Combined Effects of Endothelial Nitric Oxide Synthase Gene Polymorphism (G894T) and Insulin Resistance Status on Blood Pressure and Familial Risk of Hypertension in Young Adults: The Bogalusa Heart Study

Wei Chen, Sathanur R. Srinivasan, Abdalla Elkasabany, Darrell L. Ellsworth, Eric Boerwinkle, and Gerald S. Berenson

Impaired endothelial function with decreased nitric oxide production is shared by insulin resistance and essential hypertension. Although there are limited data on the association between the endothelial nitric oxide synthase (eNOS) G894T polymorphism and hypertension, information is absent on the combined effects of eNOS G894T genotype and insulin resistance status on blood pressure (BP) levels and the familial risk of hypertension. This aspect was examined in a community-based sample of 1021 unrelated African American and white young adults aged 19 to 38 years. African Americans displayed a lower frequency of the T894 allele than whites (0.105 vs 0.324, P < .001). After adjusting for sex, age, and body mass index (BMI), noncarriers versus carriers of the T894 allele had significantly higher systolic (SBP), diastolic (DBP) BP and mean arterial pressure (MAP) levels (111.7 vs 109.2 mm Hg for SBP; 73.6 vs 72.3 mm Hg for DBP; 86.3 vs 84.6 mm Hg for MAP), with both African Americans and whites showing similar trends. This association was modulated by insulin resistance status, measured by the homeostasis model assessment of insulin resistance (HOMA IR) using fasting insulin and glucose. Subjects with high insulin resistance (above the median HOMA IR) showed significantly greater differences in BP levels between noncarriers and carriers of the T894 allele. Furthermore, the G894T genotype and insulin resistance also showed a combined effect on the prevalence of parental hypertension, a measure of familial risk, with noncarriers versus carriers in the high insulin resistance group showing higher prevalence (70.5% vs 51.3%, P = .006, adjusted for race). Thus, the allelic variation (G894T) in the eNOS gene locus in conjunction with insulin resistance may be one factor contributing to the predisposition to hypertension. Am J Hypertens 2001;14:1046–1052 © 2001 American Journal of Hypertension, Ltd.

Key Words: Endothelial nitric oxide synthase G894T polymorphism, insulin resistance, blood pressure, parental hypertension, African American–white.

Nitric oxide, which is synthesized from L-arginine in vascular endothelial cells by nitric oxide synthase (eNOS), accounts for the biological actions of the endothelium-derived relaxing factor.1,2 Endothelial nitric oxide plays a key role in the regulation of vascular tone, blood flow, and blood pressure (BP).3–6 Individuals with essential hypertension display impaired endothelial function and related decrease in nitric oxide production.5,6

The gene encoding eNOS maps to chromosome 7q35–7q36.1 In humans, a missense variant of the eNOS gene in exon 7 shows a transversion of G to T at nucleotide position 894 (G894T) that results in a replacement of Glu by Asp at amino acid residue 298 (Glu298Asp).8 Although the relationship between nitric oxide release and BP has

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been extensively studied, information on the association between the G894T polymorphism and hypertension is scant and conflicting.9–11

Hyperinsulinemia/insulin resistance is generally considered to be an important factor in the etiology of essential hypertension.12,13 There is evidence that insulin-mediated nitric oxide synthesis and the attendant vasodilatation and increased blood flow at the level of skeletal muscle vasculature are impaired in the insulin resistance-related states of hypertension, type 2 diabetes, and obesity.14–17 It has been suggested that impaired endothelial function with decreased nitric oxide generation and skeletal muscle blood flow may be necessary for the development of insulin resistance accompanied by hypertension.14–19 Irrespective of cause and effect of their relationships, the condition of impaired endothelial function with decreased nitric oxide production is shared by insulin resistance and essential hypertension.16,17,19 In view of these physiologic interrelationships, it is pertinent to examine the combined effects of G894T polymorphism of the eNOS gene and insulin resistance status on BP levels.

As part of the Bogalusa Heart Study, a biracial (African American–white) community-based study of cardiovascular disease risk beginning in childhood,21 the present study examines 1) the African American–white difference in G894T allele frequency and 2) the independent effect of eNOS gene G894T genotype and the combined effect with insulin resistance status on BP levels and parental history of hypertension, a surrogate for familial risk, in young adults.

Methods

Study Population

Two cross-sectional surveys were conducted in 1988 to 1991 (n = 1930) and 1995 to 1996 (n = 1420) on young adults aged 18 to 38 years residing in the biracial (65% white, 35% African American) community of Bogalusa, LA. Of these young adults, 1021 genetically unrelated individuals (70.6% whites, 29.4% African Americans) aged 19 to 38 years who had eNOS G894T genotype data formed the study sample for this report. During the two cross-sectional surveys of young adults, parental history of hypertension was obtained by questionnaire. Information on their natural parents’ history of hypertension was collected. The positive parental history was defined as father or mother, or both, having had experience of hypertension, and the negative parental history as both father and mother never having had experience of hypertension.

All subjects in this study gave informed consent approved by the Institutional Review Board of the Tulane University Medical Center.

Examinations

All examinations followed the same basic protocols (procedures described elsewhere).22 Subjects were instructed to fast for 12 to 14 h before the screening, and compliance with fasting was determined by interview on the morning of the examination. Antecubital venous blood was collected to obtain serum and plasma. Replicate (six times) systolic (SBP) and diastolic (DBP) BP measurements were performed by two randomly assigned nurses on the right arm of subjects in a relaxed, sitting position using a mercury sphygmomanometer. The mean of all replicates was used in the analyses. Mean arterial pressure (MAP) was calculated as DBP + pulse pressure/3. Hypertension was defined as either SBP/DBP >140/90 mm Hg or taking antihypertensive medications. However, individuals (n = 49) taking antihypertensive medications were excluded from the calculation of mean BP levels. Height and weight were measured twice to ±0.1 cm and to ±0.1 kg, respectively, and the average values were used to calculate body mass index ([BMI] = weight in kilograms divided by the square of the height in meters).

Insulin and Glucose Analysis

A commercial radioimmunoassay kit was used for measuring plasma immunoreactive insulin (Padebas Pharmaceuticals, Piscataway, NJ). This insulin assay has 41% cross-reactivity with proinsulin, which is disproportionately low in nondiabetics, and <0.2% cross-reactivity with C-peptide. The detection limit of insulin level was 2.0 μU/mL. Plasma glucose was measured by an enzymatic method using the Beckman Instant Glucose Analyzer (Beckman Instruments, Palo Alto, CA). An approximate 10% random sample of individuals was selected daily to assess the reproducibility of laboratory analyses. Intraclass correlation coefficients, a measure of reproducibility of the entire process from blood collection to data processing, among the blind duplicate values ranged from 0.94 to 0.98 for insulin and 0.86 to 0.98 for glucose.

Insulin resistance status was assessed according to the homeostasis model assessment of insulin resistance (HOMA IR) formula described previously23: fasting insulin (in microunits per milliliter) × fasting glucose (in millimoles per liter)/22.5. This model is considered useful to assess insulin resistance in epidemiologic studies.24

Genotyping for the G894T Polymorphism

Genotyping of the eNOS G894T polymorphism was carried out using the Taqman assay. The sequences of the forward and reverse polymerase chain reaction (PCR) primers were 5′-CCCCACAGCTGCTTGAATCTA-3′ and 5′-CACCCAGTCAATCCCTTTGG-3′, respectively. Allele-specific fluorescent probes, labeled with different reporter dyes, are designed to hybridize to the target DNA in a sequence-specific manner. In this case, the two allele-specific probes were 6FAM-CCCCAGATGATCCCCCA-GAACTC and VIC-CCCCAGATGAGCCCCAGAAC. After PCR amplification, the increase in fluorescent intensity of the reporter dyes is detected by an end-point read using an ABI 7700 (Applied Biosystems, Inc, Foster City,
CA). Analysis of the fluorescent signals leads to an automated genotype determination. On the basis of the analysis of 67 pairs of blind duplicates, there was 100% concordance in G894T genotyping.

**Statistical Methods**

Statistical analyses were performed using the SAS software package (Cary, NC). Gene counting was used to estimate allele frequencies within each race. Race differences in the distribution of genotype and allele frequencies, and estimates of Hardy-Weinberg equilibrium were tested using contingency and goodness-of-fit $\chi^2$ tests, respectively. To examine the effect of the G894T polymorphism on BP and parental history of hypertension, the G894/T894 and T894/T894 genotypes were combined due to the small number of individuals homozygous for the T894 allele. Analysis of covariance (GLM) was performed to examine independent effects of the G894T polymorphism on BP and parental history of hypertension, the spectively. To examine the effect of the G894T polymorphism on BP levels between the two variables were included in the multiple linear regression model adjusting for race, sex, age, and BMI. Their independent effects were also essentially similar with respect to SBP and DBP (data not shown). Although the prevalence of hypertension in noncarriers relative to carriers was similar to the trend in BP levels, the difference was not statistically significant when adjusted for race in the total sample.

Insulin resistance status was defined as below and above the median HOMA IR. Subjects with high insulin resistance (above the median HOMA IR) showed significantly greater differences in BP levels between noncarriers and carriers of the T894 allele than those with low insulin resistance status, adjusting for race, sex, age, and BMI (Fig. 1). African Americans and whites separately showed similar trends in BP differences except for DBP in African Americans (data not shown). The differences in BP levels were also compared between the two groups stratified by median BMI, but no significant trends were seen (data not shown).

The association between the G894T polymorphism and familial predisposition to hypertension was examined in terms of prevalence of parental history of hypertension in noncarriers versus carriers of the T894 allele stratified by the median HOMA IR (Fig. 2). The prevalence of parental history of hypertension was similar in noncarriers (57.9%) versus carriers (55.9%) in the low HOMA IR group ($P = .95$, adjusted for race); whereas noncarriers showed a significantly higher prevalence of parental hypertension than carriers in the high HOMA IR group (70.5% v 51.3%, $P = .006$, adjusted for race). The total sample showed the same trend in noncarriers versus carriers (64.6% v 53.5%, $P = .059$, adjusted for race), but the difference was marginal. When the sample was stratified by median BMI, no significant trend in the association was seen (data not shown).

**Table 1.** Endothelial nitric oxide synthase G894T genotype and allele frequency in African Americans and whites

<table>
<thead>
<tr>
<th>Genotype (no. of subjects)*</th>
<th>White ($n = 721$)</th>
<th>African American ($n = 300$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G894/G894</td>
<td>325</td>
<td>241</td>
</tr>
<tr>
<td>G894/T894</td>
<td>325</td>
<td>55</td>
</tr>
<tr>
<td>T894/T894</td>
<td>71</td>
<td>4</td>
</tr>
<tr>
<td>Allele (relative frequency)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G894</td>
<td>0.676</td>
<td>0.895</td>
</tr>
<tr>
<td>T894</td>
<td>0.324</td>
<td>0.105</td>
</tr>
</tbody>
</table>

Race difference: * $P < .001$.  

| Levels of BP, HOMA IR (a surrogate for insulin resistance), and covariates in noncarriers versus carriers of the T894 allele are presented by race in Table 2. In comparisons of noncarriers and carriers, age, BMI, and HOMA IR were similar in both races and the total sample. Mean levels of SBP, DBP, and MAP differed significantly between noncarriers and carriers in whites and the total sample after adjusting for the covariates, with noncarriers showing higher BP levels. The relatively small sample of African Americans showed similar but nonsignificant trends with respect to SBP and MAP. In addition, HOMA IR ($b = 0.65$, $P = .0001$) and G894T genotype ($b = 1.52$, $P = .004$) showed independent effects on MAP when the two variables were included in the multiple linear regression model adjusting for race, sex, age, and BMI. Their independent effects were also essentially similar with respect to SBP and DBP (data not shown). Although the prevalence of hypertension in noncarriers relative to carriers was similar to the trend in BP levels, the difference was not statistically significant when adjusted for race in the total sample.

Insulin resistance status was defined as below and above the median HOMA IR. Subjects with high insulin resistance (above the median HOMA IR) showed significantly greater differences in BP levels between noncarriers and carriers of the T894 allele than those with low insulin resistance status, adjusting for race, sex, age, and BMI (Fig. 1). African Americans and whites separately showed similar trends in BP differences except for DBP in African Americans (data not shown). The differences in BP levels were also compared between the two groups stratified by median BMI, but no significant trends were seen (data not shown).

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As shown in Table 1, both genotype distribution and allele frequencies of the eNOS G894T polymorphism differed significantly between African Americans and whites, with whites showing a higher frequency of the T894 allele than African Americans (0.324 v 0.105, $P < .001$). The genotype distributions were in accordance with Hardy-Weinberg equilibrium expectations in African Americans and whites with $P = .93$ and $P = .85$, respectively.

| Levels of BP, HOMA IR (a surrogate for insulin resistance), and covariates in noncarriers versus carriers of the T894 allele are presented by race in Table 2. In comparisons of noncarriers and carriers, age, BMI, and HOMA IR were similar in both races and the total sample. Mean levels of SBP, DBP, and MAP differed significantly between noncarriers and carriers in whites and the total sample after adjusting for the covariates, with noncarriers showing higher BP levels. The relatively small sample of African Americans showed similar but nonsignificant trends with respect to SBP and MAP. In addition, HOMA IR ($b = 0.65$, $P = .0001$) and G894T genotype ($b = 1.52$, $P = .004$) showed independent effects on MAP when the two variables were included in the multiple linear regression model adjusting for race, sex, age, and BMI. Their independent effects were also essentially similar with respect to SBP and DBP (data not shown). Although the prevalence of hypertension in noncarriers relative to carriers was similar to the trend in BP levels, the difference was not statistically significant when adjusted for race in the total sample.

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Table 2.  Blood pressure levels (mean ± SD) by endothelial nitric oxide synthase (G894T) genotype and race

<table>
<thead>
<tr>
<th>T894 Allele</th>
<th>Noncarrier</th>
<th>Carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>325</td>
<td>396</td>
</tr>
<tr>
<td>Age (y)</td>
<td>29.2 ± 5.2</td>
<td>29.3 ± 4.8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.7 ± 6.0</td>
<td>26.1 ± 5.5</td>
</tr>
<tr>
<td>HOMA IR</td>
<td>2.5 ± 2.2</td>
<td>2.3 ± 1.6</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>110.3 ± 10.1</td>
<td>108.9 ± 9.9*</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>73.5 ± 8.3</td>
<td>72.1 ± 8.5††</td>
</tr>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td>85.8 ± 8.4</td>
<td>84.3 ± 8.4*‡</td>
</tr>
<tr>
<td>Hypertensives (%)</td>
<td>6.7</td>
<td>5.3</td>
</tr>
<tr>
<td>African Americans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>241</td>
<td>59</td>
</tr>
<tr>
<td>Age (y)</td>
<td>28.1 ± 5.2</td>
<td>29.1 ± 5.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.9 ± 8.1</td>
<td>29.8 ± 7.9</td>
</tr>
<tr>
<td>HOMA IR</td>
<td>3.1 ± 2.7</td>
<td>2.7 ± 1.6</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>113.7 ± 12.3</td>
<td>111.6 ± 10.0</td>
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<tr>
<td>Diastolic BP (mm Hg)</td>
<td>73.6 ± 9.5</td>
<td>73.7 ± 9.6</td>
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<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td>87.0 ± 9.7</td>
<td>86.4 ± 9.0</td>
</tr>
<tr>
<td>Hypertensives (%)</td>
<td>16.4</td>
<td>14.5</td>
</tr>
<tr>
<td>Total</td>
<td>566</td>
<td>455</td>
</tr>
<tr>
<td>Age (y)</td>
<td>28.8 ± 5.2</td>
<td>29.3 ± 4.9</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>27.6 ± 7.1</td>
<td>26.6 ± 6.0</td>
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<tr>
<td>HOMA IR</td>
<td>2.7 ± 2.4</td>
<td>2.3 ± 1.6</td>
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<tr>
<td>Systolic BP (mm Hg)</td>
<td>111.7 ± 11.2</td>
<td>109.2 ± 10.0*‡</td>
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<tr>
<td>Diastolic BP (mm Hg)</td>
<td>73.6 ± 8.8</td>
<td>72.3 ± 8.6*‡</td>
</tr>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td>86.3 ± 8.9</td>
<td>84.6 ± 8.5*‡</td>
</tr>
<tr>
<td>Hypertensives (%)</td>
<td>10.9</td>
<td>6.6</td>
</tr>
</tbody>
</table>

BMI = body mass index (kg/m²); HOMA IR = homeostasis model assessment of insulin resistance; BP = blood pressure.

Noncarriers versus carriers: *P < .05; †P < .01 (adjusted for age, sex and/or race); ‡P < .05 (adjusted for age, sex, BMI, or race).

Discussion

The present study demonstrates that the relative allele frequency of the G894T polymorphism of the eNOS gene differs between whites and African Americans. The G894T polymorphism is associated with BP levels and the prevalence of parental history of hypertension in young adults. In addition, the current study shows for the first time that this association differs by insulin resistance status.

The frequency of the T894 allele among whites in this study (0.324) was similar to that found in 68 French patients with coronary heart disease (0.316). but lower than that found in 123 French normotensive subjects (0.443). The T894 allele frequency in the current study is noteworthy in that the data are derived from a community-based sample without any selection based on cardiovascular events or risk factor levels. In the current study, African Americans compared to whites showed considerably lower frequency (0.105 v 0.324) of the T894 allele. A lower frequency (0.05 to 0.09) of the T894 allele has been reported in Japanese populations, but no corresponding African American–white data are available for comparison. Comparative studies on other African American–white populations are needed to confirm the present findings.

The relationship between nitric oxide production and BP regulation has been well established. In the present study, BP levels differed significantly between noncarriers and carriers of the T894 allele, with the noncarriers having higher BP levels than the carriers, especially in whites. To the best of our knowledge, there have been only three previous studies on this subject with contradictory results.

The current findings are in agreement with an earlier study conducted in a French population where patients with hypertension showed significantly lower frequency of the T894 allele than normotensives. In contrast, a significantly higher frequency of the T894 allele has been found in hypertensives than in normotensives in Japanese subjects. To solve these contradictory claims, an extensive association study has been conducted in a Japanese population using multiple control groups selected from a hospital, a company, and a university, and no association of the G894T polymorphism has been found with either hypertension status or BP levels. The discrepancies in the effect of the eNOS G894T polymorphism are possibly due to confounding effects of race, sex, age, and obesity on BP. Current data suggest that insulin resistance status may modify the association between G894T genotype and BP levels (discussed below).

Both insulin resistance and nitric oxide production have
been shown to be independently associated with hypertension, and these two physiologic correlates of BP are closely interrelated. There is increasing evidence that nitric oxide synthesis and the vasodilating properties of insulin are impaired in insulin-resistant states such as obesity, type 2 diabetes, and hypertension. In the current study, insulin resistance status, measured by HOMA IR, and the eNOS G894T polymorphism showed significant independent effects on BP levels. Although the eNOS G894T genotype was not related to insulin resistance status, the two factors jointly contributed to BP levels. The eNOS G894T polymorphism effect on BP was significantly stronger in individuals with high insulin resistance (Fig. 1). Because the cause–effect relationship between insulin resistance and nitric oxide production is not clear and little is known about the combined effect of insulin resistance status and G894T polymorphism on BP levels, the findings in the current study can be biologically explained either way. Studies have suggested that insulin stimulates the production of nitric oxide in endothelial cells. Insulin has been shown to increase the production of nitric oxide in cultured endothelial cells within a few minutes, indicating an activation of nitric oxide synthase by the insulin receptor. A recent study found that insulin enhances the eNOS gene expression, mediated by the activation of phosphatidylinositol-3 kinase. Taken together, there seems to be more evidence that the eNOS gene effect on the synthesis of nitric oxide in endothelial cells is dependent on insulin concentrations. The current observational data do not allow us to explore the physio-

**FIG. 1.** Difference in blood pressure levels between noncarriers and carriers of T894 allele by insulin resistance status in young adults. HOMA Insulin Resistance = homeostasis model assessment of insulin resistance; BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure; MAP = mean arterial pressure.

**FIG. 2.** The association between parental hypertension and the eNOS gene G894T polymorphism by insulin resistance status. eNOS = endothelial nitric oxide synthase; other abbreviation as in Fig. 1.
logic mechanism in the relationship between insulin resistance and the eNOS G894T genotype.

In the French population aged 18 to 74 years, the eNOS G894T polymorphism has been found to be associated with hypertension status but not with BP levels in patients having lower frequency of T894 allele.\(^\text{11}\) In our study of young adults aged 19 to 38 years, the eNOS G894T polymorphism was not associated with the prevalence of hypertension, but showed an effect on BP levels. In addition to other possible confounding variables, the relatively younger age of our study population may be an important factor responsible for the lack of association between this polymorphism and hypertension defined by clinical criteria. The difference in the prevalence of hypertension between the two generations (37.8% in parents vs. 9.0% in offspring) in our study indicated that individuals in this study, although genetically susceptible, were still relatively young for expressing clinical hypertension.

It is of interest that the eNOS G894T polymorphism in conjunction with insulin resistance is significantly associated with the prevalence of parental history of hypertension. This finding is consistent with the combined effects of eNOS gene and insulin resistance on offspring’s BP levels noted in this study. We have used parental history of hypertension as an indicator of risk in the offspring because of the familial nature of hypertension.\(^\text{32}\) Validation data from the Family Heart Study reveal that the accuracy of a reported family history of hypertension is relatively lower compared with family histories of diabetes and heart attack.\(^\text{33}\) It should be noted, however, that random misclassification of self-reported history would underestimate the true differences between groups. The results of the present study suggest that G894T polymorphism in conjunction with insulin resistance may be valuable in the assessment of hypertension risk in early life.

Nitric oxide production is genetically influenced, with 30% of the phenotypic variance due to genes.\(^\text{34}\) Very little information is available on the influence of G894T genotype on eNOS production and activity. However, the G894T genotype has been found in one study to have no appreciable effect on the enzyme activity of the eNOS,\(^\text{35}\) which does not preclude the increases in the production rate. It is also likely that this polymorphism is in linkage disequilibrium with yet unknown eNOS gene variants or some other causal loci influencing the predisposition to hypertension.

In the genetic association studies, multiple comparisons raise the issue of chance or false-positive results. However, the stratification by insulin resistance status in this study has a sound physiologic rationale in the observed relationships among BP, insulin resistance, and nitric oxide production. The stratification analyses by insulin resistance status were based on a priori hypothesis from previous reports in this aspect. Accordingly, our data showed consistent trends in differences in BP levels and the prevalence of parental hypertension by genotype and insulin resistance status in African Americans, whites, and in the total sample. Therefore, the observed association is not likely due to chance.

In conclusion, allele frequency of G894T polymorphism of the eNOS gene differed between African Americans and whites. The eNOS G894T polymorphism showed an independent association with BP levels and parental hypertension, and this association differs by insulin resistance status. These results suggest that the allelic variation (G894T) in the eNOS gene locus in conjunction with insulin resistance may be one factor contributing to the predisposition to hypertension.

Acknowledgment

The Bogalusa Heart Study is a joint effort of many investigators and staff members whose contributions are gratefully acknowledged. We especially thank participants.

References