectin I (BS-1 lectin, Vector Labs) for 1 hour. After being stained, samples were analyzed with an inverted fluorescence microscope (Leica), and double-stained cells for both BS-1 lectin and acLDL-DiI were counted as early EPCs in at least 4 randomly selected HPFs (28).

Histomorphometric Analysis and Immunohistochemistry

Tissue morphometry was performed in a blinded fashion with the Quantimet 500MC 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 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) using the digital image analyzer as described previously (29-32). Nuclei were stained with DAPI. For measurements of the cardiomyocyte cross sectional area or cardiomyocyte length only cross gated cardiomyocytes respectively longitudinally sectioned cardiomyocytes were analysed. Furthermore, length of cardiomyocytes isolated from *in situ* formalin-fixed LV tissue was measured using the KOH method as described in detail previously (33). In brief, LV samples were cut into small pieces, placed overnight into a 12.5 mol/L KOH solution and transferred into phosphate buffer. Samples were then mixed using a vortexer, centrifuged and resuspended in 10% formaldehyde-phosphate buffer. Nuclei were stained by Mayers hemalaum solution, in order to differentiate between cell fragments and intact myocytes. Myocyte length (50 structurally intact myocytes / sample) was determined by using an Olympus BX51 microscope and the software program *Analysis 5.0*.

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 anti–platelet and endothelial cell adhesion molecule-1 (CD31) rabbit polyclonal antibody (H-300, Santa Cruz) and detection of the primary antibody was performed using the avidin-biotin-peroxidase labelling system. Counterstaining was performed with hematoxylin, and results are expressed as capillaries per

cardiomyocytes. At least 6 high power fields (HPFs) of cross sectioned areas per infarct border zone were examined by an investigator blinded to the treatment group.

Echocardiographic Measurements

Echo analysis was performed under light anesthesia (ketamine 100 mg/kg, xylazine 1.25 mg/kg, and atropine 0.6 mg/kg IP) and spontaneous respiration with a commercially available ultrasound system (ATL5000 CV) 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 NAD(P)H Oxidase Activity by Electron Spin Resonance Spectroscopy Analysis

Activity of NAD(P)H oxidase was determined in remote LV myocardium (50 µg protein) by ESR spectroscopy as described previously by using the spin probe 1-hydroxy-3 carboxypyrrolidine (CP-H) and a MiniScope ESR spectrometer (Magnettech) (16,34). The ESR settings were the following: field center: 3367.67 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 NAD(P)H (obtained for each sample).

Measurement of Superoxide Production in Human Aortic Endothelial Cells by Electron Spin Resonance Spectroscopy Analysis

Human aortic endothelial cells (HAECs) purchased from Clonetics were grown according to standard procedures. Serum-starved HAECs were exposed to metoprolol (10 µmol/L), nebivolol (10 µmol/L) or the solvent after a 60 minute preincubation with various pharmacological modulators, i.e., bupranolol (10 µmol/L), or nadolol (10 µmol/L) as described in detail previously (6).

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

Blood Pressure Measurements

Systolic blood pressure (SBP) was measured by a computerized non-invasive tail cuff system (Blood Pressure Analysis System BP-200, Visitech Systems, Apex, NC, USA) as described previously (14,22).

Statistical Analysis

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

Results

Effect of Nebivolol and Metoprolol-Succinate Treatment on Endothelium-Dependent, NO Mediated Vasodilation after Myocardial Infarction

In sham-operated mice, acetylcholine produced endothelium-dependent relaxations of 93±10%. These responses were substantially impaired in aortas of WT mice 4 weeks after MI (21±4%; P<0.05; Figure 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=n.s. vs. vehicle; Figure 1A). In contrast, endothelium-independent vasodilation in response to nitroglycerin was not impaired in mice after MI and was neither changed by nebivolol nor by metoprolol treatment (Figure 1B). In $eNOS^{-/-}$ mice, no endothelium-dependent vasodilation in response to acetylcholine was observed, indicating that these responses are dependent on endothelial NO synthase (data not shown).

Effect of Nebivolol and Metoprolol-Succinate Treatment on Early EPCs after MI

Increasing evidence suggests that endothelial nitric oxide synthase activation plays a critical role for mobilization of early endothelial progenitor cells (14,35). However, it is not known whether early EPC numbers are altered by β-blocker treatment in the presence of an ischemic stimulus. Notably, nebivolol therapy markedly augmented early EPCs as compared to placebo (Figure 2A, B). In contrast, metoprolol-succinate treatment had no significant effect on early EPC numbers as compared to vehicle (Figure 2A, B). As shown in Figure 2D, nebivolol therapy did not change early EPC numbers in $eNOS^{-/-}$ mice, suggesting that this response is dependent on endothelial NO synthase.

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 (HPFs) 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 WT mice after MI (Figure 2C). In contrast, nebivolol therapy had no effect on capillary density in $eNOS^{-/-}$ mice after (Figure 2E).

ESR Spectroscopic Analysis of Myocardial NAD(P)H Oxidase Activity after MI

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

Effect of Nebivolol and Metoprolol on Cardiomyocyte Hypertrophy and Cardiomyocyte Length after MI

After myocardial infarction we observed cardiomyocyte hypertrophy as indicated by increased left ventricular weight / body weight ratio and increased cardiomyocyte length and cross sectional area (CSA) in the remote myocardium (Figure 4A-D, Table 1). Whereas both β-blocker therapies reduced cardiomyocyte hypertrophy after MI, treatment with nebivolol had a significantly more pronounced inhibitory effect on left ventricular weight / body weight and cardiomyocyte cross sectional area as compared to metoprolol ($P<0.05$ vs. metoprolol, Figure 4A; $P<0.001$ vs. metoprolol, Table 1). In $eNOS^{-/-}$ mice, the response of nebivolol therapy on LV mass and hypertrophy was attenuated as compared to wild type mice (Table 2), suggesting that this effect is at least partly dependent on endothelial NO synthase.

Our data suggest that cardiomyocyte lengthening contributed to the observed LV weight / body mass differences after myocardial infarction, i.e. measurements of cardiomyocyte length of isolated cardiomyocytes by using the KOH method indicated an increase of cardiomyocyte length after myocardial infarction that was significantly attenuated by both β-blockers after MI (Figure 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 as compared to metoprolol-succinate therapy (Figure 4C, D). 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 (Figure 4B).

Effect of Nebivolol and Metoprolol on LV Dysfunction after MI

LV ejection fraction was substantially reduced 4 weeks after MI as compared with sham-operated animals. Notably, nebivolol, but not metoprolol therapy significantly improved fractional shortening and LV ejection fraction 4 weeks after MI (Figure 5A-C, 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 myocardial infarction, suggesting that the eNOS is involved in this effect (Figure 5D, E, Table 2).

Survival post MI

Nebivolol therapy was associated with a significantly improved survival at 4 weeks after MI as compared to vehicle therapy ($P<0.05$ vs. vehicle). The impact of metoprolol was not statistically significant (Figure 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 NAD(P)H oxidase activation (36). As shown in Figure 7, pretreatment of HAECs with the $\beta_{1\text{-}2}$-blocker nadolol (10 µmol/L) had no effect on nebivolol induced reduction of endothelial superoxide production, whereas the complete $\beta_{1\text{-}2\text{-}3}$-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 β_3-receptor.

Infarct Size, Heart Rate and Blood Pressure

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

Discussion

The present study demonstrates that nebivolol, a β_1-selective adrenoreceptor antagonist with endothelial NO synthase stimulating properties attenuates LV dysfunction and cardiomyocyte hypertrophy early after myocardial infarction, associated with improved survival, that likely goes beyond the effects of conventional β-blockade. These effects of nebivolol therapy on LV dysfunction and cardiomyocyte hypertrophy early after myocardial infarction 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 augmented by cardiac NO-dependent actions which may be related, at least in part, to activation of β_3-receptors by nebivolol that may increase eNOS-dependent NO availability both, by preventing NAD(P)H oxidase activation and stimulation of endothelial NO synthase.

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 endothelial progenitor cell mobilization and myocardial neovascularisation after myocardial infarction, likely independent of its β_1-receptor blocking effects, since it was not observed with the β_1-selective adrenoreceptor antagonist metoprolol. Furthermore, in contrast to metoprolol-succinate, nebivolol inhibited myocardial NAD(P)H oxidase activation after MI. Moreover, our data suggest that the ability of nebivolol to reduce angiotensin-II induced NAD(P)H oxidase-dependent superoxide production is dependent on β_3-receptor activation. In contrast to the neutral effect of $\beta_{1\text{-}2}$-blockade by nadolol, the $\beta_{1\text{-}2\text{-}3}$-blocker bupranolol significantly inhibited nebivolol's effect on superoxide production.

Several studies have firmly established the benefical effects of conventional β-blockers on LV remodelling processes post MI (37). However, whereas 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 β_1-blockade that may be mediated by prevention of NAD(P)H oxidase activation and enhanced eNOS-dependent NO availability.

Nebivolol is a third-generation highly selective β_1-adrenoreceptor blocker. There is evidence that nebivolol in addition to its β_1-adrenoreceptor blocking effects can stimulate endothelial nitric oxide production, which has been suggested to be mediated, at least in part, by a β_3-agonistic effect (5,38).

Furthermore, nebivolol has been suggested to exert antioxidant effects, that have been attributed, at least in part, to prevent NAD(P)H oxidase activation in response to hyperlipidemia or angiotensin-II (9,12). Our data suggest that the β_3-agonistic effect of nebivolol is involved in the inhibition of endothelial NAD(P)H oxidase activation.

In the present study, nebivolol improved LV dysfunction and survival early after myocardial infarction, which was not observed with metoprolol-succinate therapy. In this respect, we and others have recently shown that both, endothelial NO synthase dependent NO production and NAD(P)H oxidase activation play a pivotal role for left ventricular dysfunction and survival early after myocardial infarction (13-17,19). In fact, statin induced improvement of LV function and survival early after MI were critically dependent on endothelial NO synthase, since 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 of 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 on top of β-blockade provides additive effects early post MI.

Moreover, in the present study we have observed that nebivolol therapy increased early endothelial progenitor cells and myocardial neovascularisation post MI in an eNOS-dependent manner, since this was not observed in eNOS-deficient mice. The observed association of an increase of early EPCs, improved myocardial neovascularisation and improved LV function after nevivolol therapy that were dependent on endothelial NO synthase 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 neovascularisation after MI and that this may contribute to an improved LV function after MI. Several recent studies have shown that administration of ex vivo expanded early EPCs increased myocardial neovascularisation at the infarct border and improved LV function (40-43). Cho et al. have reported that cardiac transplantation of early EPCs stimulated the host production of several angiogenic growth factors in the peri-infarct myocardium (43). Furthermore, Fazel et al. have observed that in mice with a mutation of the c-kit receptor, the mobilization of early endothelial progenitor cells after myocardial infarction was impaired that was associated with a reduced cardiac neovascularisation and augmented LV dysfunction early after myocardial infarction (44). 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 myocardial infarction, indicating an important role of eNOS containing cells for the effects on cardiac function after myocardial infarction (45). However, several potential local mechanisms have also been suggested whereby increased eNOS-dependent NO availability may improve myocardial neovascularisation including a reduced expression of growth inhibitors, ie, angiostatin (20), and an 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 effects of nebivolol therapy on bone marrow derived progenitor cells and stimulation of local eNOS-dependent mechanisms may have contributed to increased myocardial neovascularisation and the observed beneficial effects on LV dysfunction early after MI.

In the present study we have observed an inhibition of cardiac NAD(P)H oxidase activation early after MI in response to nebivolol, but not metorpolol therapy. Notably, we and others have recently demonstrated that prevention of NAD(P)H oxidase activation early after MI by using $p47^{phox}$ 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 infarction period and may provide potentially important beneficial cardiac effects beyond conventional β-blockade. Furthermore, our studies in endothelial cells suggest that nebivolol inhibits endothelial NAD(P)H oxidase activation via its β_3-receptor stimulating effect. Whereas prolonged β_1-receptor blockade has been shown to exert beneficial effects on LV function in patients with chronic heart failure and reduced systolic function, the present study indicates that nebivolol possesses important additional beneficial effects likely independent of its β_1-receptor blocking action, and likely related to its effects on endothelial NO synthase and the NAD(P)H oxidase system, in the early post infarction period.

Limitations of the study:

In the present study we have compared the effects of nebivolol therapy with metoprolol-succinate treatment, a selective β_1-adrenoreceptor antagonist without endothelial NO synthase stimulating or NAD(P)H oxidase inhibiting properties, on LV dysfunction in mice early after myocardial infarction, and have observed that nebivolol therapy exerts additional effects on LV dysfunction early after MI as compared to metoprolol-succinate. As described above, our findings suggest that the effects of

nebivolol treatment on LV dysfunction early after MI were dependent on endothelial NO synthase, since 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 endothelial NO synthase 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 two 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 remodelling after MI that remains to be determined in future studies and was beyond the scope of the present study.

In the present study we have performed several measurements of LV hypertrophy post myocardial infarction, including determination of LV weight / body weight ratio, cardiomyocyte length and cross sectional area, since cardiomyocyte hypertrophy post myocardial infarction 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 cross sectional area (CSA) likely need to be interpreted with caution due to its potential inherent limitations including analysis from selected representative cross sections of cardiomyocytes. While 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 reduced LV hypertrophy post MI and that this effect was more pronounced after nebivolol as compared to metoprolol-succinate treatment.

In conclusion, the present study provides novel evidence that nebivolol treatment is associated with beneficial effects on left ventricular dysfunction, cardiomyocyte hypertrophy and survival early after myocardial infarction, likely independent of β_1-receptor blocking effects, since it was not observed with metoprolol therapy. We speculate that improved NO-dependent vasodilation, mobilization of early endothelial progenitor cells and inhibition of myocardial NAD(P)H oxidase activation, as observed after nebivolol 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.

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Conflict of Interest / Disclosures

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Figure Legends

Figure 1: Endothelium-dependent and – independent Vasorelaxation

A, Endothelium-dependent, NO mediated vasodilation in response to acetylcholine (Ach) in sham-operated, vehicle, metoprolol and nebivolol treated mice 4 weeks after surgery. The significance test between groups shows a comparison at the highest concentration. **B**, Endothelium-independent vasorelaxation in response to nitroglycerine (NTG) in sham-operated, vehicle, metoprolol and nebivolol treated mice 4 weeks after surgery.

Figure 2: Early EPCs and Capillary Density

A, Early EPCs per HPF in sham-operated, vehicle, metoprolol and nebivolol treated mice 4 weeks after surgery (representative photographs are shown in **B**). **C**, Capillary density in the LV infarct border zone in vehicle, metoprolol and nebivolol treated mice 4 weeks after surgery. **D**, Early EPCs per HPF in vehicle and nebivolol treated eNOS deficient mice 4 weeks after surgery. **E**, Capillary density in the LV infarct border zone in vehicle and nebivolol treated eNOS-deficient mice 4 weeks after surgery (n=7-8).

Figure 3: Cardiac NAD(P)H Oxidase Activity

A, NAD(P)H oxidase activity in remote myocardium in sham-operated, vehicle, metoprolol and nebivolol treated mice 4 weeks after surgery as determined by ESR spectroscopy analysis (representative photographs are shown in **B**) (n=7).

Figure 4: LV Weight and Cardiomyocyte Length

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

Figure 5: LV Function

A and B, LV fractional shortening and LV ejection fraction of sham-treated and post MI 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 MI treated with vehicle or nebivolol.

Figure 6: Survival

Kaplan-Meier survival curves for mice after MI, treated with vehicle, metoprolol or nebivolol. Percentages of surviving mice were plotted.

Figure 7: Superoxide Production

ESR spectroscopy analysis of superoxide production in angiotensin-II stimulated HAECs. Effect of treatment with metoprolol (β_1), nebivolol (β_1), nadolol ($\beta_{1,2}$), bupranolol ($\beta_{1,2,3}$), n=12 in each experiment.

Table 1 **Wild type mice: Echocardiographic, Hemodynamic and Morphometric Analyses**

	Sham WT	Vehicle WT	Metoprolol WT	Nebivolol WT	*P* (Metoprolol vs. Nebivolol)	*P* (Vehicle vs. Metoprolol)	*P* (Vehicle vs. Nebivolol)
Echocardiography							
EDD [mm]	3.8 ± 0.1	6.3 ± 0.3	5.3 ± 0.3	5.2 ± 0.2	n.s.	P<0.05	P<0.01
ESD [mm]	2.4 ± 0.1	5.7 ± 0.3	4.9 ± 0.4	4.3 ± 0.2	n.s.	n.s.	P<0.01
FS [%]	36.7 ± 1.1	9.6 ± 1.7	7.6 ± 3.3	16.6 ± 2.6	P<0.05	n.s.	P<0.05
EF [%]	55.9 ± 3.1	18.6 ± 3.7	17.1 ± 5.5	31.7 ± 3.5	P<0.05	n.s.	P<0.05
Weights and Morphometric Analysis							
LV [mg/g body weight]	3.6 ± 0.2	6.2 ± 0.5	4.8 ± 0.3	4.0 ± 0.1	P<0.05	P<0.01	P<0.001
Body weight [g]	29 ± 1.6	33 ± 1.4	26 ± 0.3	29 ± 0.5	n.s.	P<0.001	P<0.05
LV [mg]	105 ± 6	205 ± 21	124 ± 9	114 ± 4	n.s.	P<0.001	P<0.001
Cardiomyocyte cross sectional area [μm^2]	259 ± 12	553 ± 70	451 ± 8	362 ± 10	P<0.001	P<0.01	P<0.001
Infarct size [%]	n.a.	42 ± 3	41 ± 14	41 ± 12	n.s.	n.s.	n.s.
Systolic RR [mmHg]	110 ± 3	112 ± 2	106 ± 2	104 ± 3	n.s.	n.s.	n.s.
Heart frequency [min^{-1}]	598 ± 20	674 ± 15	547 ± 12	553 ± 23	n.s.	P<0.01	P<0.01

WT = Wild type mice

Table 2 **eNOS-deficient mice: Echocardiographic, Hemodynamic and Morphometric Analyses**

	Sham *eNOS* $^{-/-}$	**Vehicle *eNOS* $^{-/-}$**	***Nebivolol* *eNOS* $^{-/-}$**	**Nebivolol WT**	***P* (Vehicle eNOS $^{-/-}$ vs. Nebivolol eNOS $^{-/-}$)**	**P (Nebivolol eNOS$^{-/-}$ vs. Nebivolol WT)**
Echocardiography						
EDD [mm]	3.8 ± 0.1	5.9 ± 0.5	5.9 ± 0.2	5.2 ± 0.2	n.s.	n.s.
ESD [mm]	2.3 ± 0.1	5.3 ± 0.7	5.3 ± 0.2	4.3 ± 0.2	n.s.	P<0.05
FS [%]	39.3 ± 2.6	9.6 ± 1.9	9.6 ± 1.8	16.6 ± 2.6	n.s.	P<0.05
EF [%]	60.5 ± 4.3	19.7 ± 3.4	19.6 ± 3.2	31.7 ± 3.5	n.s.	P<0.05
Weights and Morphometric Analysis						
LV [mg/g body weight]	3.8 ± 0.1	6.1 ± 0.3	5.9 ± 0.4	4.0 ± 0.1	n.s.	P<0.05
Body weight [g]	22 ± 0.8	27 ± 0.5	28 ± 0.3	29 ± 0.5	n.s.	n.s.
LV [mg]	84 ± 4	162 ± 12	167 ± 10	114 ± 4	n.s.	P<0.001
Cardiomyocyte cross sectional area [µm^2]	337 ± 26	582 ± 16	443 ± 11	362 ± 10	P<0.001	P<0.05
Infarct size [%]	n.a.	49 ± 2	49 ± 7	41 ± 12	n.s.	n.s.
Heart frequency [min^{-1}]	549 ± 19	562 ± 27	473 ± 11	553 ± 23	P<0.05	P<0.05

WT = Wild type mice