Relation of Endothelial Nitric Oxide Synthase Gene to Plasma Nitric Oxide Level, Endothelial Function, and Blood Pressure in African Americans

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Background: The role of eNOS gene polymorphisms on plasma nitrite or nitrate (NO\textsubscript{x}) level, endothelial function, and blood pressure (BP) remains unclear.

Methods: We estimated the relationship of eNOS polymorphisms (the T\textsuperscript{786} C in the 5′-flanking promoter region, T\textsuperscript{786} C; 27-bp repeat in intron 4, eNOS4; and Glu298Asp in exon 7, G894T) with plasma NO\textsubscript{x} level, brachial endothelial function assessed by ultrasound measure of brachial artery flow-mediated dilation (FMD), and BP in 60 healthy African Americans, 30 men and 30 women aged 18 to 73 years.

Results: Among them, 73.1%, 23.9%, and 3.0% carried TT, TC, and CC of T\textsuperscript{786} C, respectively, 14.5%, 27.5%, 53.6%, and 1.4% carried aa, ab, bb, and bc of eNOS4 polymorphism, respectively, and 70.4%, 23.9%, and 5.6% carried GG, GT, and TT of G894T, respectively. G894T and eNOS4 were observed in linkage disequilibrium. Mean values of age, plasma NO\textsubscript{x}, FMD, systolic and diastolic BPs were not significantly different (\(P > .05\)) by eNOS polymorphisms. Plasma NO\textsubscript{x} level was found to be associated with systolic BP (\(r = 0.51, P = .03\)), and diastolic BP (\(r = 0.41, P = .08\)), but not with FMD, in individuals with “a” allele of eNOS4 polymorphism after adjustment for age, body mass index, serum glucose, and smoking status.

Conclusions: We reveal a positive association between plasma NO\textsubscript{x} level and BP in normotensive African Americans who carry the “a” allele of eNOS4. Because the frequency of the rare allele “a” is significantly higher in African Americans than in other ethnic groups, this finding may provide a clue to understanding the genetic susceptibility to hypertension in African Americans. Am J Hypertens 2004;17:560–567 © 2004 American Journal of Hypertension, Ltd.

Key Words: endothelial nitric oxide synthase gene, nitric oxide, endothelial function, blood pressure, African Americans.

In 1980, it was first discovered that the vascular endothelium released an unstable substance named endothelium-derived relaxing factor (EDRF).\textsuperscript{1} In 1987 to 1988, Palmer and colleagues reported that nitric oxide (NO) synthesized from l-arginine by nitric oxide synthase (NOS) in endothelial cells accounted for the biological activity of EDRF.\textsuperscript{2} Two research groups, Janssens et al\textsuperscript{3} and Marsden et al.,\textsuperscript{4} isolated the cyclic DNA for the human vascular endothelial NOS in 1992. Thereupon, the endothelial nitric oxide synthase (eNOS or NOS3) gene was mapped on chromosome 7q36.\textsuperscript{5} The eNOS gene consists of 26 exons spanning approximately 21 kb of genomic DNA and encoding an mRNA of 4052 nucleotides.

Studies using cell culture and animal model revealed...
that free radical NO is important in the regulation of vasomotor tone and blood flow by inhibiting smooth muscle contraction. This NO is scavenged rapidly and acts in a paracrine fashion to transduce cellular signals. Plasma nitrite (NO$_3^-$) or nitrate (NO$_2^-$) are stable oxidation products of free radical NO. However, previous reports on the relationship of eNOS gene to plasma nitrite (NO$_3^-$) and nitrate (NO$_2^-$) levels, blood pressure (BP), or heart disease in human are controversial. Few studies reported the relationship of the eNOS genotypes to plasma NO products and clinical phenotypes in African Americans. In this study, we assessed the relationship of the selected eNOS polymorphisms of T$^{-786}$C, a single nucleotide polymorphism with T to C mutation in the promoter region, 27-bp repeat in intron 4 (eNOS4) and Glu298Asp (G894T) in exon 7 to plasma NOx, brachial endothelial function assessed by ultrasound measure of brachial flow-mediated dilation (FMD), and BP in normotensive African Americans. We also assessed the association between plasma NOx and BP, as well as plasma NOx and brachial endothelial function separately for different genotypes.

Methods

Participants and Data Collection

The study participants were recruited from shopping malls, barbershops, community centers, and health fairs in the south and southwest areas of metropolitan Atlanta. They were self-reported African Americans aged 18 years or older who did not have physician-diagnosed high BP, diabetes, coronary heart disease, stroke, or any life-threatening diseases, and who had no history of drug abuse. Participants’ eligibility was further confirmed by questionnaire and clinical examinations after enrollment in the study. The study enrolled 72 participants (38 women and 34 men). We excluded 7 subjects whose BP (systolic BP/diastolic BP ≥140/90 mm Hg) met the definition of hypertension, as reported by the Sixth Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure, and excluded 5 individuals with incomplete laboratory assays. The final participants in this study were 30 female and 30 male African American normotensives.

Individuals who agreed to participate in the study were invited to the Morehouse School of Medicine (MSM) Clinical Research Center (CRC) for interview and clinical examinations. The details of the study and informed consent were fully explained to all participants by the study coordinator. Before the enrollment, participants signed the consent form approved by the MSM Institutional Review Board. Thereafter, an interviewer-administered questionnaire was applied to collect participants’ demographic, lifestyle, socioeconomic information, as well as personal medical history and family history of hypertension, diabetes, coronary heart disease, or stroke. A physician conducted the physical examination. A trained nurse conducted participants’ height, weight, BP measurements, and electrocardiographic examination. Participant’s BP was measured in the supine position after 15 min of rest. Overnight fasting blood (30 mL) was drawn and processed (transferred to different tubes, separated by centrifugation, frozen, and packaged in −70°C freezer) by a trained technician at the CRC laboratory. Blood samples were sent to the MSM molecular genetics laboratory for genotype assays, to the MSM analytical laboratory for quantitation of plasma NOx, and to Doctors Laboratory, Inc (Valdosta, GA) for routine laboratory tests of metabolic panel, lipid profile, and hematology. All blood samples were labeled with participants’ identification numbers. Laboratory personnel were blind to participants’ information.

Genotype Assay of eNOS

Genomic DNA was extracted from 1 mL of whole blood using the QIAamp DNA blood kit (Qiagen, Valencia, CA). Genotyping of each polymorphism was performed by amplification from 30 to 50 ng of genomic DNA. The eNOS polymorphisms selected in this study were T$^{-786}$C in the promoter region, eNOS4 in intron 4, and G894T in exon 7 (see Fig. 1 for their locations). The eNOS 4a/4b genotypes was determined using primers that flank the 27-bp direct repeat using the following primers: (forward) 5'–AGGCCCTATGGTAGTGC-CCTT-3' and (reverse) 5'–TCTCTTAGTGCTGGTGCAC-3'. Primers for the G894T and T$^{-786}$C polymorphisms were as described by Tanus-Santos et al. Each assay was conducted in 25 μL containing 1.0 U Taq DNA polymerase (Qiagen), 0.5 μmol/L of the appropriate primer, and 250 μmol/L of each dNTP. The reaction for amplifying the T$^{-786}$C genotype contained 2% dimethyl sulfoxide. Each reaction was initially denatured for 1 min at 94°C. This step was followed by 34 cycles at 95°C for 25 sec, 56°C (4a/4b and Glu298Asp) or 62°C (T$^{-786}$C) for 35 sec, 72°C for 40 sec, with a final extension at 72°C for 5 min.

The eNOS alleles 4a, 4b, and 4c were identified as fragments corresponding to 393-bp, 420-bp, and 447-bp, respectively, after separation on 4% NuSieve 3:1 agarose gel (FMC Bioproducts, Rockland, ME) stained with ethidium bromide (Fig. 2). In addition, the 27-bp repeat elements were verified by DNA sequencing. Amplified

![FIG 1.](image-url) The human eNOS gene and locations of genetic polymorphisms. The eNOS has 26 exons (gray boxes). Introns that contain polymorphisms are indicated by an asterisk. The gray daggers indicate genetic variants in exons and intron. Six SNPs in the 5'-flanking region and one SNP in the 3'-flanking region are shown as an asterisk.
products of the G894T genotype were incubated with BanII or MboI restriction enzyme, whereas the T~786C genotypes were digested with Mspl before gel analysis as described above.

Quantitation of Plasma NOx
Concentrations for the nitric oxide products, total nitrate/nitrite, were determined colorimetrically by using an assay kit from the Cayman Chemical Company (Ann Arbor, MI). Briefly, plasma samples (40 µL) were incubated at room temperature for 3 hours with nitrate reductase to convert nitrates into nitrites. Greiss reagent (50 µL) was then added to convert nitrite into a chromophore. Absorbance was measured spectrophotometrically at 540 nm using a Spectramax Plus Microplate Reader (Molecular Device, Sunnyvale, CA). The concentration of nitric oxide products (in micromoles per liter) were calculated as total nitrate/nitrite from a nitrate standard curve. Each plasma sample was run in triplicate and the average was reported as the final plasma NOx level. To assess measurement variability, approximately 6% of the samples from this study and our other ongoing studies were replicates unknown to laboratory personnel. A correlation coefficient of 0.96 between samples and their replicates indicated a very good reproducibility of this measurement.

Determination of Brachial Endothelial Function
Brachial endothelial function was determined by brachial artery vasodilator response or brachial FMD. It was measured in the dominant arm according to previously validated techniques.17 An 8.0-MHz linear phased array ultrasound transducer attached to an ATL5000 (Philips Medical Systems, Bothell, WA) was used to perform diameter measurements and Doppler flow parameters of the brachial artery. Participants were imaged in the supine position. After baseline measurements of brachial artery diameter and Doppler spectral velocity, a BP cuff was inflated on the proximal portion of the arm to 200 mm Hg for 5 min, creating distal limb ischemia. After release of the cuff, reactive hyperemia occurred (flow-mediated or endothelium-dependent vasodilation). The brachial artery was imaged continuously for 2 min after cuff release and maximum diameter selected. Maximum Doppler flow velocity was similarly recorded for reactive hyperemia. Brachial flow-mediated vasodilation was calculated as (HD – BD)/BD, where HD refers to the hyperemia diameter and BD is the baseline diameter. Studies in our laboratory have confirmed the reproducibility of this technique in healthy volunteers, with intra- and interobserver variability of less than 10%.

Statistical Analysis
The SAS/Genetics version 9.0 (SAS Institute Inc., Cary, NC) was used to test Hardy-Weinberg equilibrium and linkage disequilibrium, to measure marker informativeness, and to estimate the haplotype frequency of T~786C, eNOS4, and G894T of eNOS polymorphisms with unknown gametic phase. Hardy-Weinberg equilibrium was tested by permutation version of exact test with a modified version of the Markov-chain random walk algorithm. Linkage disequilibrium between a pair of loci was tested using Weir’s χ² statistic and a likelihood-ratio test. The allele association between a pair of loci was measured by the correlation coefficient (r) and Lewontin’s D’. Marker informativeness was measured by polymorphism information content (PIC), heterozygosity and allelic diversity. Maximum-likelihood estimates of haplotype frequency were computed using an expectation-maximization (EM) algorithm. The SAS/STAT was used to compute mean and 95% confidence interval of mean for the variables of interest. The significant difference of means by the genotype of the selected eNOS polymorphisms was determined by analysis of covariance with adjustment for age, sex, body mass index (BMI), serum glucose, and smoking status. Pearson’s correlation analysis was used to estimate correlations between NOx and BP, NOx and FMD, and BP and FMD overall and stratified by genotype. To control for confounders, we estimated partial correlation coefficients controlling for age, smoking, BMI, and glucose, the factors potentially related to BP, endothelial function, and plasma NOx.16,19 Haplotype trend regression was applied to assess the association of all estimated haplotypes with NOx, FMD, systolic and diastolic BP.20

Results
Distribution of the Selected eNOS Polymorphisms
The distribution of T~786C and G894T in the sample of 60 African Americans did not deviate from the Hardy-Weinberg equilibrium. The allele frequencies of the eNOS polymorphisms and three measures of allele informativeness (PIC, heterozygosity, and allele diversity) are shown in Table 1. The alleles T of T~786C, b of eNOS4, and G of G894T are common alleles with frequency greater than 60% in this sample. The higher values of allele informativeness for eNOS4 comparing with that of T~786C and G894T suggested a higher allelic variation of eNOS4. This high allelic variation tends to be more informative and
more useful in association studies. The genotype frequencies were 73.1%, 23.9%, and 3.0% for TT, TC, and CC of T–786C, respectively, 14.5%, 27.5%, 53.6%, and 1.4% for aa, ab, bb, and bc of eNOS4, respectively, and 70.4%, 23.9%, and 5.6% for GG, GT, and TT of G894T, respectively. Maximum-likelihood estimates of haplotype frequency of eNOS T–786C, eNOS4, and G894T polymorphisms were 6.0% of CaG, 23.3% of TaG, 5.4% of CbG, 49.2% of TbG, 1.6% of TcG, 2.6% of CbT, and 11.1% of TbT. As expected, the frequencies of genotype and haplotype were not significantly different by gender (data not shown). Linkage disequilibrium test for all pairs of loci indicated an association between eNOS4 and G894T. This is consistent with the report from a previous study.16 The correlation coefficients (r) between eNOS4 alleles and G894T alleles were 0.33 of a-G, −0.33 of a-T, −0.36 of b-G, 0.36 of b-T, 0.11 of c-G, and −0.11 of c-T. The plasma NOx level was not correlated with FMD overall or stratified by the genotype of the selected eNOS polymorphisms. No association between the plasma NOx level and BP association was stratified by gender (data not shown).

Association of eNOS Genotype/Haplotype With Plasma NOx, Flow-Mediated Dilation, BP, and Cardiovascular Risk Factors

The distributions of plasma NOx, FMD, BP, and selected cardiovascular risk factors by different eNOS genotypes are listed in Table 2. We combined the homozygous bb with heterozygous bc (n = 2) of eNOS4, TC with CC (n = 2) of T–786C, and GT with TT (n = 2) of G894T, because the number of rare alleles or genotypes was too small to make comparison with others. Mean values of plasma NOx, FMD, BP, and selected cardiovascular risk factors were not significantly different by genotypes before (Table 2) and after (data not shown) adjustment for age, sex, BMI, serum glucose, and smoking status. In this sample, there were 25.4% smokers (6.8% current smokers and 18.6% former smokers). The proportions of ever smoking did not significantly differ by genotype (Table 2, similar results, data did not show, for current and former smoking). In the haplotype trend regression, in which the estimated probability of possible haplotype for each individual was the predictor, no association of any haplotype with NOx, FMD, systolic and diastolic BP was observed before or after adjustment for the selected covariates.

Table 1. Allele frequency and 95% confidence interval (CI) of selected eNOS polymorphisms

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allele</th>
<th>Frequency</th>
<th>95% CI</th>
<th>PIC</th>
<th>Heterozygosity</th>
<th>Allele Diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>T786C</td>
<td>C</td>
<td>0.167</td>
<td>0.100–0.242</td>
<td>0.24</td>
<td>0.27</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>0.833</td>
<td>0.758–0.900</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS4</td>
<td>a</td>
<td>0.292</td>
<td>0.208–0.383</td>
<td>0.35</td>
<td>0.32</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.692</td>
<td>0.600–0.775</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>0.017</td>
<td>0.000–0.042</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G894T</td>
<td>G</td>
<td>0.850</td>
<td>0.775–0.917</td>
<td>0.22</td>
<td>0.23</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>0.150</td>
<td>0.083–0.225</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

T–786C in the 5′-flanking (promoter) region; eNOS4(a/b/c) = 27-bp repeat in intron 4 (a –4 repeats, b –5 repeats, c –6 repeats); G894T = Glu298Asp in exon 7.

Correlation Between Flow-Mediated Dilation and BPs

Overall, FMD was inversely but not significantly correlated with systolic (r = −0.22, P = .08) and diastolic (r = −0.17, P = .20) BPs among the sample of normotensive African Americans. After adjusted for age, BMI, serum glucose, and cigarette smoking, the correlation coefficients decreased, r = −0.05, P = .71 for FMD correlated with systolic, and r = −0.06, P = .70 for FMD correlated with diastolic BP. None of the correlation coefficients between FMD and BP were a statistically significant departure from zero after stratified by each genotype of the three eNOS polymorphisms.

Relationship of Plasma NOx With Flow-Mediated Dilation and BP

The plasma NOx level was not correlated with FMD overall or stratified by the genotype of the selected eNOS polymorphisms. No association between the plasma NOx level and BP was observed in the 60 African Americans as a whole. Interestingly, when the assessment of the plasma NOx level and BP association was stratified by the genotype of these eNOS polymorphisms, the plasma NOx level was positively correlated with BP, especially with systolic BP in the participants who carried the a allele in 27-bp repeat of intron 4 (Table 3 and Fig. 3). The correlation coefficients in a allele carriers were r = 0.51 (P = .03) for plasma NOx and systolic BP, and r = 0.41 (P = .08) for plasma NOx and diastolic BP after adjustment for age, sex, BMI, glucose, and cigarette smoking. This correlation was not observed in those who were bb homozygotes of 27-bp repeat in intron 4. There was no relationship between plasma NOx level and BP observed in different genotypes of T–786C and G894T.

Discussion

The genotype distribution of T–786C in this study was similar to a previous report in unrelated African Americans of undetermined clinical status.16 However, the frequencies of the rare allele TT homozygotes (5.6%) of G894T and aa homozygotes (14.5%) of the 27-bp repeat in
Table 2. Mean and 95% confidence interval (CI) of selected factors by eNOS genotype

<table>
<thead>
<tr>
<th></th>
<th>T786C(TT) (n = 42)</th>
<th>T786C(TC/CC) (n = 18)</th>
<th>eNOS4(aa) (n = 9)</th>
<th>eNOS4(ab) (n = 17)</th>
<th>eNOS4(bb/bc) (n = 34)</th>
<th>G894T(GG) (n = 44)</th>
<th>G894T(GT/TT) (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOx (µM)</td>
<td>9.5 (8.3, 10.8)</td>
<td>8.1 (6.9, 9.3)</td>
<td>9.5 (7.0, 12.0)</td>
<td>8.1 (6.2, 10.0)</td>
<td>9.5 (7.7, 9.9)</td>
<td>8.8 (7.7, 9.9)</td>
<td>9.9 (8.1, 11.7)</td>
</tr>
<tr>
<td>FMD (%)</td>
<td>(13.3, 15.8)</td>
<td>(12.5, 19.1)</td>
<td>(12.4, 14.9)</td>
<td>(13.0, 19.3)</td>
<td>(13.0, 16.3)</td>
<td>(13.2, 16.1)</td>
<td>(12.6, 18.7)</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>(116.2, 122.7)</td>
<td>(1118.0, 124.2)</td>
<td>(112.5, 128.3)</td>
<td>(115.0, 122.3)</td>
<td>(114.4, 123.3)</td>
<td>(115.4, 122.2)</td>
<td>(113.9, 125.3)</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>(73.4, 77.1)</td>
<td>(73.0, 81.2)</td>
<td>(67.6, 82.2)</td>
<td>(72.0, 78.7)</td>
<td>(70.0, 77.9)</td>
<td>(70.9, 76.5)</td>
<td>(70.8, 82.7)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>(34.9, 43.5)</td>
<td>(35.6, 47.4)</td>
<td>(38.7, 50.7)</td>
<td>(30.6, 45.5)</td>
<td>(34.8, 44.3)</td>
<td>(34.0, 42.4)</td>
<td>(39.1, 49.9)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>(26.0, 31.0)</td>
<td>(24.6, 27.7)</td>
<td>(22.7, 30.1)</td>
<td>(22.7, 31.0)</td>
<td>(26.2, 31.1)</td>
<td>(25.1, 28.8)</td>
<td>(25.2, 34.9)</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>(87.1, 104.2)</td>
<td>(84.7, 94.0)</td>
<td>(81.7, 94.1)</td>
<td>(82.0, 90.7)</td>
<td>(88.7, 110.0)</td>
<td>(88.0, 97.3)</td>
<td>(76.7, 117.4)</td>
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<tr>
<td>LDL (mg/dL)</td>
<td>(110.0, 137.8)</td>
<td>(116.7, 161.0)</td>
<td>(98.4, 170.4)</td>
<td>(122.3, 164.9)</td>
<td>(103.9, 134.9)</td>
<td>(108.4, 139.3)</td>
<td>(121.3, 152.8)</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>(49.3, 54.0)</td>
<td>(45.1, 62.9)</td>
<td>(33.8, 61.8)</td>
<td>(42.9, 61.2)</td>
<td>(45.8, 55.1)</td>
<td>(45.5, 55.5)</td>
<td>(44.7, 57.6)</td>
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<tr>
<td>Triglyceride (mg/dL)</td>
<td>(86.8, 138.6)</td>
<td>(86.0, 177.7)</td>
<td>(74.5, 132.7)</td>
<td>(78.7, 111.2)</td>
<td>(97.2, 168.1)</td>
<td>(88.9, 137.7)</td>
<td>(79.9, 181.2)</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>26.8 (22.2)</td>
<td>33.3 (25.0)</td>
<td>23.5 (20.5)</td>
<td>40.0 (20.5)</td>
<td>40.0 (20.5)</td>
<td>40.0 (20.5)</td>
<td>40.0 (20.5)</td>
</tr>
</tbody>
</table>

BMI = body mass index; DBP = diastolic blood pressure; eNOS = endothelial nitric oxide synthase; eNOS polymorphisms of T786C in the 5' flanking (promoter) region; eNOS4(a/b/c) = 27-bp repeat in intron 4 (a = 4 repeats, b = 5 repeats, c = 6 repeats); FMD = brachial flow-mediated dilation; G894T = Glu298Asp in exon 7; NOx = plasma nitrite/nitrate; PBP = pulse blood pressure is SBP–DBP; SBP = systolic blood pressure.

* Smoking: ever versus never, no statistically significant difference by genotypes of each polymorphism.
intron 4 were higher in this study compared to the previous study (TT: 1.0%, aa: 6.0%). The frequency of bb homozygotes (53.3%) of the 27-bp repeat in intron 4 in our study was also higher than that in the previous report (45.0%). Distribution of all polymorphisms is under the Hardy-Weinberg equilibrium, which suggests the results of this study are unlikely to be biased by population stratification or admixture.

In this study, none of the analyzed eNOS polymorphisms or their haplotypes was significantly associated with plasma NOx level, although Tsukada et al reported high plasma NOx levels in healthy Japanese who were homozygous bb carriers, whereas Wang et al reported a contradictory result of high plasma NOx levels in healthy volunteers of European descendant who were homozygous aa of the 27-bp repeat in intron 4. Reports on the relationship of eNOS gene variants to BP alterations are controversial. In this study of normotensive African Americans, we did not observe any relationship of the selected eNOS polymorphisms or possible haplotype with BP level, or with brachial endothelial function assessed by FMD before and after adjustment for age, sex, BMI, serum glucose, and cigarette smoking. However, this study showed increased BP, especially systolic BP, with increased plasma NOx levels only in a allele carriers of the 27-bp repeat in intron 4 after adjustment for age, sex, BMI, serum glucose, and cigarette smoking, the factors known to be related to both plasma NOx level and BP. The a allele of the 27-bp repeat in intron 4 is a rare allele, but the frequency of a allele and homozygotes aa was found to be significantly higher in African Americans compared with other racial/ethnic groups. Moreover, this rare allele 4a associated with common allele G of G894T in exon 7 (r = 0.3, Lewontin’s D’ = 0.62) may be a biomarker for BP variation in African Americans. In fact, previous studies observed a relationship of the 4a variant with cardiovascular and renal diseases in African Americans.

Studies of eNOS gene knockout mouse indicated that lack of eNOS, the enzyme for NO synthesized from L-arginine, was associated with increased BP.

Table 3. Relationship of plasma nitrite/nitrate with blood pressure and FMD by eNOS4a/b/c genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>SBP</th>
<th></th>
<th></th>
<th>DBP</th>
<th></th>
<th></th>
<th>FMD</th>
<th>Adjusted for</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS4(aa/ab)</td>
<td>26</td>
<td>0.37</td>
<td>0.07</td>
<td>0.40</td>
<td>0.04</td>
<td>-0.23</td>
<td>0.27</td>
<td>-0.24</td>
<td>Age, BMI, Glucose, Smoking</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.39</td>
<td>0.05</td>
<td>0.47</td>
<td>0.02</td>
<td>-0.10</td>
<td>0.62</td>
<td>-0.22</td>
<td>All</td>
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<tr>
<td></td>
<td></td>
<td>0.47</td>
<td>0.02</td>
<td>0.40</td>
<td>0.05</td>
<td>-0.22</td>
<td>0.29</td>
<td>-0.20</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.38</td>
<td>0.06</td>
<td>0.39</td>
<td>0.06</td>
<td>-0.22</td>
<td>0.29</td>
<td>-0.20</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.37</td>
<td>0.09</td>
<td>0.34</td>
<td>0.12</td>
<td>-0.20</td>
<td>0.29</td>
<td>-0.20</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.51</td>
<td>0.03</td>
<td>0.41</td>
<td>0.08</td>
<td>-0.20</td>
<td>0.29</td>
<td>-0.20</td>
<td>All</td>
</tr>
<tr>
<td>eNOS4(bb/bc)</td>
<td>34</td>
<td>-0.05</td>
<td>0.77</td>
<td>0.04</td>
<td>0.81</td>
<td>0.05</td>
<td>0.80</td>
<td>0.04</td>
<td>All</td>
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eNOS4(aa/ab) = the combination of aa homozygous and ab heterozygous; eNOS4(bb/bc) = bb heterozygous and bb homozygous of 27-bp repeat in intron 4; other abbreviations as in Table 2.

**FIG 3.** Relationship of plasma nitric oxide (NO) with (A) systolic blood pressure (SBP), (B) diastolic blood pressure (DBP), and (C) brachial flow-mediated dilation (FMD) among 26 African American normotensives with eNOS (4aa or ab) genotypes.
Plasma NOx is the oxidation product of free radical NO. Its concentration may reflect the free radical NO level in endothelial cell. Thus, the positive association between plasma NOx and BP in our study of normotensive African Americans carrying the a allele of eNOS4 appears counterintuitive to previous studies. However, recent studies found that the chronic overexpression of eNOS in the endothelium resulted in resistance to the NO/cyclic GMP-mediated vasodilators, and that at least two distinct mechanisms might be involved: one is reduced soluble guanylate cyclase (sGC) activity, and the other is a decrease in cyclic GMP-dependent protein kinase (PKG) protein levels. Therefore, we speculate that the increased BP with increase plasma NOx levels in a allele of eNOS4 carriers may be explained by chronic overexpression of eNOS in this subgroup.

Apart from the genetics for eNOS, the corresponding increase in systolic BP with NOx might be the result of a complex interaction with free radicals, inactivating vascular relaxing effects of NO. Nitric oxide is a weak radical with a relatively long half-life that scavenges more highly reactive free radicals such as superoxides (O2-) produced, for example, by the enzyme NADPH oxidase. The interaction of superoxides with NO can result in the generation of reactive nitrogen species including peroxynitrates, nitrates, and nitrites. Furthermore, evidence indicates that an imbalance between the antioxidant effects of NO and vascular oxidative stress can result in vascular dysfunction. Thus, if NO is forced to act as an antioxidant rather than as a vasorelaxant, its efficacy would be attenuated. However, whether this process is contributing the observed variability associated with plasma NOx levels and systolic BP remains to be elucidated.

Plasma NOx level can also be influenced by administration of exogenous pharmacologic sources of NO, diet, or bacterial infection. In this study, no one was under nitroglycerin or cardiovascular disease treatment, which ruled out the pharmacologic influence. Although we were not able to control for the diet of all participants before they visited the Clinical Research Center, 15% of them had 1-week low/high salt diet at the Clinical Research Center for cardiac functional study. The correlation of plasma NOx level before and after their 7-day diet control was 0.74, which indicated diet effects on plasma NOx were minor in our study. In addition, the genotyping and NOx assay (phenotyping) were conducted independently by different technicians in different laboratories without knowing results from each other. Diet effects on the plasma NOx levels, if any, should be random across the eNOS genotype groups, unless individuals with specific variants had a predisposition to consume a diet that significantly altered their plasma NOx levels. Cigarette smoking was reported to affect both the endothelial function and plasma NOx level. Yoon and colleagues found a significantly higher concentration of plasma NOx in ever-smokers (24.4% current and 6.9% former smokers) than nonsmokers, especially in a allele of eNOS4 carriers. However, in our study, we did not find any significant difference of plasma NOx by smoking and eNOS4 genotype. Plasma NOx levels (95% confidence interval) were 7.7 µmol/L (4.9, 10.4) of ever-smokers and 9.1 µmol/L (7.2, 11.0) of nonsmokers in aa/ab of eNOS4 carriers, and were 9.2 µmol/L (5.2, 13.2) of ever-smokers and 9.6 µmol/L (8.3, 11.0) of nonsmokers in bb/bc carriers. This inconsistency between our and Yoon’s study may be due to the small proportion (6.8%) of current smokers in our sample with a limited power to detect the effect, if increased plasma NOx levels in smokers result mainly from current smoking.

This study had a small number of participants (n = 60) after excluding ineligible individuals and those with missing data, which precluded the ability to detect weak associations. Some negative results such as no association between eNOS4 and plasma NOx or BP may be due to insufficient statistical power compared to other reports. The positive result of plasma NOx associated with BP in a allele of eNOS4 carriers (n = 26) might also be chance-related (false positive). However, as both plasma NOx and BP were continuous measures, which increase statistical power, with no significant outlier (Fig. 3) in this sample, the result should be able to provide a clue for future studies.

In conclusion, we found a positive correlation between BP and plasma NOx in individuals with the rare allele a of eNOS 27-bp repeats in intron 4. Future studies in African Americans with a larger sample size are needed to confirm this result.

References


