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.[1,2] 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.[3–6] 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.

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TABLE 1. Clinical Characteristics of the Study Donors

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

In this work, we electrochemically assayed[7–9] NO, O_2^-, and $ONOO^-$ with modified electrodes into a tandem of NO/O_2^-/$ONOO^-$ 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 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 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 passaged by an enzymatic (trypsin) procedure.[10] The confluent cells (4×10^5 to 5×10^5 cells/35-mm dish) were placed with minimum essential medium containing 3 mmol/L L-arginine and 0.1 mmol/L H_4B [(6*R*)-5,6,7,8-tetrahydrobiopterin]. 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, O_2^-, and ONOO Detection

Concurrent measurements of NO, O_2^-, and $ONOO^-$ were performed with electrochemical microsensors[7–9] combined into 1 working unit with a total diameter of 3 to 4 μm. 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 μm; diameter, 0.5 μm). 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)tetrakis(3-methoxy-4-hydroxyphenyl)porphyrin for the NO sensor,[9,11] an immobilized polypyrrole/horseradish peroxidase (PPy/HRP) for the O_2^- sensor,[8] and polymeric film of Mn(III)-[2,2]paracyclophenylporphyrin for the $ONOO^-$ sensor for the $ONOO^-$ sensor.[7,12]

Measurement of NO, O_2^-, and $ONOO^-$

The tandem NO/O_2^-/$ONOO^-$ nanosensors with a platinum wire (0.1 mm) counterelectrode and saturated calomel reference electrode were applied. Differential pulse voltametry (DPV) and amperometry were performed with a computer-based Gamry VFP600 multichannel potentiostat. DPV was used to measure the basal NO, O_2^-, and $ONOO^-$ concentrations, and amperometry was used to measure changes in NO, O_2^-, and $ONOO^-$ concentrations from its basal level with time (detection limit of 1 nmol/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 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 nmol/L to 3 μmol/L before and after measurements with aliquots of NO, 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 μm from the surface of a single endothelial cell. The eNOS agonists calcium ionophore A23187 (CaI) 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^G-nitro-L-arginine methyl ester (L-NAME), an eNOS inhibitor; 0.5 mmol/L 3-morpho-linosydnonimine-*N*-ethylcarbamide (SIN-1),[13] a releaser of both NO and O_2^-; 0.01 mmol/L Mn(III)tetrakis(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 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS) or 0.1 mmol/L uric acid, both $ONOO^-$ scavengers. Immediately before the measurements of eNOS agonist–stimulated NO, 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, 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 6,8-diallyl 5,7-dihydroxy 2-(2-allyl 3-hydroxy 4-methoxyphenyl)1-*H* benzo(*b*)pyran-4-one (S17834) (Servier),[14] 0.1 oxypurinol, 0.1 rotenone, 0.01 meclofenamate, or 0.3 L-NAME.

NAD(P)H-Dependent Superoxide Production

Endothelial O_2^- production was also measured by SOD-inhibitable ferricytochrome *c* reduction assay as described previously.[15] Briefly, equal protein samples of endothelial cell homogenate were incubated in 1 mL of buffer containing ferricytochrome *c* (80 μmol/L) in the presence of NAD(P)H or NADH (100 μmol/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.

TABLE 2. Basal NO, O_2^-, and $ONOO^-$ Release From Endothelial Cells in Whites and Blacks After Inhibition of Endothelial O_2^- Generation Sources

	Whites			Blacks		
	NO	O_2^-	$ONOO^-$	NO	O_2^-	$ONOO^-$
Basal (control)	20.5±1.3†	9.1±1.0†	11.4±1.0†	9.2±1.1	17.6±1.3	18.8±1.5
+Apocynin	29.2±2.1*	4.2±1.0*	6.4±1.0*	26.9±1.8*	4.5±1.0*	7.3±1.0*
+S17834	29.8±2.2*	3.9±1.0*	6.1±1.0*	27.6±2.1*	4.3±1.0*	7.0±1.0*
+Oxypurinol	22.8±1.4†	8.0±1.0†	9.9±1.0†	11.3±1.5	15.9±1.0	16.8±1.2
+Rotenone	21.9±1.2†	8.4±1.0†	10.5±1.0†	10.7±1.2	16.2±1.1	17.2±1.4
+Meclofenamate	27.9±1.8*†	5.7±1.0*†	7.1±1.0*†	19.0±1.2*	12.2±1.0*	11.7±1.2*
+L-NAME	ND	25.3±1.4*	ND	ND	22.8±1.2*	ND

Values are given in nmol/L. ND indicates not detectable; n=12 subjects. Endothelial cells were incubated for 3 hours before NO, O_2^-, 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.

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).[16] To compare the NADPH-oxidase subunits and eNOS expressions with the expression of another protein, we analyzed the expression of 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.[17] Data are presented as mean±SEM. Means were considered significantly different at $P<0.05$.

Results

Differences Between Whites and Blacks in Basal NO, O_2^-, 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 O_2^- and $ONOO^-$ (Table 2). This suggests that in blacks, a decrease in NO bioavailability is not a result of a decrease of NO synthesis but rather of an increase of NO consumption by excess of O_2^-. To investigate the endothelial enzymatic sources of O_2^- production in both racial groups, we measured O_2^- release with concurrent measurements of NO and $ONOO^-$ in response to a range of potential oxidase inhibitors. In both blacks and whites, O_2^- 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 O_2^- release in both racial groups. However, the differences in NO, O_2^-, 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 O_2^- release in both whites and blacks because of the loss of O_2^- scavenging by NO. Intriguingly, the proportionally greater increase in O_2^- release from HUVECs in the presence of L-NAME in whites than blacks (16.2±1.5 versus 5.2±1.8 nmol/L; $P<0.01$) suggests that the net effect of NOS activity in blacks is associated with diminishing O_2^- 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 O_2^- release from endothelial cells in blacks is caused by an increase in NAD(P)H activity, we compared NADH- and NAD(P)H-dependent O_2^- production in the endothelial cells from whites and blacks. As expected, in endothelial cells, NADH stimulated O_2^- production with greater potency than NAD(P)H in both racial groups (Figure 1). NADH/NAD(P)H-stimulated O_2^- production from HUVECs was significantly greater in blacks than whites. In both racial groups, NADH/NAD(P)H-stimulated O_2^- 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

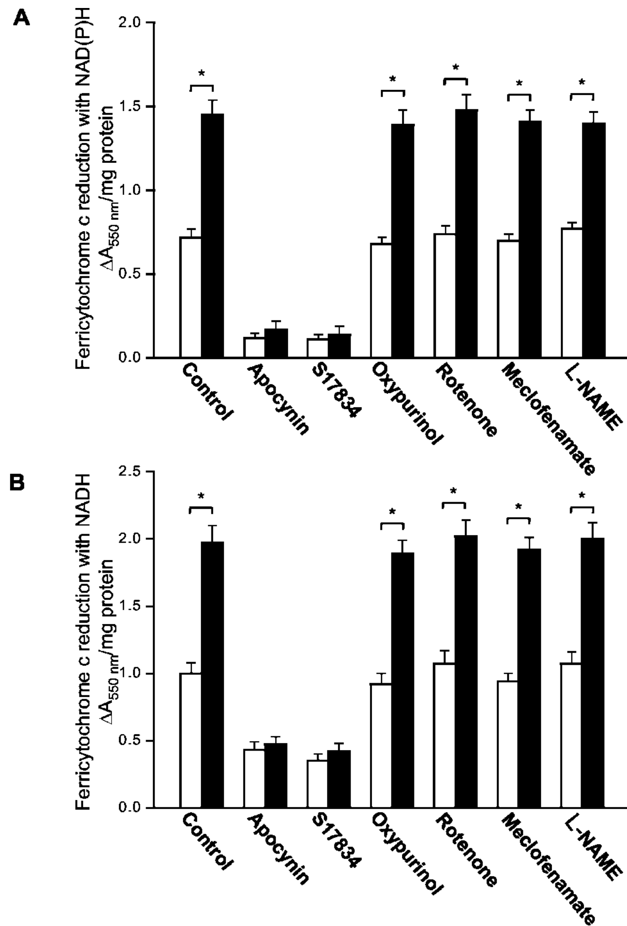

Figure 1. NAD(P)H-oxidase activity in HUVECs from whites (open bars) and blacks (solid bars). Enzyme activity was measured in response to NAD(P)H (A) or NADH (B) in endothelial cell homogenates by SOD-inhibitable ferricytochrome *c* reduction assay in presence or absence of 3 mmol/L apocynin, 50 μmol/L S17834, 100 μmol/L oxypurinol, 100 μmol/L rotenone, 10 μmol/L meclofenamate, or 300 μmol/L L-NAME. Cells were incubated with these agents for 3 hours before and during O_2^- determination. n=12 subjects; *$P<0.01$.

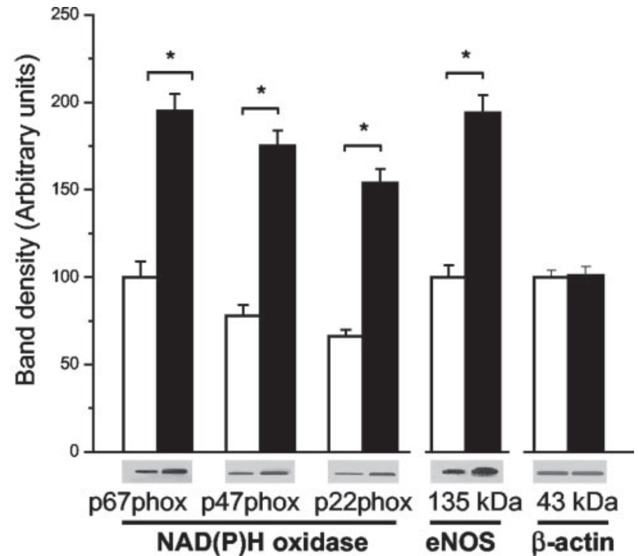

Figure 2. Differences in NAD(P)H-oxidase protein subunits and total eNOS protein expression in HUVECs between whites (open bars) and blacks (solid bars). Bars' values correspond to band chemiluminescence intensities relative to level of p67phox or total eNOS in whites. Representative blots from whites and blacks are demonstrated. Equal protein loading was confirmed with β-actin expression. n=12 subjects; *$P<0.01$.

when we used an NOS activity assay[18] 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 O_2^- but also NO, which finally leads to the formation of $ONOO^-$. To investigate further whether the racial difference in the endothelial capabilities of 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 O_2^- and $ONOO^-$ release by the tandem NO/O_2^-/$ONOO^-$ nanosensors. In both racial groups, the stimulated (by CaI) NO release from a single endothelial cell was associated with concurrent release of O_2^- and $ONOO^-$ (Figure 3). In a HUVEC from whites, the pattern of $ONOO^-$ release was similar to that for NO release, but it was delayed with a time-shift of ≈1 second. The sharp peaks of NO and $ONOO^-$ concentrations were reached at 0.9 and 1.8 seconds after CaI stimulation, respectively. In contrast, the peak value of $ONOO^-$ concentration recorded in a HUVEC from blacks was ≈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 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, O_2^-, and $ONOO^-$ release between blacks and whites from a single HUVEC. The rate of NO release was ≈5 times slower in blacks than whites (94 versus 505 nmol/L per second), whereas the rates of release were ≈2 times faster for O_2^- and ≈4 times faster for $ONOO^-$ in blacks than whites (9.4 versus 22.1 nmol/L per second for O_2^- and 209 versus 810 nmol/L per second for $ONOO^-$).

We confirmed that the release of NO, O_2^-, and $ONOO^-$ was caused by eNOS activation by the finding that eNOS inhibition by L-NAME prevented CaI-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 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 $ONOO^-$ 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 O_2^- formation that eventually reacted with NO to form $ONOO^-$. Accordingly, pretreatment of the cells with either MnTMPyP (0.01 mmol/L), a specific O_2^- scavenger, or FeTPPS (0.01 mmol/L), a specific $ONOO^-$ scavenger, abolished the differences in eNOS-dependent NO,

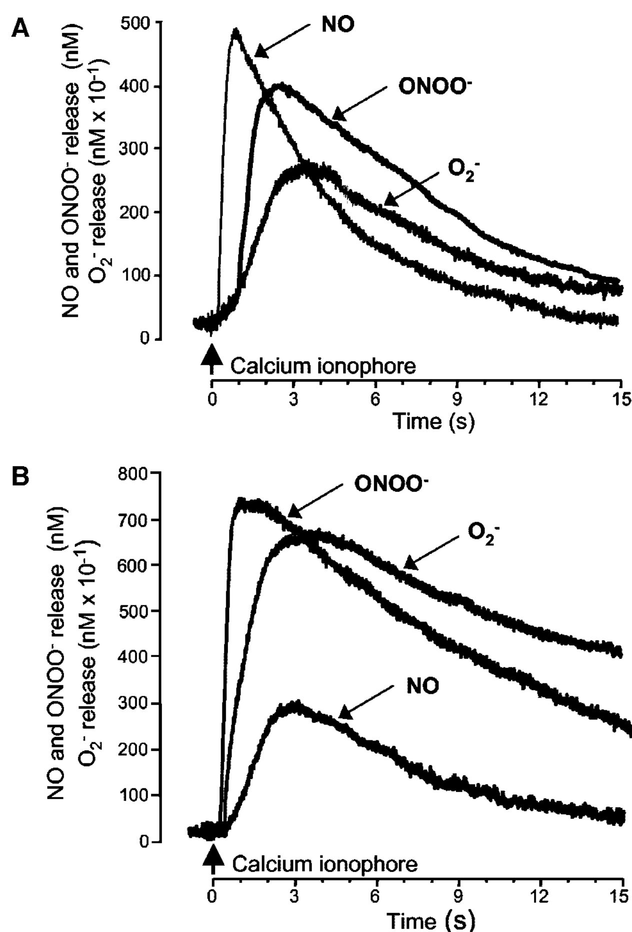

Figure 3. Representative recordings of NO, O_2^-, and $ONOO^-$ concentrations with time on surface of a single endothelial cell in whites (A) and blacks (B). Release of NO, O_2^-, and $ONOO^-$ were stimulated by 1.0 μmol/L CaI.

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 CaI, 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.[6] 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-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-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

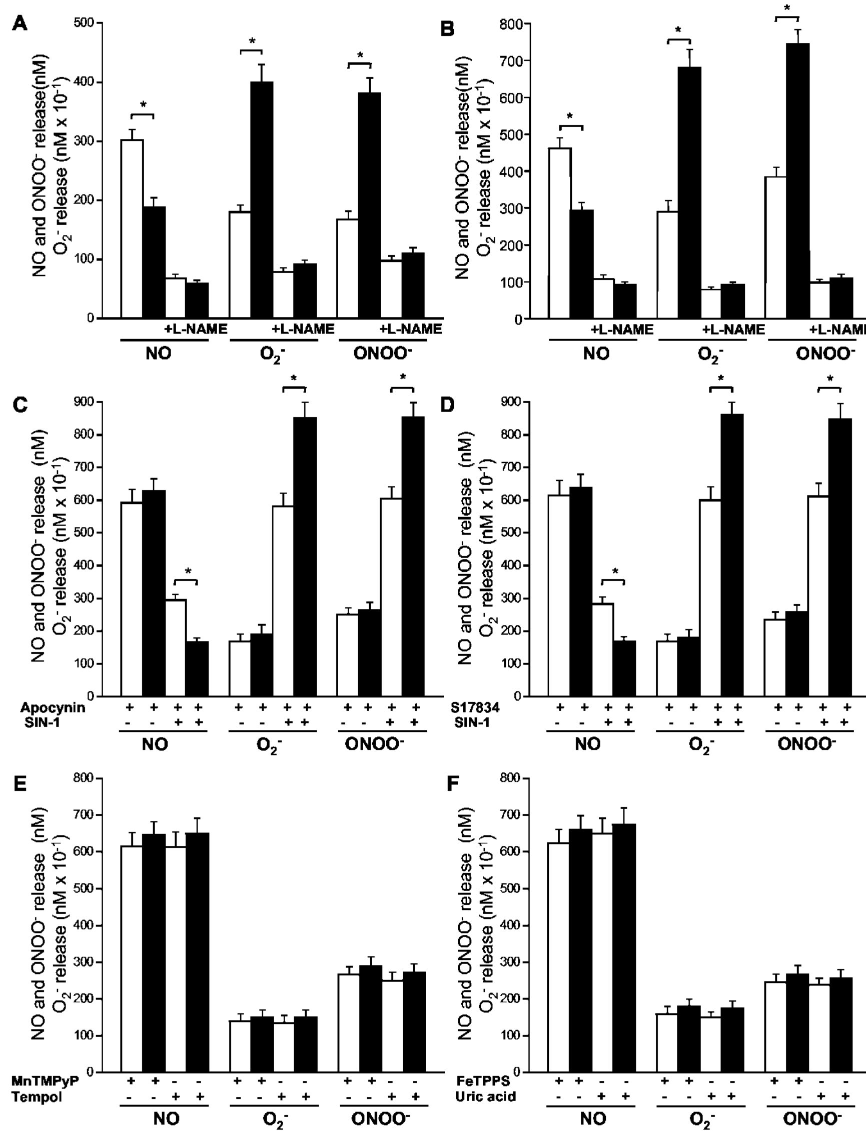

Figure 4. Differences in peak NO (A), O_2^- (B), and $ONOO^-$ (C) responses in HUVECs from whites (open bars) and blacks (solid bars) after stimulation with 1.0 μmol/L CaI. Before addition of CaI, cells were preincubated for 3 hours with 300 μmol/L L-NAME (an eNOS inhibitor), 3 mmol/L apocynin or 0.05 mmol/L S17834 [specific NAD(P)H-oxidase inhibitors], without or together with 0.5 mmol/L SIN-1 (a releaser of both NO and O_2^-), and with 0.01 mmol/L MnT-MPyP (a specific O_2^- scavenger) or 0.01 mmol/L FeTPPS (a specific $ONOO^-$ scavenger). n=12 subjects; $*P<0.01$.

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.[19–21] It is well established that tetrahydrobiopterin can be easily deactivated in fast oxidation reaction with $ONOO^-$. Recent studies[4] 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[22] 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 CaI, a receptor-independent eNOS agonist. The observation that blacks also have a reduced vasodilation in response to NO-independent stimuli[22] may be explained by the fact that lowering NO bioavailability results in downregulation of the downstream target of NO, the soluble guanylyl cyclase.[23]

In conclusion, we have provided direct evidence that, despite the increased eNOS protein level, reduced NO bioavailability in intact endothelial cells from blacks compared with whites is primarily due to increased O_2^- production mediated by upregulated NAD(P)H-oxidase activity followed by eNOS uncoupling. The racial difference in the extent of eNOS uncoupling is caused by excess $ONOO^-$ produced initially in the reaction between NO and NAD(P)H-oxidase–derived O_2^-. Compared with whites, the steady state of $NO/O_2^-/ONOO^-$ balance in the endo-

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]

Acknowledgments

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