Nebivolol Prevents Vascular NOS III Uncoupling in Experimental Hyperlipidemia and Inhibits NADPH Oxidase Activity in Inflammatory Cells

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Objectives—Nebivolol, in contrast to other selective β1-adrenergic receptor antagonists like atenolol, improves endothelial function in patients with oxidative stress within vascular tissue. With the present studies we sought to determine whether β receptor blockade with nebivolol may improve endothelial function in hyperlipidemia and whether this is attributable to reductions in vascular oxidative stress.

Methods and Results—Watanabe heritable hyperlipidemic rabbits (WHHL) were treated with nebivolol (10 mg/kg per day for 8 weeks). New Zealand white rabbits (NZWR) served as controls. Nebivolol improved endothelial function, reduced vascular superoxide and vascular macrophage infiltration, and prevented NO synthase uncoupling in WHHL. Nebivolol treatment did not modify the expression of sGC or cGK-I but improved cGK-I activity (assessed by the phosphorylation state of the Vasodilator Stimulated Phosphoprotein at serine239, P-VASP). NAD(P)H oxidase activity in whole blood and isolated neutrophils was dose-dependently inhibited by nebivolol, whereas atenolol, metoprolol, and carvedilol were markedly less effective.

Conclusions—Nebivolol therapy effectively prevents NO synthase III uncoupling and prevents activation of the neutrophil NAD(P)H oxidase and infiltration of inflammatory cells. These novel antioxidative stress actions of this compound may explain partly the beneficial effects on endothelial function in patients with enhanced vascular oxidative stress.

Key Words: nebivolol ■ NO synthase ■ superoxide ■ neutrophils ■ NADPH oxidase

β1-Adrenergic receptor blockers have become a mainstay in the management of unstable and stable angina and acute myocardial infarction as well as in the treatment of patients with hypertension and chronic congestive heart failure. Third-generation β receptor blockers comprise substances that block selectively the β1-receptor and that also have vasodilator properties attributable to simultaneous α-receptor blocking effects. Interestingly, the vasodilatory properties of the third-generation β-blocker nebivolol revealed to be mediated by the release of the endothelium-derived relaxing factor NO. Nebivolol-induced vasodilation was almost completely blocked by the inhibitors of the NO synthase L-NMMA.1 In vitro studies revealed that this phenomenon is at least in part attributable to stimulation of β1 receptors on endothelial cells by nebivolol metabolites, leading to an increase in endothelial [Ca2+] levels and subsequently to NO synthase (NOS) III activation.2

Chronic treatment with nebivolol has also been shown to improve endothelial function in patients with essential hypertension.3 Interestingly, endothelial function in the setting of hypertension has recently been shown to be improved by the antioxidant vitamin C, suggesting that this phenomenon may be partly attributable to increased production of superoxide.4 The beneficial effects on endothelial function in hypertensive patients may therefore indicate that nebivolol treatment inhibits vascular superoxide production. Indeed, recent studies revealed that antihypertensive doses of nebivolol decreased oxidative stress in healthy volunteers, reflected by a decrease in the formation of the isoprostane 8-iso-PGF2α.5 Indirect evidence for antioxidative properties of nebivolol was recently provided by in vitro experiments. Incubation of cultured endothelial as well as smooth muscle cells with nebivolol inhibited endothelin-1 expression and release6 as well as proliferation of smooth muscle cells in response to platelet-derived growth factor,6 phenomena which have been shown to be mediated by oxidative stress.7

To address these issues, we used a well-characterized model of endothelial dysfunction, the WHHL. Recent studies

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have indicated that in the setting of hypercholesterolemia, an activated NAD(P)H oxidase\(^a\) or an uncoupled NO synthase\(^b\) may contribute considerably to vascular dysfunction. The subsequent decrease in vascular NO bioavailability resulted in disturbances of NO-downstream signaling, as indicated by the marked increase in the expression but inhibition of the activity of the NO-target enzyme sGC and by a strong inhibition of the activity of the cGMP-dependent protein kinase cGK-I.\(^a\)

Based on these considerations, the present study was designed to test whether in the experimental model of hyperlipidemic WHHL, nebivolol improves endothelial function by reducing vascular superoxide production, prevents NOS III uncoupling, normalizes NO-downstream signaling, and regulates NAD(P)H oxidase activity of inflammatory cells.

**Methods**

**Animals and Protocol**

Twenty-five New Zealand White rabbits (NZWR) and twenty-five WHHL were studied. Ten of each group received concomitant treatment with the \(\beta\)-receptor blocker nebivolol, which was mixed to the diet to achieve a daily dose of 10 mg/kg per day. On the day of the study, blood samples were drawn for determination of plasma lipids.

**Vessel Preparation and Organ Chamber Studies**

Aortic rings were suspended in organ chambers, and response to acetylcholine (ACh) and nitroglycerin (NTG) was tested as described.\(^a\)

**Vascular Superoxide Production**

Vascular superoxide was estimated using lucigenin-derived chemiluminescence (LDCL, concentration 5 \(\mu\)mol/L) as previously described.\(^a\)\(^b\) To address the influence of endothelial (NOS III-derived) NO and NOS-mediated superoxide production on vascular LDCL, vessels were incubated with N\(^\circ\)-nitro-l-arginine (L-NNA, 1 \(\mu\)mol/L) for 30 minutes as described.\(^a\)

**Superoxide Generation in Whole Blood**

Venous blood was obtained from healthy subjects with 1/10 volume of 3.8% sodium citrate. The blood samples (10 to 50 \(\mu\)L) were added to 0.5 mL of 0.9% NaCl solution containing 10 mmol/L phosphate buffer (pH 7.4), 6 mmol/L KCl, and 6 mmol/L MgCl\(_2\) in the presence of 100 \(\mu\)mol/L L-012. This compound has been shown to detect superoxide more efficiently than other chemiluminescent compounds, such as luminol and lucigenin and the cypridina luciferin superoxide more efficiently than other chemiluminescent compounds.\(^a\)

**Detection of Macrophages by Slot Blot Analysis**

Protein samples from frozen aortic tissue were prepared as described for Western blotting, except the denaturation step with Laemmli’s buffer. Protein content was measured by the method of Bradford; 10 \(\mu\)g total protein of each sample was transferred to a Portran (0.45 \(\mu\)m) nitrocellulose membrane from Schleicher & Schuell (Dassel, Germany) by a Miniifold II vacuum slot blot apparatus from Schleicher & Schuell. Each slot was washed with 300 \(\mu\)L of PBS and, for a better attachment of the proteins to the membrane, dried for 15 minutes at 60°C. For detection of macrophages, the membrane was blocked for 1 hour at RT with TBST containing 3% (wt/vol) BSA and then incubated for 1 hour with mouse monoclonal anti-rabbit macrophage antibody (RAM 11, DAKO) at a dilution of 1:100, washed 3 times with TBST, and incubated for 1 hour with secondary antibody (peroxidase-labeled anti-mouse IgG, 1:10,000, Vector Laboratories). After washing with TBST, positive bands were detected by ECL and quantified by densitometry as described below.

**Detection of cGK-I, VASP, and P-VASP Expression**

Aortic segments from NZWR and WHHL with and without nebivolol treatment were frozen and homogenized in liquid nitrogen. SDS-PAGE electrophoresis and electroblotting were performed as described.\(^a\) Immunoblotting was performed with a polyclonal antibody against cGK-I and a mouse monoclonal antibody (16C2) specific for P-VASP, as described recently.\(^a\)

**Materials**

L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt) was obtained from Wako Chemicals, DEPIMPO (5-diethylxophosphoryl-5-methyl-1-pyrroline-N-oxide) from Calbiochem, Carvedilol from Boehringer, and Nebivolol from Berlin Chemie. All other materials were purchased from Sigma.
**Table 1. Plasma Lipid Levels of NZWR and WHHL With and Without Nebivolol Treatment**

<table>
<thead>
<tr>
<th></th>
<th>Total Cholesterol, mg/dL</th>
<th>LDL, mg/dL</th>
<th>HDL, mg/dL</th>
<th>Triglycerides, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZWR</td>
<td>37 ± 4</td>
<td>8 ± 4</td>
<td>23 ± 3</td>
<td>80 ± 9</td>
</tr>
<tr>
<td>NZWR + Nebivolol</td>
<td>22 ± 1</td>
<td>ND</td>
<td>13 ± 1</td>
<td>75 ± 6</td>
</tr>
<tr>
<td>WHHL</td>
<td>673 ± 45*</td>
<td>577 ± 53*</td>
<td>18 ± 3</td>
<td>403 ± 72*</td>
</tr>
<tr>
<td>WHHL + Nebivolol</td>
<td>562 ± 27*</td>
<td>447 ± 50*</td>
<td>12 ± 2</td>
<td>517 ± 108*</td>
</tr>
</tbody>
</table>

ND indicates not determined. *P < 0.05 vs control.

**Statistical Analysis**

Results are expressed as mean ± SEM. The ED50 value for each experiment was obtained by logit transformation. To compare vascular superoxide levels, P-VASP and VASP, cGK-I, sGC-dependent (ACh) and endothelium-independent (NTG) relaxation was significantly improving ACh-induced relaxation in WHHL.

**Results**

**Plasma Lipid Profile**

In WHHL, total cholesterol, LDL, and triglyceride levels were significantly higher compared with levels from control animals (Table 1). Nebivolol treatment had no significant effect on either parameter measured.

**Vasodilator Responses to ACh and NTG**

In hyperlipidemic WHHL, the sensitivity to endothelium-dependent (ACh) and endothelium-independent (NTG) relaxation was significantly reduced (Table 2). β-receptor blockade for 8 weeks had no effect on the ACh and NTG dose-response relationship of control animals while significantly improving ACh-induced relaxation in WHHL.

**Vascular Superoxide and NOS III Uncoupling in WHHL**

Hyperlipidemia led to a significant increase in LDCL compared with controls (Figure 1A). Nebivolol treatment of WHHL for 8 weeks reduced vascular superoxide levels. Incubation of vessels from WHHL with L-NNA decreased LDCL signal, compatible with an uncoupling of NOS in contrast, L-NNA incubation of vessels from WHHL treated with nebivolol increased LDCL, indicating that NOS uncoupling was prevented. Data are mean ± SEM from 5 to 7 independent experiments. *P < 0.05 vs control.

**Figure 1.** A, Effects of nebivolol treatment (10 mg/kg per day for 8 weeks). Hyperlipidemia led to a significant increase in vascular superoxide levels. Nebivolol treatment had no effect on superoxide levels of control vessels while significantly reducing oxidative stress in vessels from WHHL. Data are mean ± SEM from 5 to 7 independent experiments. *P < 0.05 vs control; †P < 0.05 vs WHHL. B, Effects of L-NNA on vascular superoxide levels from WHHL and WHHL treated with nebivolol (10 mg/kg per day for 8 weeks). Incubation of vessels from WHHL with L-NNA (1 mmol/L for 30 minutes) markedly decreased lucigenin-derived chemiluminescence, indicating a significant contribution of vascular NOS to vascular superoxide production. In contrast, incubation of vessels from WHHL treated with nebivolol increased LDCL, indicating that NOS uncoupling was prevented. Data are mean ± SEM from 5 to 7 independent experiments. *P < 0.05 vs control; †P < 0.05 vs WHHL.

**Figure 2.** Effects of nebivolol treatment (10 mg/kg per day) on vascular superoxide levels in control NZWR and hyperlipidemic WHHL, as assessed with the fluorescent dye hydroethidine.
Expression of sGC, cGK-I, and cGK-I Activity in WHHL

In WHHL, the expression of the sGC, as assessed by the determination of sGCβ₁, was not modified (Figure 4). Likewise, the expression of cGK-I was not altered. In contrast, the activity of cGK-I, as assessed by the ratio of P-VASP to VASP, was reduced (Figure 4). Treatment of WHHL for 8 weeks with nebivolol did not modify the expression of sGC and cGK-I but significantly improved the P-VASP to VASP ratio (Figure 4).

Effects of Nebivolol on Phorbolester and Angiotensin II–Stimulated Superoxide Production in Whole Blood

Incubation of human whole blood with the phorbolester PDBu increased superoxide, as assessed with L-012 chemiluminescence (CL), which was dose-dependently inhibited by nebivolol. Likewise, PDBu-induced L-012-CL was completely inhibited by gliotoxin, a specific inhibitor of neutrophil NAD(P)H oxidase (Figure 5A). Furthermore, a complete inhibition was observed in response to DPI and to the protein kinase C inhibitor chelerythrine (data not shown). Compared with carvedilol, atenolol, and metoprolol, nebivolol had stronger inhibitory effects on PDBu-induced superoxide production. Stimulation with angiotensin II increased whole-blood superoxide formation, which was also dose-dependently inhibited by nebivolol. Likewise, angiotensin II–stimulated superoxide production was inhibited by gliotoxin (Figure 5B).

Effects of Nebivolol on Phorbolester-Stimulated Production of Oxygen-Derived Free Radicals in Isolated Neutrophils

Like in whole blood, PDBu-stimulated superoxide production in isolated neutrophils was dose-dependently inhibited by nebivolol. Like demonstrated for whole blood, the inhibitory effects of carvedilol, metoprolol, and atenolol on PDBu-induced superoxide production were markedly less compared with nebivolol (Figure 6). The inhibitory effect of nebivolol on leukocyte superoxide production assessed with L-012 chemiluminescence was confirmed by EPR measurements (insert). PDBu-stimulated superoxide production of neutrophils was almost completely inhibited by preincubation with gliotoxin and PEG-SOD.

Effects of Nebivolol on L-012–Enhanced Chemiluminescence Induced by the Xanthine/Xanthine Oxidase System

In a concentration that completely inhibited superoxide production in whole blood, nebivolol (100 μmol/L) failed to...
modify superoxide production generated via the xanthine/xanthine oxidase reaction, indicating that the compound nebivolol per se is not an antioxidant (data not shown).

**Discussion**

The present studies demonstrate that the novel \( \beta_1 \)-receptor blocker nebivolol improves endothelial function and reduces vascular oxidative stress in WHHL by preventing NOS III uncoupling. Likewise, nebivolol markedly inhibited superoxide production by the phagocytic NADPH oxidase. Accordingly, cGK-I activity as assessed with the phosphorylation status of the VASP at serine239 was normalized.

**Nebivolol, a New Third-Generation \( \beta_1 \)-Receptor Blocker With Endothelium-Dependent Vasodilator Properties**

Nebivolol is a recently developed \( \beta_1 \)-receptor blocking drug that is a mixture of D- and L-enantiomers, of which D-nebivolol is considered to be a highly selective \( \beta_1 \)-receptor antagonist. In addition to its \( \beta_1 \)-receptor blocking properties, nebivolol has been shown to cause endothelium-dependent vasodilation. Nebivolol-induced vasodilation of canine coronary arteries is blocked by the NOS III inhibitor L-NNA or after removal of the endothelium. Studies in men revealed a dose-dependent vasodilation of forearm arterioles in healthy subjects as well as in patients with essential hypertension, which was inhibited by the NOS inhibitor L-NMMA. In vitro studies using cultured human endothelial cells identified a potential mechanism underlying nebivolol-induced NO release. Broeders et al showed that a nebivolol metabolite elevates endothelial free \([Ca^{2+}]\) levels via stimulation of endothelial \( \beta_2 \) receptors, leading to activation of NOS III. These effects seem to be specific for nebivolol and were not observed in response to metoprolol. In addition to its acute endothelium-dependent vasodilator properties, chronic nebivolol treatment improves endothelial function in patients with essential hypertension. An 8-week administration of 5 mg/d nebivolol enhanced basal as well as stimulated NO release in patients with essential hypertension, as assessed with L-NMMA–induced reductions and ACh-induced increases in forearm blood flow, respectively. These beneficial effects seem to be independent of its \( \beta_1 \)-receptor blocking properties, because atenolol failed to modify endothelial dysfunction in this particular patient group. Interestingly, recent studies indicated that the antioxidant vitamin C is able to improve endothelial function in forearm arterioles and coronary arteries in patients with essential hypertension, indicating that this phenomenon is closely linked to increased oxidative stress within vascular tissue. If vascular effects of nebivolol would be restricted to stimulatory effects on endothelial NO production, the formation of the NO/superoxide

![Inhibitory effects of nebivolol, carvedilol, atenolol, and metoprolol and the NADPH oxidase inhibitor diphenylene-iodonium (DPI) on phorbol ester (PDBu)-induced superoxide production in isolated neutrophils as detected with L-012–enhanced chemiluminescence and with electron paramagnetic resonance (EPR; insert). Data are mean±SEM from 3 to 5 separate experiments. *P<0.05 vs PDBu-induced CL.](image-url)
reaction product peroxynitrite would be favored, ultimately leading to a worsening rather than an improvement of endothelial function, eg, attributable to oxidation of the NOS III cofactor tetrahydrobiopterin or to a direct effect on the NOS III zinc thiolate complex. These phenomena may lead to NOS III uncoupling, which means that electrons flowing from the NOS III reductase domain to the oxygenase domain are diverted to molecular oxygen rather than to l-arginine, resulting in the production of superoxide rather than NO.

Thus, similar to statins or ACE inhibitors, nebivolol stimulates NO formation, but it may also have beneficial inhibitory effects on oxidative stress within vascular tissue. Indeed, studies with healthy volunteers indicate that nebivolol treatment for 7 days reduces the urinary excretion of the isoprostane 8-iso-PGF$_{2_\alpha}$, a specific marker for oxidative stress. Recent in vitro studies indirectly support the concept that nebivolol has inhibitory effects on oxidative stress. Using cultured human coronary smooth muscle and endothelial cells, Brehm and colleagues demonstrated that nebivolol treatment for 7 days reduces the urinary excretion of the isoprostane 8-iso-PGF$_{2_\alpha}$. Furthermore, they observed a significant inhibition of endothelin-1 liberation and mRNA production as well as proliferation of these cells in response to platelet-derived growth factor. Interestingly, recent studies have shown that endothelin expression within endothelial and smooth muscle cells as well as proliferation of smooth muscle cells in response to platelet-derived growth factor mediated by oxidative stress are partly attributable to an activation of the NAD(P)H oxidase.

### Nebivolol Treatment Reduces Oxidative Stress and Prevents NOS Uncoupling in Hyperlipidemia

To test the potential inhibitory effects of nebivolol on vascular superoxide production, the model of hyperlipidemic WHHL was chosen. Previous studies have indicated that in this particular animal model, the activation of the NAD(P)H oxidase as well as an uncoupled NOS III contribute significantly to increased vascular superoxide production. Subsequent studies in monkeys and apolipoprotein E knockout mice have confirmed these observations. As in previous studies, a significant increase in vascular superoxide, as assessed with the chemiluminescent probe lucigenin, was observed. Although nebivolol treatment for 8 weeks did not modify superoxide levels in NZWR, a significant inhibition of superoxide in vessels from WHHL was observed. DHE staining revealed that superoxide levels were reduced to some extent in the endothelium but also within the vascular media. Reductions in oxidative stress within the vasculature led to a significant improvement in endothelium-dependent and -independent relaxations to ACh and NTG, respectively. This improvement in vascular function and the reduction in vascular superoxide availability cannot be attributed to changes in total cholesterol, LDL, or HDL levels, because these parameters were not significantly modified by nebivolol treatment.

To additionally analyze the enzymatic superoxide source involved, incubation experiments with the NOS inhibitor L-NNA were performed as described recently. In vessels from WHHL, L-NNA significantly decreased LDCL, identifying NOS as a significant superoxide source. In contrast, incubation of vessels from WHHL treated with nebivolol increased LDCL levels, indicating that in these vessels baseline NO production markedly quenches the baseline chemiluminescence signal and that nebivolol treatment, similar to treatment with AT$_1$ receptor blockers, was able to prevent NOS uncoupling.

### Nebivolol Inhibits Phorbolester-Stimulated Superoxide Production in Whole Blood and Isolated Neutrophils

To address whether nebivolol has inhibitory effects on the NAD(P)H oxidase, superoxide in whole blood and in isolated inflammatory cells such as leukocytes in response to stimulation with the phorbolester PDBu with and without pretreatment with nebivolol was determined. The superoxide sensitive dye L-012 was used to determine relative rates of superoxide production. Incubation of whole blood with phorbolester PDBu increased L-012 chemiluminescence. Incubation with increasing concentrations of nebivolol dose-dependently inhibited the CL signal (Figure 5A). A complete inhibition was also observed in response to gliotoxin, a specific inhibitor of the NAD(P)H oxidase, with chelerythrine, an inhibitor of PKC, and with DPI, an inhibitor of flavin-dependent oxidoreductases. Compared with other $\beta$-blockers, such as carvedilol, atenolol, and metoprolol, nebivolol was more effective in inhibiting phorbolester-stimulated superoxide production. Likewise, angiotensin II-induced superoxide production of whole blood was inhibited by nebivolol as well as by the NAD(P)H oxidase inhibitor gliotoxin. In isolated neutrophils, similar phenomena were observed. These findings may indicate that nebivolol may prevent a protein kinase C-dependent activation of the NAD(P)H oxidase. The observed inhibitory effect of nebivolol on superoxide release from leukocytes and on vascular infiltration with macrophages (see Figure 3) represents important additional mechanisms for the antioxidant properties of this compound. Phagocyte-NAD(P)H oxidase is considered a key source for superoxide in vascular inflammation. In fact, superoxide formation in vascular tissue and NAD(P)H oxidase subunits has been shown to colocalize with NAD(P)H oxidase subunits and evaded macrophages. By inhibiting tissue infiltration by inflammatory cells and by suppressing NAD(P)H oxidase activity of inflammatory cells, nebivolol may not only directly increase vascular NO bioavailability but also reduce the formation of the potent oxidant peroxynitrite, which has been hypothesized to play a key role in NOS III uncoupling. In addition, a magnitude of additional oxidative stress depends on phagocyte NAD(P)H oxidase, eg, activation of proatherogenic myeloperoxidase by superoxide-derived hydrogen peroxide. Myeloperoxidase, which is increasingly expressed in PMN of patients with coronary artery disease, has been shown to oxidize NO, thereby impairing vascular NO bioavailability and thus illustrating why the antioxidative stress properties of nebivolol may be so profound.

Compared with other $\beta$-blockers such as carvedilol, metoprolol, and atenolol, nebivolol had markedly stronger inhibitory effects on agonist-superoxide production in whole blood and isolated neutrophils. This observation may explain, at
least in part, why β-blockade with nebivolol and not atenolol24 possesses potent antiatherosclerotic effects

Nebivolol Improves NO Downstream Signaling in Vessels From WHHL

The effects of nebivolol treatment on NO-cGMP downstream signaling was assessed by determining the expression of the sGC subunit β1 and by determining the activity and expression of the cGMP-dependent kinase cGK-I. Surprisingly, in vessels from WHHL, the expression of the subunit sGC β1 was not modified at all. This finding is in contrast to recent studies where in cholesterol-fed animals a marked upregulation of both sGC subunits has been reported.25 Cholesterol feeding usually results in total cholesterol exceeding 1000 mg/dL by far, whereas in WHHL, lipid levels are more closer to the pathophysiological range. Thus, the lack of changes in sGC expression despite total cholesterol levels in the range of 500 mg/dL challenge the concept that high cholesterol levels per se lead to a modulation of the expression of the enzyme.

Although we did not establish changes in the expression of the cGK-I, a strong inhibition of the activity of CGK-I as assessed by the phosphorylation was observed, as described previously.9 Treatment of NZWR with nebivolol did not modify the P-VASP to VASP ratio but normalized the P-VASP to VASP ratio in vessels from WHHL. Thus, similar to previous observations with AT1-receptor blocker treatment, the improvement of vascular NO bioactivity is likely attributable to reductions in oxidative stress within vascular tissue, thereby improving endothelium-dependent and -independent NO-induced relaxations and normalizing NO-cGMP downstream signaling.5

Conclusions

With the present studies, we demonstrate for the first time that the novel β-receptor blocker nebivolol has potent inhibitory effects on vascular superoxide production in an animal model of hypercholesterolemia. These beneficial effects seem to be partly the result of the prevention of NOSIII uncoupling and the subsequent normalization of NO/cGMP/cGK signaling. The observed inhibitory effects on NAD(P)H oxidase activity of inflammatory cells represent an important additional mechanism by which nebivolol may beneficially influence the progression of the atherosclerotic process.

Acknowledgments

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