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

R. Preston Mason, PhD; Leszek Kalinowski, MD, PhD; Robert F. Jacob, PhD; Adam M. Jacoby, BS; Tadeusz Malinski, PhD

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

Received April 15, 2005; revision received August 8, 2005; accepted September 19, 2005.
From the Department of Biochemistry, Ohio University, Athens, Ohio (L.K., A.M.J., T.M.); Elucida Research, Beverly, Mass (R.P.M., R.F.J.); and Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Mass (R.P.M.). Dr Kalinowski was on sabbatical leave from the University of Gdansk.
Guest Editor for this article was Thomas F. Lüscher, MD.
Correspondence to Tadeusz Malinski, PhD, Department of Biochemistry, Ohio University, 350 W State St, Athens, OH 45701. E-mail malinski@ohiou.edu
© 2005 American Heart Association, Inc.
Circulation is available at http://www.circulationaha.org

DOI: 10.1161/CIRCULATIONAHA.105.556233
Clinical Characteristics of the Study Donors

<table>
<thead>
<tr>
<th></th>
<th>Whites</th>
<th>Blacks</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>22 ± 1</td>
<td>22 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>57 ± 2</td>
<td>58 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22.6 ± 1.4</td>
<td>23.1 ± 1.7</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>121 ± 2</td>
<td>123 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>74 ± 2</td>
<td>77 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking, yes/no</td>
<td>0/12</td>
<td>0/12</td>
<td>NS</td>
</tr>
<tr>
<td>Family history of hypertension, yes/no</td>
<td>7/5</td>
<td>8/4</td>
<td>NS</td>
</tr>
<tr>
<td>Family history of diabetes, yes/no</td>
<td>1/11</td>
<td>4/8</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma glucose, mmol/L</td>
<td>5.1 ± 0.1</td>
<td>5.2 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.74 ± 0.14</td>
<td>2.68 ± 0.13</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.30 ± 0.08</td>
<td>1.24 ± 0.06</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.22 ± 0.08</td>
<td>1.12 ± 0.09</td>
<td>NS</td>
</tr>
</tbody>
</table>

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 hyper-tensive 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-purinocceptor–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 cells. All cell culture donors were healthy, with no pregnancy or prenatal complications. There were no meaningful differences in the contraceptives used between black and white subjects: approximately 50% of blacks and 60% of white donors did not use contraceptives at least 2 months before conception. None of the donors took any drugs regularly, and all were nonsmokers and consumed regular calorie/content diet. The donors were between 20 and 25 years of age, with a mean body mass index (in kg/m²) of 22.6 ± 1.4 for white donors and 23.1 ± 1.7 for black donors (Table). The identification of race was defined by self-report. Reproducibility of the measurements with nanosensors was high (5% to 12%).

However, reproducibility can be influenced by the distance of nanosensors from the surface of a single endothelial cell, and thus, to achieve high reproducibility, it is crucial to keep the distance from the cell constant (in the present studies: 4 ± 1 μm). Iliac artery endothelial cells (IAECs) were isolated (postmortem) into primary 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 placed 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) tetrakis (3-methoxy-4-hydroxyphenyl) porphyrin for the NO sensor, an immobilized polyppyrrole/horseradish peroxidase (PP/HRP) for the O₂⁻ sensor, and polymeric film of Mn(III) [2,2] paracyclophanylporphyrin for the ONOO⁻ sensor.
μmol/L nebulol or atenolol), 0.3 mmol/L L-NAME (N'-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 O₂⁻, 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 O₂⁻ and ONOO⁻ release with tandem nanosensors. We compared these measurements to the levels of NO/O₂⁻/ONOO⁻ obtained from age-matched white donors as a function of nebulol treatment. In both groups, stimulated NO release from a single endothelial cell with calcium ionophore (CaI) was associated with concurrent release of O₂⁻ 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 NO and ONOO⁻ 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, O₂⁻, 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 release were faster by ~2 times for O₂⁻ and ~4 times for ONOO⁻ (22.1±1.5 versus 9.4±0.7 nmol · L⁻¹ · s⁻¹ for O₂⁻ and 810±50 versus 209±15 nmol · L⁻¹ · s⁻¹ for ONOO⁻). Pretreatment of the cells from blacks with 1.0 μmol/L nebulol significantly improved NO release with concurrent reduction of O₂⁻ 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 nebulol from blacks (Figure 1). However, the improvement of NO bioavailability in the presence of nebulol was demonstrated in endothelium from white and black donors after stimulation with either ACh, a receptor-dependent eNOS agonist, or CaI, a receptor-independent eNOS agonist. Figure 2 (A and B) shows the peak release of NO with nebulol pretreatment after stimulation with either CaI 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 CaI and ACh. Nebivolol significantly increased release of NO throughout the concentration range in response to these agonists. The favorable effect of nebulol was dose-dependent, reaching maximal effect at a concentration of 5 μmol/L. The effect of nebulol 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 O$_2^\cdot$ and ONOO$^\cdot$ 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 O$_2^\cdot$ and ONOO$^\cdot$ release. In contrast, when the cells were pretreated up to 180 minutes with atenolol, levels of NO/O$_2^\cdot$/ONOO$^\cdot$ were not affected by the drug treatment. In addition, we confirmed that the release of NO, O$_2^\cdot$, and ONOO$^\cdot$ 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/O$_2^\cdot$/H$_2$O$_2$/ONOO$^-$/H$_2$O$_2$ 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 O$_2^\cdot$/H$_2$O$_2$ and low ONOO$^-$/H$_2$O$_2$ 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$^-$/H$_2$O$_2$ produced by HUVECs before and after treatment with $\beta$-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 $1.30 \pm 0.18$ in the endothelium of whites and much lower ($0.50 \pm 0.07$) in the endothelium of blacks. Nebivolol treatment significantly increased the NO/ONOO$^-$ ratio to similar levels ($3.45 \pm 0.58$ in whites and $3.32 \pm 0.65$ 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.\(^2\) 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.\(^18\) 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.\(^19\) 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.\(^3\)

The study presented here indicates that 2 enzymes, NAD(P)H oxidase and uncoupled eNOS, contribute to increased oxidative and nitrosative 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 \(\beta_1\)-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 \(O_2^-\) or NO. The release of NO and \(O_2^-\) 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 \((k=9.6\times10^{9}\text{ mol}^{-1}\cdot L^{-1}\cdot s^{-1})\). At low concentrations, peroxynitrite rapidly \((t_{1/2}\leq1\text{ s})\) isomerizes to harmless \(NO_2^-\). However, protonated ONOO\(^-\) generated at high concentrations can diffuse and undergo heterogeneous or homogenous cleavage to \(NO_2^-\), \(OH^-\), or \(NO_2^\cdot\) and \(OH\).\(^20\) Three products of this cleavage reaction (\(NO_2^-\), \(OH^-\), and \(NO_2^\cdot\)) are highly reactive molecules that can trigger a cascade of potentially harmful actions, including DNA strand breakage and oxidation of protein tyrosine, sulphydryl groups, and lipids. Thus, peroxynitrite formation represents an important means of propagating NO/\(O_2^-\)-mediated nitrosative 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 \(O_2^-\) 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 \(O_2^-\).\(^14\) The concentration of nebulol in plasma can be as high as 0.7 to 0.9 \(\mu\text{mol/L}\). 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 \(O_2^-\) through electron stabilization mechanisms. Therefore, both inhibition of NAD(P)H oxidase and direct \(O_2^-\) 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,\(^12,13\) as well with a very recent study that demonstrated that nebivolol decreases oxidative stress in patients with essential hypertension.\(^21\) However, it has been demonstrated that an acute infusion of antioxidant (ascorbic acid) improved vasodilation equally in black and white subjects.\(^22\) 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.\(^23\) 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 \(O_2^-\). Thus, agents that enhance NO bioavailability while reducing nitrosative stress may have therapeutic potential in this population, especially under disease conditions.

**Conclusions**

We have demonstrated that nebivolol, a lipophilic \(\beta_1\)-selective agent with direct vasodilating properties, enhanced NO bioavailability and caused a reduction of nitrosative 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 nitrosative 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 (\(\approx90\%\) increase) than in that of whites (\(\approx40\%\) 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 β1-selective blockade.

Acknowledgments
This work was supported by the Marvin and Ann Dilley White Endowment and Biomimetic Nanoscience and Nanotechnology Research Grant at Ohio University.

Disclosures
Dr Mason has received research funding from and has served as a consultant to Mylan Pharmaceuticals.

References
Nebivolol Reduces Nitrooxidative Stress and Restores Nitric Oxide Bioavailability in Endothelium of Black Americans
R. Preston Mason, Leszek Kalinowski, Robert F. Jacob, Adam M. Jacoby and Tadeusz Malinski

_Circulation_. 2005;112:3795-3801; originally published online December 5, 2005; doi: 10.1161/CIRCULATIONAHA.105.556233
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/112/24/3795

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/