Endothelial NO Synthase Genotype and Risk of Preeclampsia

A Multicenter Case-Control Study

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Abstract—Polymorphisms in the endothelial NO synthase (eNOS) gene have been evaluated as risk factors for preeclampsia. However, data from small studies are conflicting. We assessed whether eNOS genotypes alter the risk of preeclampsia in a population in which the incidence of this disorder is high. A total of 844 young pregnant women (322 preeclamptic and 522 controls) were recruited from 5 cities. Genotyping for the Glu298Asp, intron-4 and –786T→C polymorphisms in the eNOS gene was conducted. Multivariate odds ratios (ORs) were obtained to estimate the association of individual polymorphisms and haplotypes with preeclampsia risk. No increase in the risk of preeclampsia for the intron-4 or –786T→C polymorphisms was observed under any model of inheritance. In contrast, in women homozygous for the Asp298 allele, the adjusted OR for preeclampsia was 4.60 (95% confidence interval [CI], 1.73 to 12.22) compared with carriers of the Glu298 allele. After a multivariate analysis, carriage of the "Asp298–786C-4b" haplotype was also associated with increased risk of preeclampsia (OR, 2.11 [95% CI, 1.33 to 3.34]) compared with carriers of the "Glu298–786T-4b" haplotype. The eNOS Glu298Asp polymorphism and the Asp298–786C-4b haplotype are risk factors for preeclampsia. **(*Hypertension*. 2004;44:702-707.)**

Key Words: preeclampsia ■ nitric oxide synthase ■ polymorphism ■ haplotypes ■ case-control studies

Preeclampsia, a major cause of maternal and neonatal morbidity and mortality, affects 5% to 7% of pregnancies in the Western world but can have up to 3-fold greater incidence in other geographic areas with different ethnic or social characteristics.[1] In Colombia, 42% of maternal deaths are attributed to this disorder, which is also the major reason for premature delivery.[2]

Systemic arteriolar vasodilatation, probably dependent on endothelial NO,[3,4] is responsible for the hemodynamics of the first half of the pregnancy (increased blood volume and cardiac output and decreased blood pressure).[5,6] Deficiencies in the vasodilatory, antithrombotic, and atheroprotective effects of NO[7,8] have been implicated in the pathogenesis of cardiovascular disease, for which preeclampsia is also a risk factor.[9,10] Therefore, the gene that encodes endothelial NO synthase (eNOS), the enzyme that regulates endothelial NO availability, is a candidate gene for preeclampsia.[11]

A single nucleotide polymorphism in exon 7 (G894T), which encodes an amino acid substitution (Glu298Asp), and a variable number of tandem repeats in intron-4 have been evaluated in preeclampsia.[12–16] However, results have been inconsistent, possibly because of low incidence of the disease, low prevalence of the gene variants in the studied populations, and relatively small study sizes. Furthermore, it is not clear whether genotypic risks reported for Glu298Asp and intron-4 a/b polymorphisms are independent or reflect carriage of common risk haplotypes. The present study assessed the independent contribution of the Glu298Asp, intron-4, and –786T→C polymorphisms and also that of eNOS haplotypes to the risk of preeclampsia in a population with high incidence of this disorder.

Methods

Subjects

A case-control study was performed in 844 unrelated young pregnant women recruited from 5 Colombian cities from January 2000 to November 2003. At the time of admission for labor and delivery, a verbal interview was conducted to ascertain maternal age, gestational

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The University College London holds a patent related to ADMA (asymmetrical dimethylarginine), an endogenous NO synthase inhibitor.

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TABLE 1. Maternal and Neonatal Characteristics of the Sample Studied

Variable	Cases (n=322)	Controls (n=522)	*P* Value
Age (years)	19.2±2.9	18.9±2.6	0.279
Systolic blood pressure (mm Hg)	147.1±12.8	111.4±9.3	<0.001
Diastolic blood pressure (mm Hg)	95.8±8.0	68.4±7.5	<0.001
Low SES	261/300 (87.0)	445/497 (89.5)	0.275
Ethnic background (%)*			
Hispanic–White	39 (12.2)	74 (14.3)	
African–Caribbean	68 (21.3)	119 (23.0)	
Other mixture	213 (66.5)	324 (62.7)	0.495
Current smoking	8/317 (2.5)	21/522 (4.0)	0.249
History of UTIs or vaginal infections†	159/316 (50.3)	247/520 (47.5)	0.430
Maternal history of PE	45/322 (14.0)	32/522 (6.1)	<0.001
History of sister with PE	25/322 (7.8)	20/522 (3.8)	0.014
Gestational age at delivery (weeks)	36.4±4.0	39.1±1.3	<0.001
Multiple pregnancy	6/322 (1.9)	2/512 (0.4)	0.040
Newborn weight (g)	2526±769	3110±453	<0.001
Newborn height (cm)	46.4±5.1	49.6±2.4	<0.001
Low Apgar (<7) at first minute	49/321 (15.3)	45/510 (8.8)	<0.001

Data are present as n (%) or mean±SD.
SES indicates socioeconomic status; UTI, urinary tract infections; PE, preeclampsia.
*In 2 cases and 5 controls, there was no information regarding ethnic background.
†Only those infections reported during the current pregnancy were evaluated (urinary and vaginal).

age, parity, smoking status, family history of preeclampsia, ethnic background, and socioeconomic status. Blood pressure was measured in the right arm after a 5-minute period of rest according to recommendations of the American Heart Association.[17]

A total of 322 preeclamptic patients and 522 healthy pregnant women were included. A case was a primigravid woman <26 years old with a blood pressure of ≥140/90 mm Hg and proteinuria ≥0.3 g in 24 hours, or ≥2+ reading on dipstick in a random urine determination with no evidence of urinary tract infection after 20 weeks of gestation.[18] A control was defined as a primigravid woman <26 years of age without preeclampsia and in labor after 37 weeks of pregnancy. Patients with a previous autoimmune, metabolic, renal, or cardiac diseases including hypertension were excluded. All participants signed the informed consent document approved by the ethics committee from the Universidad Autónoma de Bucaramanga.

DNA Extraction and Genotyping

Blood was drawn from the antecubital vein into EDTA and samples stored at −50°C. DNA was extracted by means of the QIAamp DNA blood minikit (Qiagen). Polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis was used for genotyping Glu298Asp and –786T→C polymorphisms, whereas genotypes for intron-4 polymorphism were determined by PCR.[19,20] Details are outlined in additional material (available online at http://hypertensionaha.org). Genotyping was conducted in a blinded fashion. A total of 10% of samples were subject to repeat PCR and genotyping, and no discrepancies were detected.

Statistical Analysis

Means, proportions, and SDs (±) were used for descriptive purposes. To evaluate differences between groups, unpaired Student *t*, χ^2, or Mann–Whitney tests were used as appropriate. Tests for Hardy–Weinberg equilibrium were performed by χ^2 analysis, and linkage disequilibrium coefficients between polymorphisms were estimated by log-linear analysis among control subjects.[21] As an a priori hypothesis, the association between eNOS polymorphisms and preeclampsia was evaluated under a recessive model of inheritance based on previous results from the eNOS genotype on ischemic heart disease risk.[22] Codominant and dominant models were also evaluated. Multivariate analysis using logistic regression methods and goodness-of-fit analysis were also conducted.[23,24] All statistical analyses were conducted using SPSS software version 11.0 (SPSS). To construct the haplotypes and test their association with preeclampsia, THESIAS software (version 2.0) was used. This software used the Stochastic-EM (expectation maximization) algorithm to infer haplotype, as has been described previously.[25] For each polymorphism and haplotype, the odds ratio (OR), *P* value, and 95% confidence interval (CI) were obtained. A *P* value <0.05 was considered significant.

Results

Demographic Characteristics of Study Groups

There were no significant differences in age, smoking status, ethnic background, and socioeconomic status between cases and controls (Table 1). As expected, systolic and diastolic blood pressure was significantly higher in the preeclamptic women ($P<0.001$). Other maternofetal characteristics are outlined in Table 1.

eNOS Polymorphisms and Haplotypes

Allele Frequencies and Linkage Disequilibrium of eNOS Polymorphisms

For the control group, genotype frequencies were as predicted by Hardy–Weinberg equilibrium for Glu298Asp ($P=0.86$) and intron-4 ($P=0.38$) but not –786T→C ($P=0.02$). For cases, the intron-4 polymorphism was in Hardy–Weinberg equilibrium ($P=0.1$), but the Glu298Asp ($P<0.001$) and –786T→C ($P=0.003$) polymorphisms were not. Pairwise linkage disequilibrium coefficients (Δ) were calculated for the 3 polymorphisms studied. All comparisons were statistically significant ($P<0.05$). However, allelic associations between the Glu298Asp and –786T→C and intron-4 variants were weak (Δ=0.19

TABLE 2. Genotype and Allele Frequencies for eNOS Polymorphisms Evaluated in the Study According to Ethnic Background

	Ethnic Background							
	All Ethnic Groups*		Hispanic–White		African–Caribbean		Other†	
Gene Variant	Cases (n=322)	Controls (n=522)	Cases (n=39)	Controls (n=74)	Cases (n=68)	Controls (n=119)	Cases (n=213)	Controls (n=324)
Glu298Asp-genotype								
Glu/Glu	217 (67.4%)	403 (77.2%)	23 (59.0%)	62 (83.8%)	57 (83.8%)	92 (77.3%)	135 (63.4%)	244 (75.3%)
Glu/Asp	84 (26.1%)	113 (21.6%)	13 (33.3%)	11 (14.9%)	9 (13.2%)	27 (22.7%)	62 (29.1%)	75 (23.1%)
Asp/Asp	21 (6.5%)	6 (1.1%)	3 (7.7%)	1 (1.4%)	2 (2.9%)	0 (0%)	16 (7.5%)	5 (1.5%)
Allele frequency								
Glu	518 (80.4%)	919 (88.0%)	59 (75.6%)	135 (91.2%)	123 (90.4%)	211 (88.7%)	332 (77.9%)	563 (86.9%)
Asp	126 (19.6%)	125 (12.0%)	19 (24.4%)	13 (8.8%)	13 (9.6%)	27 (11.3%)	94 (22.1%)	85 (13.1%)
Intron-4 genotype								
b/b	253 (78.6%)	393 (75.3%)	31 (79.5%)	49 (66.2%)	42 (61.8%)	83 (69.7%)	179 (84.0%)	256 (79.0%)
b/a	51 (15.8%)	103 (19.7%)	4 (10.3%)	18 (24.3%)	21 (30.9%)	26 (21.8%)	25 (11.7%)	59 (18.2%)
b/c	4 (1.2%)	12 (2.3%)	1 (2.6%)	4 (5.4%)	0 (0%)	6 (5.0%)	3 (1.4%)	2 (0.6%)
a/a	13 (4.0%)	12 (2.3%)	3 (7.7%)	3 (4.1%)	4 (5.9%)	4 (3.4%)	6 (2.8%)	5 (1.5%)
a/c	0 (0%)	2 (0.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (0.6%)
c/c	1 (0.3%)	0 (0%)	0 (0%)	0 (0%)	1 (1.5%)	0 (0%)	0 (0%)	0 (0%)
Allele frequency								
a	77 (12.0%)	129 (12.4%)	10 (12.8%)	24 (16.2%)	29 (21.3%)	34 (14.3%)	37 (8.7%)	71 (11.0%)
b	561 (87.1%)	901 (86.3%)	67 (85.9%)	120 (81.1%)	105 (77.2%)	198 (83.2%)	386 (90.6%)	573 (88.4%)
c	6 (0.9%)	14 (1.3%)	1 (1.3%)	4 (2.7%)	2 (1.5%)	6 (2.5%)	3 (0.7%)	4 (0.6%)
–786T→C-genotype								
TT	167 (51.9%)	303 (58.0%)	18 (46.2%)	41 (55.4%)	51 (75.0%)	87 (73.1%)	97 (45.5%)	170 (52.5%)
TC	146 (45.3%)	208 (39.8%)	21 (53.8%)	31 (41.9%)	17 (25.0%)	31 (26.1%)	107 (50.2%)	146 (45.1%)
CC	9 (2.8%)	11 (2.1%)	0 (0%)	2 (2.7%)	0 (0%)	1 (0.8%)	9 (4.2%)	8 (2.5%)
Allele frequency								
T	480 (74.5%)	814 (78.0%)	57 (73.1%)	113 (76.4%)	119 (87.5%)	205 (86.1%)	301 (70.7%)	486 (75.0%)
C	164 (25.5%)	230 (22.0%)	21 (26.9%)	35 (23.6%)	17 (12.5%)	33 (13.9%)	125 (29.3%)	162 (25.0%)

*In 2 cases and 5 controls, there was no information regarding ethnic background.

†Other category is composed of individuals of mixed percentage: White–indigenous, White–African-Caribbean, and indigenous–African-Caribbean.

[$P<0.001$] and $\Delta=0.11$ [$P=0.013$], respectively). Similar results were obtained for the association between the intron-4 and the –786T→C variant ($\Delta=0.13$ [$P=0.002$]).

Association Between Genotype and Risk of Preeclampsia

The genotype distribution of the Glu298Asp polymorphism differed significantly among preeclamptic and normotensive women (Table 2). In a univariate analysis under a recessive model of inheritance, women homozygous for the Asp298 allele were 6.0× (95% CI, 2.30 to 18.34; $P<0.001$) more likely to develop preeclampsia compared with carriers for the Glu298 allele. After adjusting for possible confounding variables (ethnic origin, site of recruitment, age, smoking status, history of urinary tract or vaginal infections, and socioeconomic status) using multiple regression analyses, the association between homozygosity for Asp298 allele remained significant (OR, 4.6 [95% CI: 1.73 to 12.22]; $P=0.002$; Table 3). When a codominant model was evaluated, only the Asp/Asp versus Glu/Glu comparison was significant (OR, 5.24 [95% CI, 1.68 to 12.16]; $P=0.003$), whereas heterozygosity (Glu/Asp versus Glu/Glu) was not associated with an increase in risk of preeclampsia (OR, 1.31 [95% CI, 0.91 to 1.87]; $P=0.14$).

Under a recessive model of inheritance, no significant differences among cases and controls were observed for the intron-4 (OR, 1.93 [95% CI: 0.82 to 4.57]; $P=0.101$) and –786T→C (OR, 1.34 [95% CI, 0.50 to 3.51]; $P=0.524$) polymorphisms. Similar results were obtained under a codominant model of inheritance for those polymorphisms (Table 3).

After constructing the haplotypes and estimating their frequencies in cases and controls, there was a global significant difference in the haplotype frequency in cases compared with controls (adjusted P value=0.01; Table 4). Analysis of individual haplotypes adjusting for possible confounding variables revealed that with reference to the common Glu298–786T-4b, the rarer Asp298–786C-4b haplotype was associated with an increased risk of preeclampsia (OR, 2.11 [95% CI: 1.33 to 3.34]; $P=0.001$). No other haplotypes were associated with an increase in risk (Table 4).

Discussion

The main finding of this study was that young Colombian women homozygous for the Asp298 allele were at increased

TABLE 3. Estimate of the Effects of the eNOS Polymorphisms on Preeclampsia Risk Modeled With Logistic Regression

Polymorphism	Unadjusted OR (95% CI)	*P*	Adjusted OR* (95% CI)	*P*
Recessive model				
Glu298Asp				
Asp/Asp vs (Glu/Asp+Glu/Glu)	6.00 (2.30–18.34)	<0.001	4.60 (1.73–12.22)	0.002
Intron-4				
a/a vs (b/a+b/b+b/c+c/c)	1.93 (0.82–4.57)	0.218	2.14 (0.90–5.10)	0.086
–786T→C				
C/C vs (C/T+T/T)	1.34 (0.50–3.51)	0.536	0.80 (0.30–2.17)	0.664
Codominant model				
Glu298Asp				
Asp/Asp vs Glu/Glu	6.50 (2.48–19.93)	<0.001	5.24 (1.68–12.16)	0.003
Glu/Asp vs Glu/Glu	1.38 (0.98–1.94)	0.053	1.31 (0.91–1.87)	0.146
Intron-4				
a/a vs b/b	1.68 (0.71–4.01)	0.198	2.09 (0.86–5.05)	0.102
a/b vs b/b	0.77 (0.52–1.13)	0.165	0.77 (0.51–1.15)	0.201
b/c vs b/b	0.52 (0.12–1.73)	0.251	0.51 (0.16–1.69)	0.272
a/c vs b/b	0	0.371	NA	(0–8.31)
c/c vs b/b	NA		0.393	NA
–786T→C				
C/C vs T/T	1.48 (0.55–3.95)	0.388	1.13 (0.54–4.18)	0.819
C/T vs T/T	1.27 (0.97–1.73)	0.095	1.50 (0.64–4.29)	0.434

*Adjusted for ethnic background, site of recruitment, multiple pregnancy, maternal age, socioeconomic status, smoking, and urinary or vaginal infections during pregnancy. In the comparisons of the eNOS genotype, the reference value was set to 1.0.

risk of preeclampsia (OR, 4.60 [95% CI, 1.73 to 12.12]; $P=0.002$; after adjustment for other risk factors). This increase in risk may reflect the presence of a high-risk Asp298–786C-4b haplotype, which was associated with an OR for preeclampsia of 2.11 (95% CI, 1.33 to 3.34; $P=0.001$) compared with the common Glu298–786T-4b haplotype, after adjustment for potential confounders.

It has been clearly demonstrated that during normal pregnancy, the NO pathway is activated, leading to an increased NO availability.[26] This increase in NO availability is thought to be responsible for maternal vasodilation required to accommodate the increased circulating volume during pregnancy without a rise in blood pressure. In preeclampsia, this adaptation fails, endothelial dysfunction occurs,[27] blood pressure rises, and proteinuria develops. Moreover, maternal endothelial dysfunction persists after an episode of preeclampsia.[28] This is of interest because endothelial dysfunction is a key feature of a number of cardiovascular disorders, and preeclampsia itself is a risk factor for future cardiovascular disease, with women who experience preeclampsia exhibiting an up to 2-fold excess risk of cardiovascular disease in later life.[29]

A likely mechanism by which eNOS Asp298 might reduce NO bioavailability has also been reported. Two recent studies have shown that eNOS Asp298 is subject to selective proteolytic cleavage in endothelial cells and vascular tissues, and this could account for reduced vascular NO generation in subjects homozygous for this variant,[30,31] although these findings have been debated.[32] The findings from molecular studies have received some support from physiological stud-

TABLE 4. Frequency of the eNOS Haplotypes and Risk of Preeclampsia

Glu298Asp	–786T→C	Intron-4	Frequency in Controls*	Frequency in Cases	Unadjusted OR (95% CI)	*P* Value	Adjusted† OR (95% CI)	*P* Value
Glu	T	b	0.624	0.552	Reference	—	Reference	—
Glu	T	a	0.087	0.091	1.16 (0.80–1.67)	0.42	1.11 (0.75–1.65)	0.57
Glu	C	b	0.120	0.123	1.20 (0.83–1.75)	0.32	1.21 (0.82–1.79)	0.31
Glu	C	a	0.047	0.037	0.90 (0.50–1.60)	0.72	0.89 (0.48–1.62)	0.71
Asp	T	b	0.067	0.101	1.60 (1.07–2.40)	0.02	1.37 (0.89–2.11)	0.14
Asp	C	b	0.051	0.093	2.07 (1.33–3.23)	0.001	2.11 (1.33–3.34)	0.001

*Haplotypes with a frequency of <5% in the control population were not listed.

†OR adusted by age, ethnic background, recruitment place, smoking, and urinary or vaginal infections during pregnancy.

ies in vivo. We demonstrated that healthy pregnant women who carried the common Glu298Asp polymorphism in eNOS gene had reduced flow-mediated dilatation of the brachial artery, an NO-dependent response.[33] More recently, we showed that an impairment in the endothelial function is an early feature of women who subsequently developed preeclampsia.[27] Thus, these findings suggest that women homozygous for the Asp298 allele generate low NO in vivo and may be more susceptible to endothelial dysfunction. This might account for the increased risk of preeclampsia observed in the present study. Associations between the intron-4 variant and differences in NO pathway activity have also been described,[34] but the data are conflicting.[35] Because this variant is intronic, it is unlikely to be functional in its own right. A functional effect for the –786T→C promoter polymorphism has also been proposed from in vitro reporter gene assays, with promoters carrying the –786C allele having a significantly reduced luciferase reporter activity compared with promoters carrying the –786T allele; and recently, the –786T→C variant has been associated with reduced placental eNOS mRNA levels.[36,37] Additionally, lower serum nitrite/nitrate levels have been found in individuals with the –786C variant in some[36] but not all studies.[35] Data from the current study suggest that there may be a risk haplotype (Asp298–786C-4b) that confers the increase in risk, but this finding will require confirmation in larger studies because the power was limited as a result of the very low frequency for some of the individual haplotypes.

One important limitation of the current study relates to the ethnic mix of the population evaluated (white, African–Caribbean, and mixed population). This raises the issue of whether the positive association observed with eNOS reflects confounding by ethnicity attributable to population stratification.[38] We tested this possibility in a variety of ways. First, no significant differences in ethnic background were observed among cases and controls. Second, there was no significant difference in frequencies of the Asp/Asp genotype among the control samples from different ethnic groups. Third, the increased risk of preeclampsia among women homozygous for the Asp298 allele was unaffected by adjustment for ethnicity or for geographic location after multivariate analysis. Additionally, in recent studies, bias from population stratification was quantified, and it was concluded that its impact is likely to be small and decrease as the number of ethnic strata increase, as was the case in the present study.[39,40] However, in future studies, residual confounding by ethnicity could be further evaluated by typing highly polymorphic nonfunctional genetic markers, the allele frequencies of which differ by ethnic group.[38]

With the exception of the special case of population stratification described, residual confounding by other risk factors for preeclampsia is not anticipated because genotype for an allele is assigned randomly at conception according to Mendel's second law, and other preeclampsia risk factors (such as maternal obesity) should be distributed equally between carriers and noncarriers of the allele in a manner analogous to situation in the treatment and placebo arms of a clinical trial.[41]

In conclusion, our study suggests that young Colombian women homozygous for the Asp298 allele are at increased risk of developing preeclampsia, but very large studies or meta-analysis will be required to confirm these findings and refine estimates of the effect size.

Perspectives

Preeclampsia has a partial genetic basis, but the genes involved are unresolved. Because of the low sibling recurrence risk described for preeclampsia, several candidate genes with small to moderate effect is the more likely model to explain such genetic susceptibility.[42] Studies with very large sample sizes evaluating gene variants with potentially functional effects, as well as haplotype analysis, would be the ideal scenario to confirm or exclude candidate genes. Unless genetic effects are large, the utility of genetic association studies is likely to be the identification of disease mechanism rather than new predictive tools.[41] The current study supports the hypothesis that endothelial dysfunction attributable to decreased NO bioavailability plays a role in the pathogenesis of preeclampsia.[27,33]

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