Nebivolol Inhibits Superoxide Formation by NADPH Oxidase and Endothelial Dysfunction in Angiotensin II–Treated Rats

Matthias Oelze, Andreas Daiber, Ralf P. Brandes, Marcus Hortmann, Philip Wenzel, Ulrich Hink, Eberhard Schulz, Hanke Mollnau, Alexandra von Sandersleben, Andrei L. Kleschyov, Alexander Mülsch, Huige Li, Ulrich Förstermann, Thomas Münzel

Abstract—Nebivolol is a β-receptor antagonist with vasodilator and antioxidant properties. Because the vascular NADPH oxidase is an important superoxide source, we studied the effect of nebivolol on endothelial function and NADPH oxidase activity and expression in the well-characterized model of angiotensin II–induced hypertension. Angiotensin II infusion (1 mg/kg per day for 7 days) caused endothelial dysfunction in male Wistar rats and increased vascular superoxide as detected by lucigenin-derived chemiluminescence, as well as dihydroethidine staining. Vascular NADPH oxidase activity, as well as expression at the mRNA and protein level, were markedly upregulated, as well as NOS III uncoupled, as evidenced by NO synthase III inhibitor experiments and dihydroethidine staining and by markedly decreased hemoglobin–NO concentrations. Treatment with the β-receptor blocker nebivolol but not metoprolol (10 mg/kg per day for each drug) normalized endothelial function, reduced superoxide formation, increased NO bioavailability, and inhibited upregulation of the activity and expression of the vascular NADPH oxidase, as well as membrane association of NADPH oxidase subunits (Rac1 and p67phox). In addition, NOS III uncoupling was prevented. In vitro treatment with nebivolol but not atenolol or metoprolol induced a dissociation of p67phox and Rac1, as well as an inhibition of NADPH oxidase activity assessed in heart membranes from angiotensin II–infused animals, as well as in homogenates of Nox1 and cytosolic subunit–transfected and phorbol ester–stimulated HEK293 cells. These findings indicate that nebivolol interferes with the assembly of NADPH oxidase. Thus, inhibitory effects of this β-blocker on vascular NADPH oxidase may explain, at least in part, its beneficial effect on endothelial function in angiotensin II–induced hypertension. (Hypertension. 2006;48:677-684.)

Key Words: angiotensin II ■ nitric oxide synthase ■ endothelium ■ oxidative stress ■ vasodilation

Both arterial hypertension and coronary artery disease are associated with an activation of the circulating and local renin–angiotensin system and increased oxidative stress within the vascular wall. Angiotensin II (Ang II) treatment has been shown to cause endothelial dysfunction, which is at least in part mediated by increased vascular superoxide levels. Superoxide sources may include the NADPH oxidases and an uncoupled endothelial NO synthase (NOS III). Increased vascular superoxide production and endothelial dysfunction are also accompanied by increased NOS III expression but decreased vascular NO production, downregulation of the target enzyme soluble guanylyl cyclase, and by a decrease in the activity of the cGMP-dependent kinase (cGK-I). Interestingly, these phenomena seemed to be linked, at least in part, to an activation of protein kinase C (PKC), because the inhibition of increased vascular PKC activity in vitro and in vivo partially inhibited superoxide production and improved NO/soluble guanylyl cyclase/cGK-I signaling.

Adrenergic β-receptor antagonists are standard drugs for the treatment of hypertension and coronary artery disease. Some of these β-blockers, such as carvedilol and nebivolol, have been shown recently to exhibit antioxidative properties. In vitro stimulation of the β2-receptors on endothelial cells by nebivolol metabolites increased endothelial [Ca2+] levels and, accordingly, NOS III activity. We have shown recently that chronic treatment with nebivolol could normalize vascular superoxide formation, as well as endothelial dysfunction in Watanabe heritable hyperlipidemic rabbits, which served as a model of hyperlipidemia and early stage atherosclerosis. Nebivolol also exerted this antioxidative effect in vitro when whole blood, as well as isolated neutrophils or macro-

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The first 2 authors contributed equally to this study and should, therefore, be considered as joint first authors.

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phages, were stimulated by a phorbol ester derivative. This indicates that the protective mechanism was mainly based on suppression of the phagocytic NADPH-dependent superoxide formation by either direct inhibition of the enzyme activity or inhibition of its activation by PKC. The antioxidative effect was not stereoselective, because it was mediated by D- as well as L-nebivolol excluding an involvement of the β receptor and pointing toward a free-radical scavenging effect of the molecule itself for these protective effects.

Chronic treatment with nebivolol has also been shown to improve endothelial function in patients with essential hypertension. This protective effect was explained by a nebivolol-mediated decrease in oxidative stress as shown by decreased levels of the isoprostane 8-iso-prostaglandin F₂α in patients and decreased expression of endothelin-1 in endothelial and smooth muscle cells. It was shown recently that Nox1 overexpression in transgenic mice potentiates Ang II–induced hypertension, which likewise improved in Nox1-deficient mice. With the present study, we tested whether the antioxidative effects of the β-blocker nebivolol also comprises inhibitory effects on the nonphagocytic, vascular NADPH oxidase in the setting of Ang II infusion. We also tested whether this may lead to a preservation of the NO signaling pathway and endothelial function. In vitro experiments using cardiac membrane preparations from Ang II–infused animals and HEK cells overexpressing NADPH oxidases were performed to address whether nebivolol may interfere with the process of NADPH oxidase assembly.

We show here for the first time that nebivolol improves endothelial function in Ang II–induced hypertension by preventing increases in NADPH oxidase activity and expression and by preventing NOS III uncoupling.

**Methods**

An expanded Methods section is available in an online supplement available at http://www.hypertensionaha.org.

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Effects of in vivo nebivolol and metoprolol treatment (+Nebi and +Meto, each 10 mg/kg per day for 7 days) on the concentration response relationship to acetylcholine (ACh) (A) and on NO/cGMP-signaling detected by the phosphorylation state of VASP (B; densitometric quantification and original blots shown below) in aortic rings from Ang II–infused (Ang II, 1 mg/kg per day for 7 days) rats. Data are the mean±SEM of n=9 to 18 (A) and 5 (B) experiments. *P<0.05 vs control (Ctr); †P<0.05 nebivolol-treated vs untreated.
Results

Effects of Nebivolol on NO/cGMP Signaling and Endothelial Function

Ang II treatment caused severe endothelial dysfunction (Figure 1) and a marked impairment of the NO/cGMP signaling assessed by the phosphorylation of the vasodilator stimulated phosphoprotein ([P-VASP] Figure 1B). In vivo treatment with nebivolol markedly improved endothelial dysfunction and increased P-VASP levels in Ang II–treated animals, while having no effects on these parameters in control animals. Metoprolol in a concentration of 10 and 100 mg/kg had no significant effects on these parameters. Ang II infusion also slightly but significantly decreased potency of the endothelium-independent vasodilator nitroglycerin, which was not corrected by nebivolol treatment (Table I available online).

Effects of Nebivolol Treatment on Vascular Superoxide Production and NOS III Uncoupling Assessed by Dihydroethidine-Derived Fluorescence and Plasma Nitrite/Hemoglobin—NO Levels

Dihydroethidine (DHE) staining revealed vascular superoxide throughout the vessel wall in vessels from Ang II–treated animals (Figure 2). Although vascular superoxide in control rats was not modified by nebivolol treatment, a marked reduction was observed in vessels from hypertensive rats treated with nebivolol (Figure 2A). To assess the contribution of NOS III to superoxide formation because of NOS III uncoupling, rat aortic tissue was incubated with a NOS III inhibitor, N\textsuperscript{G}-nitro-L-arginine (L-NNA). L-NNA increased DHE-derived fluorescence within the endothelial monolayer from control rats (marked with E in Figure 2A). This finding indicates that in normotensive rats, NOS III–derived NO quenches basal levels of superoxide by concomitant formation of, for example, peroxynitrite, which is not detected by DHE. In contrast, incubation of vessels from Ang II–treated rats with L-NNA decreased DHE-derived fluorescence exclusively within the endothelium, thereby identifying NOS III as a significant superoxide source. This effect of L-NNA was not observed in vessels from Ang II–and nebivolol-treated rats, indicating that nebivolol treatment was able to prevent NOS III uncoupling (Figure 2A).

Ang II treatment also markedly decreased NO bioavailability (measured as plasma nitrite and whole blood hemoglobin [Hb]–NO), which was substantially corrected even above control levels by in vivo nebivolol treatment (Figure 2B and
The nitrate/nitrite ratio was increased in response to Ang II and normalized by nebivolol cotreatment (Figure II and serum parameters, available online).

Effects of Nebivolol Treatment on Vascular Superoxide Assessed by Lucigenin- and L-012–Enhanced Chemiluminescence

Ang II treatment increased vascular superoxide 4-fold as assessed by lucigenin-derived chemiluminescence (LDCL) 5 μmol/L. Treatment with nebivolol decreased LDCL by 50%, whereas superoxide production of control vessels was not modified at all by nebivolol (Figure 3A). Likewise, Ang II infusion significantly increased NADPH oxidase activity in heart membranes, which was significantly inhibited by treatment with nebivolol but not with metoprolol (Figure 4A).

In heart membranes isolated from Ang II treated rats, L-012–enhanced chemiluminescence was markedly increased, and nebivolol cotreatment did not modify the L-012 signal in control subjects but markedly decreased the signal in the Ang II infusion group (Table III).

Effects of Nebivolol Treatment on Vascular Expression and Distribution of the NADPH Oxidase Subunits p67phox and Rac1

Ang II treatment upregulated the expression of p67phox and Rac1 in aortic membrane fractions and in heart membranes, all of which was normalized by in vivo nebivolol treatment (Figures 3B and 4B). In addition, we found significant increases in the expression of Nox1 in heart membranes that were decreased by treatment with nebivolol. By RT-PCR measurements we established significant increases in Nox2 (gp91phox), p47phox, and p22phox mRNA in aortic and heart tissue ranging from 2- to 4-fold, all of which was almost normalized by nebivolol but not metoprolol cotreatment in vivo (Figure I).

Effects of In Vitro Nebivolol on Superoxide Production and Membrane Association of NADPH Oxidase Subunits p67phox and Rac1 in Heart Membrane From Ang II–Treated Animals

Superoxide production (NADPH oxidase activity) in heart membranes of Ang II–treated animals was concentration-dependently inhibited by nebivolol (Figure 5A). With respect to other β-blockers, carvedilol but not atenolol and metoprolol were able to inhibit superoxide production to a similar extent (Figure 5A). Likewise, membrane association of NADPH-oxidase subunits p67phox and Rac1 was dose-dependently inhibited by 1 to 100 μmol/L of nebivolol (Figure 5B) resulting in significant inhibition at 100 μmol/L. Although carvedilol showed a similar inhibitory effect on LDCL, the inhibition of membrane association was less pronounced. Atenolol and metoprolol clearly had no effect at all on this parameter (Figure 5B).

Effects of In Vitro Nebivolol on P-VASP Levels in Vessels Exposed to Phorbol Ester

Incubation of rat aortic vessels with a phorbol ester derivative (10 μmol/L) dramatically decreased NO/cGMP signaling as assessed by the P-VASP at serine239, all of which was concentration-dependently prevented by preincubation with nebivolol and normalized at 100 μmol/L of nebivolol (Figure 5C).

Effects of Nebivolol and Other β-Blockers on Reactive Oxygen Species Production and Rac1 Translocation in Transfected HEK293 Cells

HEK293 cells were transfected with Nox1, Nox1a, and Nox1b (the analogs of p47phox and p67phox), and basal reactive oxygen species (ROS) production was measured using a chemiluminescence-based luminol-peroxidase assay. Nebivolol, as well as carvedilol, significantly decreased the chemiluminescence signal when used at 100 μmol/L, whereas atenolol and metoprolol had...
no effect (Figure 6). Nebivolol (100 μmol/L) inhibited the phorbol myristate (PMA)–induced translocation of Rac1 in the transfected cells (Figure 6B).

**Discussion**

The present studies demonstrate that the novel β1-receptor blocker nebivolol but not metoprolol improves endothelial function and reduces vascular oxidative stress in the experimental model of Ang II–induced hypertension and that these effects were associated with a normalization of the expression of the NADPH oxidase subunits Nox1, Nox2, p22phox, p47phox, p67phox, and Rac1 and an inhibition of NOS III uncoupling. Furthermore, nebivolol but not metoprolol inhibited activation of the vascular NADPH oxidase and was able to dissociate an already assembled and active membrane-associated NADPH oxidase complex. These observations indicate that nebivolol, in addition to its β1-receptor–blocking and NO-releasing effects, possesses substantial inhibitory effects on vascular oxidative stress, all of which may beneficially influence endothelial dysfunction because of oxidative stress in the setting of arterial hypertension.

We have shown recently that in vivo treatment with the β-blocker nebivolol markedly improved endothelial dysfunction and NO signaling in vessels from hypercholesterolemic Watanabe heritable hyperlipidemic rabbits, which was at least in part secondary to a normalization of vascular superoxide production. Importantly, in this particular study, nebivolol was able to inhibit superoxide production by macrophages and neutrophils activated in vitro by Ang II and by phorbol ester. Both compounds induce the oxidative burst in these inflammatory cells mainly by activating the NADPH oxidase. Interestingly, other β-blockers, such as atenolol and metoprolol, were not able to inhibit superoxide production, which may explain why nebivolol but not metoprolol is able to reduce the progression of atherosclerosis in the setting of hypercholesterolemia.

Up to now, there are no data available on whether β-blockers are able to inhibit superoxide production by the vascular NADPH oxidase, enzymes that have been identified to represent some of the major superoxide-producing enzymes in the setting of atherosclerosis, diabetes mellitus,
heart failure,22 and arterial hypertension.23 In animals infused with Ang II, we have demonstrated recently that there is a marked degree of endothelial dysfunction, increased oxidative stress because of upregulation of the activity and expression of the NADPH oxidase and by NOS III uncoupling, and that there is a strong inhibition of NO signaling as evidenced by the marked reduction of the vascular levels of the P-VASP, a surrogate marker for the activity of the cGK-I.4,24

To address the possibility that nebivolol treatment is able to prevent NOS III uncoupling, vessels from Ang II–infused animals with and without nebivolol were incubated with the NOS inhibitor L-NNA. In vessels from Ang II–infused animals, the superoxide signal within endothelial cells was almost eliminated, identifying NOS III as a significant superoxide source. In vessel from Ang II–infused animals treated with nebivolol, L-NNA increased the superoxide signal like in control animals, providing evidence, as shown before,21,22,24 that under these conditions, NOS III is re-
coupled and that NO produced by NOS III metabolizes superoxide produced by the endothelium under baseline conditions. This hypothesis is further strengthened by the observed decrease in plasma nitrite levels (indirect measure of NO) and Hb–NO in response to Ang II, which is compatible with NOS III uncoupling, and the correction of these parameters by in vivo treatment with nebivolol.

Importantly, the increase in superoxide production induced by Ang II was not only restricted to the endothelium but also involved the media, as shown by the DHE experiments. Treatment with nebivolol was able to drastically reduce superoxide production throughout the vessel wall. One of the candidate enzymes responsible for the increased superoxide production throughout the vessel wall is the vascular NADPH oxidase.25 Importantly, in vivo nebivolol treatment resulted in a normalization of the NADPH oxidase activity in membrane fractions from the heart, whereas metoprolol was virtually ineffective (Figure 4A). In

![Figure 5.](https://example.com/figure5.png)
experiments performed in HEK293 overexpressing Nox1-containing NADPH oxidase, facilitating a direct analysis of the effects of β-blockers on NADPH oxidase function. Also, in these experiments nebivolol strongly inhibited oxidase activity, and this effect was associated with a dissociation of Rac1 from the activated enzyme complex.

Based on these observations, it might be valid to speculate regarding the underlying mechanism. The interaction of Rac1 and the cytosolic phox subunits with the membrane-bound main oxidase complex depends on the association of the PX domain of p47phox with the plasma membrane and the Rac anchoring in the membrane via its geranyl-geranylated tail (for review see Reference 26). It, therefore, seems probable that nebivolol at the concentrations reached in these studies may have direct effects on membrane properties (such as influencing the fluidity) attenuating the anchoring of cytosolic proteins in the process of cellular activation. This concept could also nicely account for the beneficial effects of nebivolol on endothelial NO synthase activity.11

Using HEK293 cells, we could demonstrate that nebivolol, but not metoprolol or atenolol, decreased the basal ROS formation and prevented translocation of Rac1 to the membrane. In addition, we were also able to show that when the NADPH oxidase is already assembled, such as in hearts from Ang II–infused animals, nebivolol, but not metoprolol or atenolol, was able to dissociate the NADPH oxidase subunits Rac1 and p67phox from the membrane and simultaneously reduced NADPH oxidase activity (Figure 5A and 5B).

### Perspectives

The present study indicates that the selective β1-receptor blocker nebivolol is able to prevent the adverse effects of Ang II hypertension with respect to NO/cGMP/cGK-I signaling and endothelial function by inhibiting increases in oxidative stress. Nebivolol treatment prevented increases in the expression of the NADPH-oxidase subunits Nox1, Nox2, p22phox, p47phox, and p67phox, as quantified by quantitative RT-PCR and Western blotting technique (in the heart and aorta). In contrast, in vivo treatment with metoprolol failed to demonstrate inhibitory effects on NADPH oxidase expression (please see the online data supplement).

As mentioned before, nebivolol has been demonstrated to suppress the NADPH oxidase–mediated oxidative burst in inflammatory cells in vitro.11 It remains to be established whether it may have similar inhibitory effects on superoxide production by the vascular NADPH oxidase in vitro. In the present studies, we have chosen several methods to approach this point. First we incubated isolated rings with phorbol ester (Figure 5C), a PKC-dependent activator of the vascular NADPH oxidase, and subsequently quantified the activity of the cGK-I by measuring P-VASP levels in vascular tissue. The phorbol ester–triggered activation of NADPH oxidase–dependent superoxide formation impaired NO signaling by 70%, all of which was prevented when aortic rings were coincubated with nebivolol. These data indicate that nebivolol may be able to inhibit the activation of the NADPH oxidase. These observations are further strengthened by addition, nebivolol therapy prevented the upregulation of the NADPH oxidase subunit expression, such as p22phox, gp91phox (Nox2), Nox1, p47phox, and p67phox, as quantified by quantitative RT-PCR and Western blotting technique (in the heart and aorta). In contrast, in vivo treatment with metoprolol failed to demonstrate inhibitory effects on NADPH oxidase expression (please see the online data supplement).

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Disclosures
None.

References
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METHODS

All animals were treated in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health and was granted by the Ethics Committee of the University Hospital Eppendorf. Nebivolol was obtained from Berlin-Chemie AG, Berlin, Germany. All other chemicals were purchased from Sigma-Aldrich, Merck or Fluka.

Animal model, in vivo infusion of angiotensin II and nebivolol

Male wistar rats (75 in total; 300-330 g; Charles River, Sulzfeld, Germany) were anesthetized by isoflurane inhalation and treated with a subcutaneous osmotic minipump (Durect Corp., Cupertino, CA 95014) containing either angiotensin-II (Ang-II) or NaCl (0.9 % NaCl) for 7 days. Ang-II infusion rate averaged 1 mg/kg/d, as described previously. Animals from both groups were randomized to receive either nebivolol (10 mg/kg/d), low dose metoprolol (10 mg/kg/d) or the vehicle (DMSO 90%, ethanol 10%) via an additional subcutaneous osmotic minipump. After 7 days the rats were sacrificed under isoflurane anesthesia.

Assessment of NO/cGMP-signaling

NO/cGMP-signaling was assessed by the phosphorylation of the vasodilator Stimulated Phosphoprotein (VASP) at serine 239 (P-VASP) using immunoblotting techniques as described previously.

Vascular reactivity studies

Vasodilator responses to the endothelium-dependent vasodilator acetylcholine (ACh) and the endothelium-independent vasodilator nitroglycerin (NTG) were determined in organ chambers by isometric tension studies, as previously described.
Preparation of membrane fractions for assessment of superoxide formation and p67phox and Rac1 translocation

Membrane fractions were prepared and measured as previously described. NADPH oxidase activity (200 µM NADPH) of the membrane suspensions (0.2 mg/ml protein in PBS) was measured by the luminol analogue L-012 (100 µM) and by lucigenin (5µM). Chemiluminescence was detected in a Lumat LB 9507 (Berthold Techn., Bad Wildbad, Germany). Results were expressed as counts/min after 5 min and normalized for protein content.

To assess the effects of β-blockers on NADPH oxidase activity and membrane translocation of its subunits in vitro the membrane fractions (in Tris buffer with DTT) were incubated with either DMSO (0.5 % v/v), nebivolol (1-100 µM from stocks in DMSO), carvedilol, atenolol or metoprolol (all 100 µM from stocks in DMSO) for 30 min at 37 °C. Following additional centrifugation (1h, 100,000g, 4°C) to remove potentially dissociated NADPH oxidase subunits the pellet was resuspended in Tris buffer without DTT and subjected to chemiluminescence measurements and Western blot analysis.

Assessment of vascular superoxide production

- Oxidative fluorescent microtopography

The fluorescent dye dihydroethidine (DHE) was used to detect O₂•− in situ. To address the influence of endothelial (NOS III-derived) O₂•−, vessels were incubated with N⁰-nitro-L-arginine (L-NNA, 1 mM) for 30 minutes as described.

- Lucigenin-Enhanced Chemiluminescence

Vascular O₂•− was estimated using lucigenin-enhanced chemiluminescence (LDCL, concentration 5 µmol/L) as previously described.
Expression and membrane-location of NADPH-oxidase subunits

Sample preparation and Western blotting was performed as described previously. Briefly, rat aortic tissue and heart was homogenized in liquid nitrogen and cytosolic and membrane fractions obtained by ultracentrifugation (1h, 100,000xg, 4°C) were subjected to SDS-PAGE and electro-blotting on nitrocellulose membranes (BioRad). The blots were developed with a mouse monoclonal antibody against p67\textsuperscript{phox} (dilution 1:500), Rac1 (dilution 1:1000) (Transduction Laboratories, Rockford, IL, USA) and Nox1 (dilution 1:100; Santa Cruz Biotechnologies). Immunodetections were accomplished with either SuperSignal Substrate (Pierce) or ECL Reagent (Amersham). The bands were evaluated by densitometry.

Quantification of mRNA by reverse transcription real-time PCR (qRT-PCR)

mRNA expression was analyzed with quantitative real-time RT-PCR using an iCyclerTM iQ system (Bio-Rad Laboratories, Munich, Germany). Briefly, total RNA from rat aorta and heart was isolated according to the manufacturer's protocol (RNeasy Fibrous Tissue Mini Kit; Qiagen, Hilden, Germany). 0.5 µg of total RNA was used for real-time RT-PCR analysis with the QuantiTect™ Probe RT-PCR kit (Qiagen). TaqMan® Gene Expression assays (Applied Biosystems, Foster City, CA) for p47\textsuperscript{phox} and TBP were purchased as probe and primer sets. Nox2 (gp91\textsuperscript{phox}) and p22\textsuperscript{phox} were obtained from MWG-Biotech (Ebersberg, Germany). Sequences of the primers and TaqMan® probes were (forward, reverse and probe) CTTCTTGGGTAGCTGACTG, GCAGCAAGATACAGCATGCAG and CACCTGACAGGGAATTTTCA for Nox2; TACCTGACCCTGGAAG, GCAGTAAGGGAGCAGAAGCCC and TGTTGGGCCCCTCCACCAGAAATTAC for p22\textsuperscript{phox}. The comparative Ct method was used for relative mRNA quantification. Gene expression was normalized to the endogenous control, TBP mRNA, and the amount of target gene mRNA expression in each sample was expressed relative to that of control.
Determination of NO synthesis as plasma nitrite and Hb-NO in rat blood

Nitrite, the oxidation product of NO, was assayed in rat plasma and serum as a measure of NO synthesis. Nitrite was determined by ozone chemiluminescence after chemical reduction to NO using a NOATM 280 Nitric Oxide Analyzer (Sievers) \(^{10, 11}\). Nitrite plasma levels correlate with the NO biosynthesis \(^{12, 13}\). The nitrate/nitrite ratio is often used as an indirect marker of oxidative stress or more specifically peroxynitrite formation and was increased in Ang-II treated and normalized in nebivolol co-treated rats. This would point to mixed NO/O\(_2\)\(^{-•}\) synthesis in Ang-II treated rats and accordingly to partial NOS uncoupling.

NO synthesis/bioavailability was also assessed by EPR-based detection of Hb-NO. Samples of venous blood for Hb-NO/EPR studies were obtained by the puncture of the right heart of anaesthetized rats; blood samples were immediately frozen/stored in liquid nitrogen. The EPR measurements were carried out at 77K using an X-band table-top spectrometer MS200 (Magnettech, Berlin). The instrument settings were as follows: 10 mW microwave power, 0.5 mT amplitude modulation, 100 kHz modulation frequency, 330 mT center field, 40 mT sweep width, 60 s sweep time and 3 scans. The assessment of Hb-NO levels was performed by the method of subtraction of the EPR signal of the Hb-NO-depleted blood from the EPR spectrum of immediately frozen blood \(^{14}\). The Hb-NO-depleted blood was obtained by the leaving of blood sample at room temperature for 6 hours.

Systemic blood Hb-NO levels measured by EPR method were considered to be an index of endothelial function \(^{14}\). Additionally, recent studies suggest that circulating Hb-NO is an important NO reservoir, which may release the physiologically active NO under low oxygen pressure \(^{15, 16}\).

Rac1 expression and ROS formation in HEK293 cells
HEK293 cells were seeded into 12 wells dishes and transfected with 0.3 µg mouse Nox1, Noxa1 and Noxo1 (the analogs of p47\textsuperscript{phox} and p67\textsuperscript{phox}) using lipofectamine. Radical formation was determined by luminol (200 µM) and horse radish peroxidase (1 U/ml)-driven chemiluminescence in trypsinized cells in 500 µl HEPES-modified Tyrode’s solution using a chemiluminescence reader (LB9505 Berthold, Wildbad, Germany) as reported previously \(^1\). ROS formation was determined in the presence or absence of β-blockers at the concentrations indicated and normalized to the signal obtained in the presence of solvent.

**Statistical Analysis**

Results are expressed as means±SEM. EC\(_{50}\) values were obtained by logit-transformation. One way ANOVA was employed to compare NADPH oxidase subunit protein and mRNA expression and distribution, P-VASP, eNOS, nitrite, Hb-NO and O\(_2^-\) production. Vascular responses were compared by multivariate analysis of variance. A Scheffe’s post hoc test was used to examine differences between groups when significance was indicated. P values <0.05 were considered significant.

**References**


Table I. Effects of treatment with nebivolol and metoprolol on the potency and efficacy of endothelium-dependent (ACh) and -independent (NTG) vasodilators in isolated aortic segments from Ang-II-infused rats.

<table>
<thead>
<tr>
<th>In vivo Treatment</th>
<th>Potency, $EC_{50}$ (-log M)</th>
<th>Efficacy, % Maximal relaxation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ACh</td>
<td>NTG</td>
</tr>
<tr>
<td>Control</td>
<td>7.27±0.07</td>
<td>7.61±0.06</td>
</tr>
<tr>
<td>Ang-II</td>
<td>6.74±0.05 *</td>
<td>7.37±0.06 *</td>
</tr>
<tr>
<td>Ang-II + nebivolol</td>
<td>7.05±0.06 *†</td>
<td>7.31±0.07 *</td>
</tr>
<tr>
<td>Ang-II + metoprolol</td>
<td>6.74±0.04 *</td>
<td>nd</td>
</tr>
</tbody>
</table>

Each value (mean±SEM) has been calculated from 9-18 experiments.

*P<0.05 vs. Control. †P<0.05 Nebivolol-treated vs. untreated. nd = not determined.
Table II. Effects of treatment with nebivolol on the angiotensin-II induced decrease in NO bioavailability measured as Hb-NO in whole blood.

<table>
<thead>
<tr>
<th>In vivo</th>
<th>Treatment</th>
<th>Relative Intensity of the EPR Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>1100±64</td>
</tr>
<tr>
<td></td>
<td>Ang-II</td>
<td>761±97 *</td>
</tr>
<tr>
<td></td>
<td>Ang-II + nebivolol</td>
<td>1605±258 †</td>
</tr>
</tbody>
</table>

Each value (mean±SEM) has been calculated from 3 experiments.

*P<0.05 vs. Control. †P<0.05 vs. Ang-II infusion.

Table III. Effects of treatment with nebivolol on the angiotensin-II induced increase in L-012-enhanced chemiluminescence in heart membrane fractions as a measure of NADPH oxidase activity.

<table>
<thead>
<tr>
<th>In vivo</th>
<th>Treatment</th>
<th>Chemiluminescence [counts/min/mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>96614±22277</td>
</tr>
<tr>
<td></td>
<td>Control + nebivolol</td>
<td>84111±11334, p=0.64</td>
</tr>
<tr>
<td></td>
<td>Ang-II</td>
<td>177619±54048, p=0.23</td>
</tr>
<tr>
<td></td>
<td>Ang-II + nebivolol</td>
<td>104611±11887, p=0.74</td>
</tr>
</tbody>
</table>

Each value (mean±SEM) has been calculated from 8-13 experiments.

Significance values vs. control group.
Legend to figure I: mRNA expression of \( \text{gp}91^{\text{phox}} \), \( \text{p}47^{\text{phox}} \) and \( \text{p}22^{\text{phox}} \) was determined in aortic and cardiac tissue from differently treated Wistar rats: control (Ctr, sham-treated), angiotensin II-infused (Ang-II), angiotensin II- and nebivolol co-infused (Ang-II + Nebi), angiotensin II- and metoprolol co-infused (Ang-II + Meto). *, p<0.05 vs. control and #, p<0.05 vs. Ang-II. Data are mean ± SEM from 3-4 independent measurements.
**Supplementary information – NO bioavailability**

**Nitrite and Nitrate in Plasma**

![Nitrite and Nitrate in Plasma graph]

**Nitrite and Nitrate in Serum**

![Nitrite and Nitrate in Serum graph]

**Legend to figure II:** Nitrite levels in plasma and serum was measured using an NO analyzer. Nitrate levels were determined upon reduction of nitrate to nitrite by nitrate reductase *, p<0.05 vs. control and #, p<0.05 vs. Ang-II. Data are mean ± SEM from 4-6 independent measurements.