Race-Specific Differences in Endothelial Function
Predisposition of African Americans to Vascular Diseases

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Background—The prevalence of the endothelium-impaired function disorders, such as hypertension and diabetes mellitus, and the severity of their complications are considerably greater in blacks than whites. Evidence has accumulated that superoxide (O$_2^-$) production and its interaction with nitric oxide (NO), yielding the strong oxidant peroxynitrite (ONOO$^-$), play central roles in vascular pathophysiology. We hypothesized that the differences in endothelial NO/O$_2^-$/ONOO$^-$ metabolism may highlight the potential predisposition to endothelial dysfunction and cardiovascular complications prevalent in blacks.

Methods and Results—Highly sensitive tandem electrochemical NO/O$_2^-$/ONOO$^-$ nanosensors were positioned in single human umbilical vein endothelial cells (HUVECs) isolated from blacks and whites, and the kinetics of NO/O$_2^-$/ONOO$^-$ release were recorded in vitro. HUVECs were also analyzed by Western immunoblotting and enzyme activity assays for NAD(P)H-oxidase and endothelial NO synthase (eNOS). Compared with whites, HUVECs from blacks elicited reduced release of bioactive NO with an accompanying increase in the release of both O$_2^-$ and ONOO$^-$. The greater potency of NO production because of eNOS upregulation in HUVECs from blacks is associated with a decrease in the NO bioavailability. This is due to increased NO degradation by excess O$_2^-$ produced primarily by 2 enzymatic sources: NAD(P)H-oxidase and uncoupled eNOS.

Conclusions—Compared with whites, the steady-state NO/O$_2^-$/ONOO$^-$ balance in endothelial cells from blacks is kept closer to the redox states characteristic for the endothelium-impaired function disorders. This may explain the differences in racial predisposition to the endothelium dysfunction during ongoing vascular disturbances with the hallmark of enhanced NO inactivation within the endothelium by oxidative stress. (Circulation. 2004;109:2511-2517.)

Key Words: nitric oxide ■ endothelium ■ risk factors ■ African Americans ■ ethnic groups

In the past few decades, the excess of hypertension and diabetes mellitus among people of African descent (blacks) has been recognized as a substantial portion of the apparent black health disadvantage. This is especially true for African Americans, who have one of the highest rates of hypertension and diabetes mellitus in the world. Cardiovascular complications associated with these diseases, such as stroke and heart and renal failures, are responsible for the greater rates of mortality in blacks compared with whites.

Endothelium-derived NO is a physiological mediator of numerous cellular and organ functions. One important physiological role of NO is to protect the cardiovascular system against pathophysiological insults. Besides being the most potent endogenous vasodilator, NO also inhibits smooth muscle cell proliferation and migration, adhesion of leukocytes to the endothelium, and platelet aggregation. An impairment of the NO signaling pathway, ie, endothelial dysfunction, is one of the earliest events in vascular diseases. The reduced bioavailability of NO observed in the pathogenesis of vascular diseases may occur by a reduction in NO synthesis and an increase in O$_2^-$ generation. O$_2^-$ reacts rapidly with NO, reducing NO bioactivity and producing ONOO$^-$, a strong oxidant. The increased O$_2^-$ production within endothelium accounts for a significant portion of the relative NO deficit in the vascular diseases, including hypertension and diabetes. In the majority of cases, the source of O$_2^-$ excess is uncertain, although involvement of NAD(P)H-dependent oxidases, xanthine oxidase, cyclooxygenase, mitochondrial oxidases, and endothelial nitric oxide synthase (eNOS) and neuronal NOS have been suggested. It should be noted that the net effect of the reaction between NO and O$_2^-$ compromises reduction of concentration of both substrates as well as biological effects of ONOO$^-$ itself. Peroxynitrite induces the oxidation of proteins, DNA, and lipids in vascular walls. Thus, increased production of a potentially deleterious metabolite, ONOO$^-$, by eNOS may shift the balance between oxidative and reductive states of the endothelial cell and may alter the beneficial effects of increasing NO activity. Modulation of either production or removal of NO and O$_2^-$ is reflected in changes of ONOO$^-$ formation and makes fluctuations in their levels transient.
In this work, we electrochemically assayed \( \text{NO}, \text{O}_2^-, \text{and ONOO}^- \) with modified electrodes into a tandem of \( \text{NO/O}_2^-/\text{ONOO}^- \) nanosensors, for the first time allowing us to measure concurrently the molecules in real time in a single endothelial cell. This approach is extremely favorable toward understanding the processes and mechanism of pathogenesis in vascular diseases at the molecular level. We sought to determine whether the predisposition to vascular complications in blacks implicates a reduction of NO bioavailability in endothelium. If so, what mechanism underlies the endothelium-determined race-specific diversity? We found that decreased NO availability in blacks compared with whites is due to excess \( \text{O}_2^- \) produced by NAD(P)H-oxidase that finally yields enhanced formation of ONOO\(^-\) after stimulation of eNOS. This, in turn, leads to the eNOS uncoupling, which produces \( \text{O}_2^- \) and NO, and may very well contribute to oxidative stress and endothelial dysfunction.

### Methods

#### Subjects and Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated into primary cultures from 12 white and 12 black female donors by Clonetics and purchased as proliferating cells. All cell culture donors were healthy, and none had pregnancy or perinatal complications. The clinical characteristics of the donors are reported in Table 1. None of the donors took any drugs regularly, and all were nonsmokers and consumed regular caloric/content diet. Before selection for the study, donors of each group were screened by clinical history, physical examination, routine chemical analyses, and ECG. Exclusion criteria were history or evidence of present or past hypertension, diabetes mellitus, renal disease, cardiac disease, peripheral vascular disease, vasculitis, coagulopathy, or any other disease predisposing the donors to vascular complications. The local Research Ethics Committee approved collection of tissue specimens, and all donors gave written informed consent.

The HUVEC culture was incubated in 95% air/5% CO\(_2\) at 37°C and passed over by an enzymatic (trypsin) procedure. The confluent cells (4x10\(^5\) to 5x10\(^5\) cells/35-mm dish) were placed with minimum essential medium containing 3 mmol/L l-arginine and 0.1 mmol/L H\(_2\)B \(\{6(9)\}-5.6,7,8\text{-tetrahydrobipterin}\}. Before the experiments, the cells (from the second or third passage) were rinsed twice with Tyrode's solution–HEPES buffer with 1.8 mmol/L CaCl\(_2\). All experiments were blinded to the race of the endothelial cell donors and study treatment.

#### Preparation of the Triple Sensor for NO, \( \text{O}_2^- \), and ONOO\(^-\) Detection

Concurrent measurements of NO, \( \text{O}_2^- \), and ONOO\(^-\) were performed with electrochemical microsensors\(^{7-9}\) combined into 1 working unit with a total diameter of 3 to 4 \(\mu\)m. Their design was based on previously developed and well-characterized chemically modified carbon-fiber technology. Each of the sensors was made by depositing a sensing material on the tip of carbon fiber (length, 4 to 5 \(\mu\)m; diameter, 0.5 \(\mu\)m). The fibers were sealed with nonconductive epoxy and electrically connected to copper wires with conductive silver epoxy. We used conductive film of polymeric nickel[II]tetrasik-(3-methoxy-4-hydroxyphenyl)porphyrin for the NO sensor,\(^{11,11}\) an immobilized poly-pyrrole/horseradish peroxidase (PP/HRP) for the \( \text{O}_2^- \) sensor,\(^8\) and polymeric film of Mn(III)-(2,2)paracyclophosphorylporphyrin for the ONOO\(^-\) sensor for the ONOO\(^-\) sensor.\(^{11,12}\)

#### Measurement of NO, \( \text{O}_2^- \), and ONOO\(^-\)

The tandem NO/O\(_2^-\)/ONOO\(^-\) nanosensors with a platinum wire (0.1 mm) counter electrode and saturated calomel reference electrode were applied. Differential pulse voltammetry (DPV) and amperometry were performed with a computer-based Gamry VFP600 multichannel potentiostat. DPV was used to measure the basal NO, \( \text{O}_2^- \), and ONOO\(^-\) concentrations, and amperometry was used to measure changes in NO, \( \text{O}_2^- \), and ONOO\(^-\) concentrations from its basal level with time (detection limit of 1 mmol/L and resolution time <50 ms for each sensor). The DPV current at the peak potential characteristic for NO (0.65 V) oxidation and ONOO\(^-\) (−0.45 V) or \( \text{O}_2^- \) (−0.23 V) reduction was directly proportional to the local concentrations of these compounds in the immediate vicinity of the sensor. Linear calibration curves were constructed for each sensor from 5 mmol/L to 3 mmol/L before and after measurements with aliquots of NO, \( \text{O}_2^- \), and ONOO\(^-\) standard solutions, respectively. The tandem system of NO/O\(_2^-\)/ONOO\(^-\) nanosensors was lowered with the help of a computer-controlled micromanipulator until it reached the surface of the cell membrane (a small piezoelectric signal, 6 to 8 pA, of 1 to 3 ms duration was observed at this point). The sensors were slowly raised 4±1 mm from the surface of a single endothelial cell. The eNOS agonist calcium ionophore A23187 (Cal) and acetylcholine were then injected with a nanoinjector that was also positioned by a computer-controlled micromanipulator. In the experiments with eNOS agonist stimulation, endothelial cells were pretreated for 3 hours with 0.3 mmol/L \( N^\text{-nitro-l-arginine methyl ester} \) (L-NAME), an eNOS inhibitor; 0.5 mmol/L 3-morpho-linosydnominine-N-ethylcarbamide (SIN-1),\(^{13}\) a releaser of both NO and \( \text{O}_2^- \); 0.01 mmol/L Mn(III)tetrais-[1-methyl-4-pyridyl]porphyrin pentachloride (MnTMPyP) or 1.0 mmol/L tempol (4-hydroxytetramethylpiperidine-1-oxyl), both cell-permeable superoxide dismutase (SOD) mimetics, or 0.01 mmol/L Mn(III)tetrais-(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTTPPS) or 0.1 mmol/L uric acid, both ONOO\(^-\) scavengers. Immediately before the measurements of eNOS agonist stimulated NO, \( \text{O}_2^- \), and ONOO\(^-\) release, the incubation was stopped by washing the cells twice with the buffer free of the test substances. In the experiments for determination of basal NO, \( \text{O}_2^- \), and ONOO\(^-\), the cells were preincubated for 3 hours before and during the measurements with various oxidase inhibitors (mmol/L): 3.0 apocynin,\(^3\) 0.05 6-dialyl 5,7-di-hydroxy 2-[2-allyl 3-hydroxy 4-methoxyphenyl]1-\(H\) benz(\(\phi\))pyran-4-one (S17834) (Servier), 0.1 oxypurinol, 0.1 rotenone, 0.01 meclofenamate, or 0.3 L-NAME.

#### NAD(P)H-Dependent Superoxide Production

Endothelial \( \text{O}_2^- \) production was also measured by SOD-inhibitable ferricytochrome c reduction assay as described previously.\(^{18}\) Briefly, equal protein samples of endothelial cell homogenate were incubated in 1 mL of buffer containing ferricytochrome c (80 \(\mu\)mol/L) in the presence of NAD(P)H or NADH (100 \(\mu\)mol/L) at 37°C for 45 minutes, and then absorbance was measured at 550 nm. All experiments were performed with or without SOD (400 U/mL). Superoxide production was calculated as the portion of ferricytochrome c reduction inhibited by SOD.

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**TABLE 1. Clinical Characteristics of the Study Donors**

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<td>22±1</td>
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<td>Systolic blood pressure, mm Hg</td>
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<td>NS</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
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<td>77±2</td>
<td>NS</td>
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<td>0/12</td>
<td>NS</td>
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<td>Family history of hypertension, yes/no</td>
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<td>8/12</td>
<td>NS</td>
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<tr>
<td>Family history of diabetes, yes/no</td>
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<td>4/12</td>
<td>NS</td>
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<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>
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<tr>
<td>Triglycerides, mmol/L</td>
<td>1.22±0.08</td>
<td>1.12±0.09</td>
<td>NS</td>
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Western Immunoblotting

Samples of endothelial cell homogenate, equalized for protein content, were separated by SDS-PAGE (5% gels) and transferred to PVDF membranes. NADPH-oxidase components were detected with goat polyclonal antibodies against p67phox or p47phox or p22phox, and eNOS was detected with polyclonal anti-eNOS antibody (Santa Cruz Biotechnology). To compare the NADPH-oxidase subunits and eNOS expressions with the expression of another protein, we used antibodies against β-actin by Western blot using a monoclonal anti-β-actin antibody. Bands were detected by horseradish peroxidase–conjugated secondary antibodies and visualized by chemiluminescence.

All chemicals were purchased from Sigma-Aldrich, unless otherwise noted.

Calculations and Statistical Analysis

When applicable (comparison between 2 values), statistical analysis was done with Student’s t test. For multiple comparisons, results were analyzed by ANOVA followed by Bonferroni’s and Dunn’s correction. Data are presented as mean±SEM. Means were considered significantly different at P<0.05.

Results

Differences Between Whites and Blacks in Basal NO, O2−, and ONOO− Release From HUVECs

Compared with whites, endothelial cells from blacks elicited reduced release of biologically active (diffusible) NO with an accompanying increase in the release of both O2− and ONOO− (Table 2). This suggests that in blacks, a decrease in NO bioavailability is not a result of a decrease in NO synthesis but rather of an increase of NO consumption by excess of O2−. To investigate the endothelial enzymatic sources of O2− production in both racial groups, we measured O2− release with concurrent measurements of NO and ONOO− in response to a range of potential oxidase inhibitors. In both blacks and whites, O2− production was inhibited by apocynin and S17834, NAD(P)H-oxidase inhibitors. Also, both apocynin and S17834 completely abolished differences between whites and blacks in release of the detected molecules. When the cells were treated with oxypurinol, meclofenamate, or rotenone, only meclofenamate appreciably suppressed O2− release in both racial groups. However, the differences in NO, O2−, and ONOO− release from HUVECs between whites and blacks were still maintained in the presence of each of the 3 inhibitors. The presence of L-NAME resulted in a significant increase of O2− release in both whites and blacks because of the loss of O2− scavenging by NO. Intriguingly, the proportionally greater increase in O2− release from HUVECs in the presence of L-NAME in whites than blacks (16.2±1.5 versus 5.2±1.8 mmol/L; P<0.01) suggests that the net effect of NOS activity in blacks is associated with diminishing O2− scavenging by eNOS-derived NO. In both groups, NO and ONOO− release in the presence of L-NAME were suppressed below the detection limits.

Increased NAD(P)H-Oxidase Activity and Protein Subunits in HUVECs From Blacks

To ascertain whether the increased O2− release from endothelial cells in blacks is caused by an increase in NAD(P)H activity, we compared NADH- and NAD(P)H-dependent O2− production in the endothelial cells from whites and blacks. As expected, in endothelial cells, NADH stimulated O2− production with greater potency than NAD(P)H in both racial groups (Figure 1). NADH/NAD(P)H-stimulated O2− production from HUVECs was significantly greater in blacks than whites. In both racial groups, NADH/NAD(P)H-stimulated O2− production was inhibited by apocynin or S17834 but not by oxypurinol, rotenone, meclofenamate, or L-NAME. We also investigated the relative abundance of NAD(P)H-oxidase protein subunits in the endothelial cells. Relative quantification of protein bands, normalized to β-actin, revealed increased levels of the p22phox membrane-bound subunit and the p67phox and p47phox cytosolic subunits in HUVECs from blacks compared with whites (Figure 2).

Increase in Both eNOS Protein Level and Uncoupling in HUVECs From Blacks

As in NAD(P)H-oxidase protein subunits, Western blot analysis revealed a parallel increase in eNOS expression in HUVECs from blacks (Figure 2). Also, the NOS activity was significantly greater in HUVEC lysates in blacks than whites.

### Table 2. Basal NO, O2−, and ONOO− Release From Endothelial Cells in Whites and Blacks After Inhibition of Endothelial O2− Generation Sources

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<thead>
<tr>
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<tr>
<td></td>
<td>NO</td>
<td>O2−</td>
<td>ONOO−</td>
<td>NO</td>
<td>O2−</td>
<td>ONOO−</td>
</tr>
<tr>
<td>Basal (control)</td>
<td>20.5±1.3†</td>
<td>9.1±1.0†</td>
<td>11.4±1.0†</td>
<td>9.2±1.1</td>
<td>17.6±1.3</td>
<td>18.8±1.5</td>
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<tr>
<td>+ Apocynin</td>
<td>29.2±2.1*</td>
<td>4.2±1.0*</td>
<td>6.4±1.0*</td>
<td>26.9±1.8*</td>
<td>4.5±1.0*</td>
<td>7.3±1.0*</td>
</tr>
<tr>
<td>+ S17834</td>
<td>29.8±2.2*</td>
<td>3.9±1.0*</td>
<td>6.1±1.0*</td>
<td>27.6±2.1*</td>
<td>4.3±1.0*</td>
<td>7.0±1.0*</td>
</tr>
<tr>
<td>+ Oxypurinol</td>
<td>22.8±1.4†</td>
<td>8.0±1.0†</td>
<td>9.9±1.0†</td>
<td>11.3±1.5</td>
<td>15.9±1.0</td>
<td>16.8±1.2</td>
</tr>
<tr>
<td>+ Rotenone</td>
<td>21.9±1.2†</td>
<td>8.4±1.0†</td>
<td>10.5±1.0†</td>
<td>10.7±1.2</td>
<td>16.2±1.1</td>
<td>17.2±1.4</td>
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<tr>
<td>+ Meclofenamate</td>
<td>27.9±1.8†</td>
<td>5.7±1.0†</td>
<td>7.1±1.0†</td>
<td>19.0±1.2*</td>
<td>12.2±1.0*</td>
<td>11.7±1.2*</td>
</tr>
<tr>
<td>+ L-NAME</td>
<td>ND</td>
<td>25.3±1.4*</td>
<td>ND</td>
<td>ND</td>
<td>22.8±1.2*</td>
<td>ND</td>
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</table>

Values are given in mmol/L. ND indicates not detectable; n=12 subjects. Endothelial cells were incubated for 3 hours before NO, O2−, and ONOO− determination with various oxidase inhibitors: 3 mmol/L apocynin or 0.05 mmol/L S17834 [both inhibitors of NAD(P)H oxidase], 100 μmol/L oxypurinol (an inhibitor of xanthine oxidase), 100 μmol/L rotenone (an inhibitor of mitochondrial oxidases), 10 μmol/L meclofenamate (an inhibitor of cyclooxygenase), or 300 μmol/L L-NAME (an inhibitor of eNOS).

*P<0.01 vs basal; †P<0.01 vs blacks.
when we used an NOS activity assay18 based on conversion of \(^3\)H-L-arginine to \(^3\)H-L-citrulline (data not shown). This suggested that endothelial cells in blacks compared with whites overproduce not only \(\text{O}_2^-\) but also NO, which finally leads to the formation of \(\text{ONOO}^-\). To investigate further whether the racial difference in the endothelial capabilities of \(\text{O}_2^-\) and NO production results in a disparity in NO bioavailability, we studied the eNOS-stimulated kinetics of free NO release with simultaneous detection of the kinetics of \(\text{O}_2^-\) and \(\text{ONOO}^-\) release by the tandem NO/O\(\text{O}_2^-/\text{ONOO}^-\) nanosensors.

In both racial groups, the stimulated (by Ca\(\text{I}\)) NO release from a single endothelial cell was associated with concurrent release of \(\text{O}_2^-\) and \(\text{ONOO}^-\) (Figure 3). In a HUVEC from whites, the pattern of \(\text{ONOO}^-\) release was similar to that for NO release, but it was delayed with a time-shift of \(\approx\)1 second. The sharp peaks of NO and \(\text{ONOO}^-\) concentrations were reached at 0.9 and 1.8 seconds after Ca\(\text{I}\) stimulation, respectively. In contrast, the peak value of \(\text{ONOO}^-\) concentration recorded in a HUVEC from blacks was \(\approx\)2 seconds before reaching the maximum for NO concentration, and it was at the time of the peak NO concentration in a HUVEC from whites. The blunted peaks of \(\text{O}_2^-\) concentrations were observed in the same time span (3 to 4 seconds) in HUVECs from both racial groups. There were significant differences in the kinetics of the initial rates of NO, \(\text{O}_2^-\), and \(\text{ONOO}^-\) release between blacks and whites from a single HUVEC. The rate of NO release was \(\approx\)5 times slower in blacks than whites (94 versus 505 nmol/L per second), whereas the rates of release were \(\approx\)2 times faster for \(\text{O}_2^-\) and \(\approx\)4 times faster for \(\text{ONOO}^-\) in blacks than whites (9.4 versus 22.1 nmol/L per second for \(\text{O}_2^-\) and 209 versus 810 nmol/L per second for \(\text{ONOO}^-\)).

We confirmed that the release of NO, \(\text{O}_2^-\), and \(\text{ONOO}^-\) was caused by eNOS activation by the finding that eNOS inhibition by L-NAME prevented Ca\(\text{I}\)-stimulated release of the detected molecules in both racial groups (Figure 4). In contrast to L-NAME, pretreatment with the specific NAD(P)H-oxidase inhibitors in concentrations that significantly inhibited NAD(P)H-dependent \(\text{O}_2^-\) production in the endothelial cells (Figure 2), apocynin 3 mmol/L or S17834 0.05 mmol/L, abolished the difference in the eNOS-dependent release of the detected molecules from HUVECs between whites and blacks (Figure 4). The slow continuous generation of \(\text{ONOO}^-\) by SIN-1 (0.5 mmol/L) during pretreatment with apocynin or S17834 caused the differences between whites and blacks to be maintained. This suggested that the either apocynin or S17834 was effective in abolishing the difference in the eNOS function in HUVECs between both groups by preventing \(\text{O}_2^-\) formation that eventually reacted with NO to form \(\text{ONOO}^-\). Accordingly, pretreatment of the cells with either MnTMPyP (0.01 mmol/L), a specific \(\text{O}_2^-\) scavenger, or FeTPPS (0.01 mmol/L), a specific \(\text{ONOO}^-\) scavenger, abolished the differences in eNOS-dependent NO,
O$_2^-$, and ONOO$^-$ release. The effect of abolishing racial differences in NO, O$_2^-$, and ONOO$^-$ release was also observed when the cells were pretreated in the same way with other specific scavengers of O$_2^-$ and ONOO$^-$, tempol (1 mmol/L), or uric acid (0.1 mmol/L), respectively (data not shown). As in the studies with Cal, the differences between whites and blacks in the peak NO, O$_2^-$, and ONOO$^-$ concentrations were also revealed in HUVECs in response to acetylcholine (1 μmol/L), a receptor-independent eNOS agonist (data with acetylcholine are not shown). Likewise, the racial differences were abolished by pretreatment with the NAD(P)H-oxidase inhibitors (SIN-1 inhibited this effect) and the scavengers of O$_2^-$ or ONOO$^-$. Of note, the racial differences in NO/O$_2^-$/ONOO$^-$ balance and both eNOS and NAD(P)H regulation were revealed even in prolonged cell cultures when the cells from the eighth passages were investigated.

**Discussion**

Despite the enormous recent efforts devoted to genetic studies of hypertension and diabetes, it is now clear that the genetic determinants of the diseases from the variable racial populations are exceedingly complex, and the degree of anticipated interindividual and intergroup heterogeneity may lie beyond the possibility of definition. Moreover, in the absence of important breakthroughs in epidemiological methods, studies that focus on the cause of vascular complications related to hypertension and diabetes in blacks seem to provide the best clues to the pathogenesis. Recently, evidence has accumulated that O$_2^-$ production and its interaction with NO, yielding the strong oxidant ONOO$^-$, play central roles in vascular pathophysiology. The present study demonstrates race-specific differences in endothelial NO bioavailability and ONOO$^-$ formation. These differences arise because endothelial cells from blacks generate significantly more O$_2^-$, primarily from 2 enzymatic sources: NAD(P)H-oxidase and eNOS. These findings are of paramount importance, because they reveal that NO/O$_2^-$/ONOO$^-$ balance in intact endothelium may operate in a diverted steady state among ethnic groups. They also explain the differences in racial predisposition to the endothelial dysfunction during ongoing vascular disturbances with the hallmark of enhanced NO inactivation within the endothelium by O$_2^-$. The increased levels of NAD(P)H-oxidase protein subunits and total eNOS protein in the endothelial cells from blacks compared with whites, in association with increased enzymatic activities, suggest that upregulated gene expression or posttranscriptional upregulation of protein levels contributes to the enhancement of basal NO/O$_2^-$/ONOO$^-$ steady-state concentrations in the endothelium from blacks. NAD(P)H-oxidase-derived O$_2^-$ production in the cells not only enhanced degradation of NO in blacks but also altered eNOS function, as illustrated by abolishing the differences in NO/O$_2^-$/ONOO$^-$ balance between whites and blacks after eNOS activation in the presence of the specific NAD(P)H-oxidase inhibitors apocynin and S17834. The proportionally greater effect of both inhibitors in reduction of O$_2^-$ and ONOO$^-$ generation with concomitant lesser inactivation of NO in blacks compared with whites most likely resulted from the prevention of O$_2^-$ production by eNOS, in addition to inhibition of NAD(P)H-oxidase in the endothelial cells in blacks. It seems that this effect of both apocynin and S17834 is not caused by a direct action on eNOS but rather the inhibition of NAD(P)H-oxidase-derived O$_2^-$ production and subsequently diminishing ONOO$^-$ in the cells. Accordingly, the difference in eNOS function between whites and blacks was abolished under conditions favoring the reduction of ONOO$^-$ formation in the endothelial cells by using O$_2^-$ or ONOO$^-$ scavengers. Also, the action of the NAD(P)H-oxidase inhibitors on eNOS function was prevented in blacks when the cells were exposed additionally to excess ONOO$^-$ generated by SIN-1.

Our data provide direct evidence that the uncoupled eNOS in human endothelium under physiological conditions is probably the most significant source of O$_2^-$. Also, the degree of enzyme uncoupling is different in the endothelial cells of whites and blacks. We found that activation of eNOS is associated not only with NO release but also with concomitant release of O$_2^-$ and ONOO$^-$. This was demonstrated in the most accurate method by using the NO/O$_2^-$/ONOO$^-$ nanosensors, which offer the advantage of allowing measurements to be made in situ (in the close vicinity of the
membrane-bound eNOS) and in real time. Inhibition of NO production by L-NAME with concurrent inhibition of O$_2^-$ and ONOO$^-$ formation during activation of eNOS also confirms the concept that uncoupling of eNOS occurs in intact endothelial cells. The eNOS uncoupling may be caused by local (in the close proximity of the enzyme) L-arginine and/or tetrahydrobiopterin concentration. It is well established that tetrahydrobiopterin can be easily deactivated in fast oxidation reaction with ONOO$^-$. Recent studies on isolated eNOS enzyme also suggested that the region containing paired cysteine residues that coordinates binding of zinc sulfate and the integrity of tetrahydrobiopterin binding may be controlled by the redox status of the cell. Thus, exposure of eNOS to a strong oxidant, such as ONOO$^-$, may destabilize eNOS in the active dimeric form, ultimately leading to enzymatic “uncoupling.” Our studies suggest that ONOO$^-$ may represent not only a pathogenic but also a physiological mediator of uncoupling eNOS and maintaining this process on different steady-state levels.

The previously reported blunted vasodilator response to acetylcholine in healthy blacks compared with whites can be explained by the diminished NO bioavailability within endothelial cells, a mechanism that is not restricted to the muscarinic cell receptors. The release of diffusible (biologically active) NO from the endothelial cells was blunted in blacks in response to either acetylcholine or Ca$^+$, and with 0.01 mmol/L MnTMPyP (a specific O$_2^-$ scavenger) or 0.01 mmol/L FeTPPS (a specific ONOO$^-$ scavenger). n=12 subjects; *P<0.01.
thelial cells from blacks is kept closer to the redox state that had been documented in the endothelium-impaired function disorders.3,6,15,23

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