Race-Specific Differences in Endothelial Function

Predisposition of African Americans to Vascular Diseases

Leszek Kalinowski, MD, PhD; Iwona T. Dobrucki, MS; Tadeusz Malinski, PhD

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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From the Department of Biochemistry, Ohio University, Athens. Dr Kalinowski was on sabbatical leave from the Department of Clinical Biochemistry, Medical University of Gdansk, and Laboratory of Cellualr and Molecular Nephrology of the Polish Academy of Science, Poland.

Correspondence to Tadeusz Malinski, Department of Biochemistry, Ohio University, Athens, OH 45701. E-mail malinski@ohiou.edu

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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/m²	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$_4$B [(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