Nebivolol Reduces Nitroxidative Stress and Restores Nitric Oxide Bioavailability in Endothelium of Black Americans

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Background—Alterations in endothelial function may contribute to increased susceptibility of black Americans to cardiovascular disease. The ability to pharmacologically reverse endothelial dysfunction in blacks was tested with nebivolol, a β1-selective agent with vasodilating and antioxidant properties.

Methods and Results—The effects of nebivolol on endothelial nitric oxide (NO), superoxide (O2−), and peroxynitrite concentration (ONOO−) release were studied in human umbilical vein endothelial cells and iliac artery endothelial cells isolated from age-matched black and white donors. Kinetics and concentrations of NO/O2−/ONOO− were measured simultaneously with nanosensors from single cells and shown to have significant interracial differences. The rate of NO release was 5 times slower in blacks than in whites (94 versus 505 nmol·L−1·s−1), whereas the rates of release were faster by 2 times for O2− and 4 times for ONOO− (22.1 versus 9.4 nmol·L−1·s−1 for O2− and 810 versus 209 nmol·L−1·s−1 for ONOO−). Pretreatment with 1.0 to 5.0 μmol/L nebivolol restored NO bioavailability in endothelial cells from black donors with concurrent reductions in O2− and ONOO− release, similar to levels in the endothelium of whites. The effects of nebivolol were dose-dependent and not observed with atenolol; similar effects were observed with apocynin, an NAD(P)H oxidase inhibitor.

Conclusions—Reduced endothelial NO bioavailability in American blacks is mainly due to excessive O2− and ONOO− generation by NAD(P)H and uncoupled endothelial NO synthase. Nebivolol decreased O2− and ONOO− concentrations and restored NO bioavailability in blacks to the level recorded in cells from whites, independently of β1-selective blockade. (Circulation. 2005;112:3795-3801.)

Key Words: cardiovascular diseases • endothelium • nitric oxide • beta-blockers • ethnic groups

Epidemiological studies indicate a higher prevalence of cardiovascular risk factors, such as hypertension and diabetes mellitus, among American blacks.1 The complications associated with these diseases, such as stroke, heart, and renal failure, contribute to greater mortality rates in this population. It has been proposed that these observed differences in cardiovascular risk may be attributed to changes in vascular physiology, including reduced nitric oxide (NO)–dependent vasodilatation of the microvasculature. In support of this concept, a clinical evaluation of brachial artery activity demonstrated reduced responsiveness of conductance vessels to both endogenous and exogenous NO in healthy blacks compared with age-matched whites.2 To understand the basis for this difference, we recently reported low bioavailability of NO from endothelium of blacks despite much higher levels of endothelium-dependent NO synthase (eNOS).3 The cellular basis for this paradox was the finding that excessive O2− generation by NAD(P)H oxidase and uncoupled eNOS resulted in the loss of functional NO owing to its reactivity with O2−, which results in formation of peroxynitrite (ONOO−), a potent oxidant. The ONOO− radical can be protonated to form ONOOH, which along with O2− represents the primary components of nitroxidative and oxidative stress in the cardiovascular system.

The endothelium modulates vascular tone through release of NO, a potent vasodilator that regulates regional blood flow.4,5 An essential role of NO is to protect the cardiovascular system against pathophysiological insults; NO inhibits smooth muscle cell proliferation and migration, adhesion of leukocytes to the endothelium, and platelet aggregation.6 Loss of normal endothelium-dependent NO production has been linked to various vascular diseases, including atherosclerosis, hypertension, and diabetes mellitus.7,8 Reduction in the bioavailability of NO contributes to the pathogenesis of vascular disease and is specifically linked to mechanisms of eNOS uncoupling.4,5
Nebivolol is a β1-receptor–blocking drug that consists of a 1:1 mixture of d- and l-enantiomers, of which d-nebivolol is a highly selective β1-receptor antagonist. In addition to its β1-receptor–blocking properties, nebivolol (including both of its optical enantiomers) has been shown to cause endothelium-dependent vasodilation in both normotensive and hypertensive subjects.9–11 In experimental models, nebivolol has been demonstrated to effect vasodilation through endothelial β1-adrenergic receptor–mediated NO production and/or ATP efflux with consequent stimulation of P2Y–purinoceptor–mediated NO release.11,12 It has also been reported that nebivolol inhibits NO synthase uncoupling13 and produces systemic antioxidant effects.14

The present study was conducted to evaluate the effect of nebivolol on NO release in endothelial cells from American blacks and the mechanisms of eNOS coupling/uncoupling. It was hypothesized that nebivolol restores NO bioavailability in blacks by interfering with mechanisms of NO synthase uncoupling that lead to increased O2•− levels. To test this hypothesis, we conducted measurements of NO synthase products (NO and O2•−), along with ONOO−, using tandem electrochemical nanosensors. The activity of nebivolol was compared with atenolol (another β1-selective antagonist) in endothelial cells from black and white donors with similar cardiovascular risk factors, including family histories of hypertension and diabetes.

**Methods**

**Subjects and Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were isolated into primary cultures from 12 white and 12 black American female donors by Clonetics (San Diego, Calif) and purchased as proliferating cultures from white and black donors. The donors were between 18 and 32 years of age. The mean age was 25.2 ± 2.0 years for white and 22.7 ± 2.6 years for black donors. The Local Research Ethics Committee approved collection of tissue specimens. The cultured cells were incubated in 95% air/5% CO2 at 37°C and passaged by an enzymatic (trypsin) procedure. The confluent cells (4 to 5 × 10⁶ cells/35-mm dish) were plated with minimum essential medium containing 3 mmol/L l-arginine and 0.1 mmol/L BH4 [(6R)-5,6,7,8-tetrahydrobiopterin]. Before the experiments, the cells from the second or third passage were rinsed twice with Tyrode-HEPES buffer with 1.8 mmol/L CaCl2.

**Preparation of Nanosensors for NO, O2•−, and ONOO− Detection**

Concurrent measurements of NO, O2•−, and ONOO− were performed with 3 electrochemical nanosensors combined into 1 working unit with a total diameter of 2.0 to 2.5 μm. Their design was based on previously developed and well-characterized chemically modified carbon-fiber technology.15,16 Each of the nanosensors was made by depositing a sensing material on the tip of a carbon fiber (length 4 to 5 μm, diameter 200 to 500 μm). The fibers were sealed with nonconductive epoxy and electrically connected to copper wires with conductive silver epoxy. We used a conductive film of polymeric Ni(II) tetraakis (3-methoxy-4-hydroxyphenyl) porphyrin for the NO sensor, an immobilized polypropylene/horseradish peroxidase (PP/HRP) for the O2•− sensor, and polymeric film of Mn(III) [2.2] paracyclophanylporphyrin for the ONOO− sensor.

**Measurement of NO, O2•−, and ONOO−**

A module of NO/O2•−/ONOO− nanosensors (working electrodes) with a common wire (0.1 mm) counter electrode and saturated calomel reference electrode was applied. Differential pulse voltammetry and amperometry were performed with a computer-based Gamry VFP600 multichannel potentiostat. Differential pulse voltammetry was used to measure basal NO, O2•−, and ONOO− concentrations, and amperometry was used to measure changes in NO, O2•−, and ONOO− concentrations from the basal level with time. The differential pulse voltammetry current at the peak potential characteristic for NO (0.65 V) oxidation and ONOO− (−0.45 V) or O2•− (−0.23 V) reduction was directly proportional to the local concentrations of these compounds in the immediate vicinity of the sensor. Linear calibration curves (current versus concentration) were constructed for each sensor from 10 nmol/L to 2 μmol/L before and after measurements with aliquots of NO, O2•−, and ONOO− standard solutions, respectively. The detection limit of the sensors was 10⁻⁹ mol/L. The quantification of each analyte (concentration in nmol/L) was performed with a maximum current from amperograms and standard calibration curves. At a constant distance of the sensors from the surface of the endothelial cell, reproducibility of measurements is relatively high (5% to 12%). The position of nanosensors (x, y, and z coordinates) versus the endothelial cell was established with the help of a computer-controlled micromanipulator. To establish a constant distance from cells, the module of sensors was lowered until it reached the surface of the cell membrane (a small piezoelectric signal, 0.1 to 0.2 pA, of 1- to 3-ms duration was observed at this point). After that, the sensors were slowly raised 4 ± 1 μm (z coordinate) from the surface of a cell culture. The sensors were then moved horizontally (x, y coordinates) and positioned above a surface of randomly chosen single endothelial cells (at least 10 mm apart from the endothelial cell, which was used to establish the z coordinate). The eNOS agonists, calcium ionophore A23187 (Cal) or acetylcholine (ACH), were then injected with a nanoinjector that was also positioned by a computer-controlled micromanipulator. In experiments conducted with eNOS agonist stimulation, endothelial cells were pretreated for 180 minutes with β-blockers (1.0 or 5.0...
μmol/L nebivolol or atenolol), 0.3 mmol/L L-NAME (N^ω-nitro-L-arginine methyl ester), an eNOS inhibitor, or 3.0 mmol/L apocynin, an NAD(P)H oxidase inhibitor.

Calculations and Statistical Analysis
To control intraexperimental variations, the measurements of endothelial O$_2^\cdot$-, NO, and ONOO$^-$ of each subject were performed with a single cell randomly selected from 4 to 8 cell culture dishes. The mean was calculated for each subject (n=4 to 8) and for all subjects (n=12 HUVECs and n=7 IAECs). When applicable (comparison between 2 values), statistical analysis was done by the Student t test. For multiple comparisons, results were analyzed by ANOVA followed by Bonferroni and Dunn’s correction. Data are presented as the mean±SEM. Mean values were considered significantly different at P<0.05.

Results
To investigate whether a potential reduction in NO bioavailability is associated with the endothelium of black donors, we characterized the eNOS-stimulated kinetics of free NO release with simultaneous detection of the kinetics of O$_2^\cdot$- and ONOO$^-$ release with tandem nanosensors. We compared these measurements to the levels of NO/O$_2^\cdot$/ONOO$^-$ obtained from age-matched white donors as a function of nebivolol treatment. In both groups, stimulated NO release from a single endothelial cell with calcium ionophore (CaI) was associated with concurrent release of O$_2^\cdot$- and ONOO$^-$ (Figure 1). In HUVECs from whites, the pattern of ONOO$^-$ release was similar to that for NO release, but it was delayed with a time shift of ≈1 s. The sharp peaks of O$_2^\cdot$- concentrations were observed in the same time span (3 to 4 s) from both racial groups.

There were significant differences in the kinetics of the initial rates of NO, O$_2^\cdot$-, and ONOO$^-$ release between black and white donors from single cells. Specifically, the rate of NO release was ≈5 times slower in blacks than in whites (94±6 versus 505±25 nmol · L$^{-1}$ · s$^{-1}$), whereas the rates of O$_2^\cdot$- release were faster by ≈2 times for O$_2^\cdot$- and ≈4 times for ONOO$^-$ (22.1±1.5 versus 9.4±0.7 nmol · L$^{-1}$ · s$^{-1}$ for O$_2^\cdot$- and 810±50 versus 209±15 nmol · L$^{-1}$ · s$^{-1}$ for ONOO$^-$). Pretreatment of the cells from blacks with 1.0 μmol/L nebivolol significantly improved NO release with concurrent reduction of O$_2^\cdot$- and ONOO$^-$ release in response to eNOS activation by CaI. Remarkably, there were not any significant differences in kinetics of the released species after stimulation with CaI between the nonpretreated cells from whites and the cells pretreated with nebivolol from blacks (Figure 1). However, the improvement of NO bioavailability in the presence of nebivolol was demonstrated in endothelium from white and black donors after stimulation with either ACh, a receptor-dependent eNOS agonist, or Cal, a receptor-independent eNOS agonist. Figure 2 (A and B) shows the peak release of NO with nebivolol pretreatment after stimulation with either Cal or ACh over a broad range of concentrations (0.1 nmol/L to 10 μmol/L) administered to individual cell culture wells. The NO levels were elevated with increasing concentrations of the eNOS agonists and reached a maximum at a concentration of 1.0 μmol/L for both Cal and ACh. Nebivolol significantly increased release of NO throughout the concentration range in response to these agonists. The favorable effect of nebivolol was dose-dependent, reaching maximal effect at a concentration of 5 μmol/L. The effect of nebivolol on eNOS agonist–stimulated
NO release was disproportionately greater in cells from black than from white donors with either CaI or ACh (Figure 2).

As in HUVECs, nebivolol also potentiated endothelial NO availability stimulated by the eNOS agonists in IAECs in both racial groups; the effect of nebivolol was more pronounced in cells from blacks than from whites (Figure 3). In contrast to nebivolol, pretreatment of the cells with the same concentrations of atenolol failed to modify NO availability after addition of the eNOS agonist in both HUVECs and IAECs (Figure 3). This study identified a potent effect of nebivolol in the restoration of eNOS function and a physiologically relevant level of bioavailable NO produced by endothelial cells from different vascular beds. The extent of eNOS uncoupling in the endothelial cells from black donors was much greater than that observed in cells from whites, as evidenced by higher \( \text{O}_2^- \) and \( \text{ONOO}^- \) production and lower levels of bioavailable NO (Figure 4).

The early effect of nebivolol on NO production can be observed within 10 minutes after treatment (Figure 4). Differences in measured levels of NO bioavailability between the groups were eliminated in the presence of nebivolol after its maximal effect was achieved with 180 minutes of treatment. Additionally, the marked improvement in NO production over time with nebivolol was associated with large reductions in both \( \text{O}_2^- \) and \( \text{ONOO}^- \) release. In contrast, when the cells were pretreated up to 180 minutes with atenolol, levels of NO/O\( _2^- \)/ONOO\(^-\) were not affected by the drug treatment. In addition, we confirmed that the release of NO, \( \text{O}_2^- \), and \( \text{ONOO}^- \) was related to eNOS activation, because eNOS inhibition of the enzyme by L-NAME blocked CaI- and ACh-stimulated release in both racial groups. Thus, it appears that nebivolol not only favorably restored the level of bioavailable NO in endothelial cells from blacks but also reversed eNOS uncoupling and improved its function.
Nebivolol also eliminated functional differences in the NO/O₂⁻/ONOO⁻ balance between cells from white and black donors. To test whether the benefit observed with nebivolol was related to specific inhibition of superoxide formation, we compared its effects with those of apocynin, a specific NAD(P)H oxidase inhibitor. Treatment of the cells for 180 minutes with apocynin effectively abolished differences between the racial groups, as evidenced by the formation of high NO and O₂⁻/ONOO⁻ and low ONOO⁻/H₂O₂ concentrations after activation of eNOS (Figure 4). This effect of apocynin was reproduced by other potential oxidase inhibitors, such as oxypurinol and rotenone (data not shown).

Figure 5 depicts the concentration ratio of NO to the concentration of ONOO⁻ produced by HUVECs before and after treatment with β-blockers. The NO concentration indicates NO bioavailability, whereas ONOO⁻ concentration reflects the extent of nitroxidative stress in these cells. Nebivolol treatment favorably improved the level of bioavailable NO while reducing nitroxidative stress. The effect of nebivolol was evident in the endothelium from both white and black donors. The NO/ONOO⁻ ratio was initially 1.30±0.18 in the endothelium of whites and much lower (0.50±0.07) in the endothelium of blacks. Nebivolol treatment significantly increased the NO/ONOO⁻ ratio to similar levels (3.45±0.58 in whites and 3.32±0.65 in blacks). Atenolol treatment did not change the NO/ONOO⁻ ratio in the endothelium of either whites or blacks.

**Discussion**

Cardiovascular disease is the leading cause of death among black Americans. Hypertension has been identified as the overwhelming risk factor that leads to cardiovascular events in this population, including heart failure, stroke, renal disease, and coronary artery disease. Epidemiological studies...
also indicate that the clinical manifestations of atherosclerosis are more severe in the black population, but the mechanism is not fully understood. Over the past decade, there has been growing appreciation of potential differences in endothelium-dependent mechanisms of vasodilation among blacks. It was demonstrated that postischemic vasodilatation, an index of endothelial function, was reduced significantly in young healthy blacks compared with age-matched healthy whites, as determined by mean postischemic dilation, an index of endothelial function. Furthermore, no gender difference was observed in the postischemic vasodilatory response of blacks, unlike that observed in whites. The basis for these differences in activity may be related to mechanisms of endothelial NO metabolism. In particular, interethnic genetic differences between whites and blacks have been observed, including the distribution of genetic variables for 3 clinically relevant eNOS polymorphisms. It has also been demonstrated recently that there is a phenotypic difference in NO bioavailability in blacks that can be attributed to eNOS uncoupling, despite an increase in eNOS levels.

The study presented here indicates that 2 enzymes, NAD(P)H oxidase and uncoupled eNOS, contribute to increased oxidative and nitrooxidative stress along with a loss of functional NO in the endothelium of blacks. We observed a marked difference in NO bioavailability in endothelial cells from black donors after stimulation with either a receptor-dependent (ACh) or -independent (CaI) agonist compared with age-matched white donors with similar age and family history of hypertension and diabetes. Remarkably, treatment with nebivolol effectively eliminated differences in NO bioavailability between blacks and whites in a highly reproducible and dose-dependent fashion. The benefits of nebivolol were observed in 2 distinct vascular beds and were not reproduced by another β1-selective agent (atenolol). The mechanism to explain the disproportionate enhancement of endothelial NO bioavailability in blacks may be related to reductions in enzymatic sources of superoxide. The data presented here strongly indicate that an elevated concentration of superoxide, generated by NAD(P)H oxidase, can be an initial cause of eNOS uncoupling in the endothelium of blacks. The uncoupled eNOS can, alternatively, generate O2•− or NO. The release of NO and O2•− in close proximity to each other increases the chance of their collision to produce ONOO•. ONOO• can be produced in a diffusion-controlled rapid reaction (k = 9.6 × 106 mol−1·L−1·s−1). At low concentrations, peroxynitrite rapidly (t1/2 ≤ 1 s) isomerizes to harmless NO2•. However, protonated ONOO• generated at high concentrations can diffuse and undergo heterogeneous or homogenous cleavage to NO2•, OH•, or NO2 and OH•. The products of this cleavage reaction (NO2•, OH•, and NO2) are highly reactive molecules that can trigger a cascade of potentially harmful actions, including DNA strand breakage and oxidation of protein tyrosine, sulfhydryl groups, and lipids. Thus, peroxynitrite formation represents an important means of propagating NO/O2•−-mediated nitrooxidative stress.

The studies presented here indicate the effect of nebivolol resembles closely that of apocynin, a well-characterized inhibitor of NAD(P)H oxidase. The relatively short time (minutes) and low concentration (micromolar) suggest that the inhibition of O2•− generation by NAD(P)H oxidase is a primary factor in the restoration of NO bioavailability by nebivolol in the endothelium of blacks. However, nebivolol is also a good scavenger of O2•−. The concentration of nebivolol in plasma can be as high as 0.7 to 0.9 μmol/L. The location of the lipophilic nebivolol molecule in the membrane of endothelial cells at a relatively high (micromolar) concentration in the tissue may amplify the scavenging process of O2•− through electron stabilization mechanisms. Therefore, both inhibition of NAD(P)H oxidase and direct O2•− scavenging by nebivolol may result in its beneficial effects on the restoration of NO bioavailability and endothelial function in blacks. The action of nebivolol on the restoration of endothelial functions was identical in HUVECs and IAECs. This is consistent with earlier studies that indicated an antioxidant benefit for nebivolol through inhibition of NAD(P)H oxidase activity, as well as with a very recent study that demonstrated that nebivolol decreases oxidative stress in patients with essential hypertension. However, it has been demonstrated that an acute infusion of antioxidant (ascorbic acid) improved vasodilation equally in black and white subjects. This suggests that the difference in oxidative stress alone may not be sufficient to explain the differences in endothelial function of blacks and whites. We demonstrated this with direct measurements of NO and oxidative species using nanotechnological approaches. The recognition that endothelial dysfunction and increased oxidative stress contribute disproportionately to mechanisms of heart failure in blacks led to the design of the A-HeFT study (African American Heart Failure Trial). That trial showed that the addition of isosorbide dinitrate and hydralazine to conventional therapy for heart failure reduced relative 1-year mortality by 43% among blacks with advanced disease. Isosorbide dinitrate is an organic nitrate that directly increases vascular NO levels, whereas hydralazine is a vasodilator with antioxidant activity that may scavenge oxyradical species, including O2•−. Thus, agents that enhance NO bioavailability while reducing nitrooxidative stress may have therapeutic potential in this population, especially under disease conditions.

**Conclusions**

We have demonstrated that nebivolol, a lipophilic β1-selective agent with direct vasodilating properties, enhanced NO bioavailability and caused a reduction of nitrooxidative stress in vascular endothelium. Nebivolol prevented eNOS uncoupling and restored NO bioavailability in endothelial cells from black donors. The most beneficial action of nebivolol on endothelial function is the reduction of nitrooxidative stress by inhibition of NAD(P)H oxidase activity and/or direct antioxidant properties of nebivolol. Nebivolol increased NO bioavailability in endothelial cells from both black and white donors. However, the relative increase in NO with nebivolol was much more pronounced in the endothelium of blacks (≈90% increase) than in that of whites (≈40% increase). Nebivolol treatment restored the bioavailable NO and decreased ONOO•, bringing the ratio of concentration of these 2 molecules in the endothelium of black and white Americans to the same level. These findings indicate a novel role for nebivolol in the treatment of higher-risk populations.
characterized by endothelial dysfunction, independent of \( \beta_1 \)-selective blockade.

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