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Glu298Asp Endothelial Nitric Oxide Synthase Polymorphism Is a Risk Factor for Erectile Dysfunction in the Mexican Mestizo Population

HAYDEE ROSAS-VARGAS,* RAMON M. CORAL-VAZQUEZ,* ROSARIO TAPIA,† JOSE L. BORJA,* RICARDO A. SALAS,† AND FABIO SALAMANCA*

*From the *Unidad de Investigacion Medica en Genetica Humana, Hospital de Pediatria; and †Sección de Andrología, Hospital de Especialidades, Centro Medico Nacional Siglo XXI-IMSS, Mexico, D.F., Mexico.*

ABSTRACT: Penile erection depends on the balanced action between antagonist vasoactive molecules such as nitric oxide (NO) and angiotensin. Endothelial nitric oxide synthase (eNOS) and angiotensin-converting enzyme (ACE) polymorphisms have been associated with endothelial dysfunction, which is described as a cause of erectile dysfunction (ED). Endothelial NOS and ACE are both regulators of vascular and corporal smooth muscle tone, which are connected by interaction between the NO-cyclic guanosine monophosphate pathway and the renin-angiotensin system. We analyzed the frequencies of 894 G/T (Glu298Asp) eNOS and ACE I/D polymorphisms in Mexican patients with ED (n = 53) and in an age-matched control group (n = 62). The populations analyzed were in Hardy Weinberg equilibrium. We found significant differences in allelic (χ^2 = 4.42; P = .03) and genotypic frequencies (χ^2 = 3.96; P = .04) between patients and controls for the 894 G/T eNOS polymorphism. Presence of the 894T allele in carriers increased the risk of ED (odds ratio [TT + GT versus GG] = 2.37; 95% confidence interval, 1.08 to 5.21; P = .02). Multiple logistic regression analysis showed that the Glu298Asp polymorphism was an independent factor for ED, as was diabetes mellitus, hypertension, cardiac disease, and cigarette smoking. No association was found between ACE I/D polymorphism and ED in the population studied. Therefore, our results suggest that Glu298Asp eNOS polymorphism plays a role as a genetic susceptibility factor for ED.

Key words: Angiotensin-converting enzyme, endothelial dysfunction, impotence.

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According to their etiology, causes of erectile dysfunction (ED) can be broadly classified as organic, psychogenic, or mixed. Among organic ED cases, 40% involve vascular problems derived from arterial flow disorders, or impaired veno-occlusion, which is commonly a consequence of poor relaxation of corporal smooth muscle (Miller 2000; Shabsigh and Anastasiadis, 2003). Nitric oxide (NO) is an important vasoactive molecule and neurotransmitter essential for relaxation of smooth muscle cells of the corpus cavernosum (Rajfer et al, 1992). NO is produced by a group of enzymes termed nitric oxide synthases (NOSs) that use L-arginine as their substrate. Three major NOS isoforms are recognized: inducible NOS (iNOS); neuronal NOS (nNOS), and endothelial NOS (eNOS) (Alderton et al, 2001), each one coded by a separate gene. Endothelium-derived NO plays an important role in the regulation of vascular tone, besides its capacity to inhibit leukocyte adhesion to vessel walls and to block platelet adhesion. Several polymorphisms in the eNOS gene have been described, some associated with development of cardiovascular diseases (ie, a 27-bp repeat in intron 4), and as a smoking-dependent risk for coronary artery disease (Wang et al, 1996); and a T-786C variant in the promoter region of the eNOS gene that reduces transcription of the gene is strongly associated with coronary spastic angina and myocardial infarction (Nakayama et al, 1997). In particular, 894 G/T polymorphism, located at exon 7, has been determined as a risk factor for coronary artery disease (Yoshimura et al, 1998, Hingorani et al, 1999, Liao et al, 1999), essential hypertension (Shoji et al, 2000; Jachymova et al, 2001), and myocardial infarction (Shimasaki et al, 1998). This single base substitution results in replacement of an Asp residue instead of Glu at position 298 (Glu298Asp) of mature eNOS. An association between the Glu298Asp eNOS polymorphism and an increased protein lability has been observed in acidic conditions ex vivo (Tesauro et al, 2000; Fairchild et al, 2001); however, the functional significance of this polymorphism in vivo is still not clear.

Angiotensin II (Ang II), the primary effector of the renin-angiotensin system (Unkelbach et al, 1998; Roks et al, 1999), is a multifunctional hormone that plays an important role in vascular function. NO and Ang II are con-

Correspondence to: Haydee Rosas-Vargas, Unidad de Investigacion Medica en Genetica Humana, Hospital de Pediatria, Centro Medico Nacional Siglo XXI-IMSS, Av Cuauhtemoc No 330, Col Doctores, Delegacion Cuauhtemoc. 06725 Mexico, D.F., Mexico (e-mail: hayrov@hotmail.com).

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nected by interaction between the NO-cyclic guanosine monophosphate (cGMP) pathway and the renin-angiotensin system, and they participate in the regulation of vascular and corporal smooth muscle tone; however, whereas NO functions as a vasodilator, Ang II plays a role as a vasoconstrictor (Bloch et al, 1998; Andersson, 2001; Yan et al, 2003). Moreover, there is evidence of participation of Ang II in contraction of human cavernosum smooth muscle during detumescence (Becker et al, 2001). Ang II is obtained from its precursor, Ang I, by catalytic reaction of angiotensin-converting enzyme (ACE) (Turner and Hooper 2002). The ACE gene contains a polymorphism in intron 16 that consists of the presence (insertion [I]) or absence (deletion [D]) of a 287-bp region. Codominant association of the I/D polymorphism has been reported with interindividual variability in plasma ACE concentration. ACE plasma activity is duplicated in persons with the DD genotype in contrast to Ang II, whereas the DI genotype is related to intermediate activity (Rigat et al, 1990, 1992). Although the ACE I/D polymorphism does not induce any direct change in the enzyme structure or activity, the ACE gene acts as a quantitative trait locus that modulates ACE levels, and the ACE I/D polymorphism is a marker that is in linkage disequilibrium with functional variants located in the ACE gene (Villard and Soubrier, 1996). In consequence, it is not surprising that the ACE DD genotype has been extensively associated with some cardiovascular (Cambien et al, 1992; Lindpaintner et al, 1995; Montgomery et al, 1997) and renal disorders (Yoshida et al, 1995; Marre et al, 1997). Because eNOS and ACE play an important role in the process of penile erection, we analyzed the frequency of the eNOS Glu298Asp and the ACE/ID polymorphisms in a group of Mexican Mestizo patients with ED.

Patients and Methods

Patients

Fifty-three patients with ED and 62 healthy controls without ED were recruited for this study. Patients and controls were Mexican Mestizos. Mestizo populations in Mexico are descendents of the mixing between Amerindian, Spanish, and African populations, and their genetic pool is clearly different from those ethnic groups that do not have this admixture. Patients with ED were analyzed by andrological clinical evaluation, hormone profile, and intracavernosal injection with a vasoactive agent (Caverject, Pharmacia, and Upjohn, Kalamazoo, Mich) test. Psychogenic cases of ED were excluded from the study. Ages of men in both the ED and control groups were 30–59 years of age (mean 46 $\pm$ 8.7 years for the ED group; mean 43.17 $\pm$ 7.49 years for the control group). Diabetes mellitus, hypertension, cardiac disease, and cigarette smoking were evaluated in all subjects. Prior consent was requested from patients and control subjects, and the Research and Ethical Committees of the Instituto Mexicano del Seguro Social approved the protocol.

Polymorphism Determination

A sample of peripheral blood was obtained from all individuals, and genomic DNA was purified using the method developed by Kempter and Grossbadern (1992). Genotyping of the 894 G/T (Glu298Asp) eNOS polymorphism of each individual was determined by polymerase chain reaction (PCR) followed by enzyme restriction, whereas the ACE I and D alleles were differentiated only by PCR followed by size fractionation. All PCR assays were carried out in the presence of 200 ng of genomic DNA, 1 U of toq DNA polymerase 10 pmol of each primer, 0.2 mM deoxynucleotide triphosphates, 3 mM magnesium chloride, 50 mM potassium chloride, 20 mM Tris-hydrochloric acid (pH 8.4), and autoclaved distilled water was added to a volume of 25 μL. The sequence of flanking intronic primers used for amplification of eNOS exon 7 was 5′-CATGAGGCTCAGCCCCAGAAC-3′ (forward) and 5′-AGTCAATCCCTTTGGTGGTCAC-3′ (reverse) (Hingorani et al, 1999). The thermal cycling procedure consisted of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, repeated for 30 cycles performed in a Biometra, thermal cycler (Whatman Biometra, Gottingen, Del). The PCR product was divided into equal parts for endonuclease digestion with enzymes *Ban*II and *Mbo*I, respectively, during 16 hours at 37°C. The 206-bp PCR product was cleaved into two fragments of 124 bp and 82 bp with *Ban*II in the presence of a G nucleotide base at nucleotide 894 but not in its absence. As a confirmatory complement, *Mbo*I generated 119-bp and 87-bp fragments in the presence of a T nucleotide base at nucleotide 894. Restriction products were resolved by electrophoresis on a 2.5% agarose gel stained with ethidium bromide.

ACE I/D polymorphism analysis was modified from the method described by Lindpaintner et al (1995). A first PCR reaction (see above) with primers hace3s 5′-GCCCTGCAGGTGTCTGCAGCATGT-3′ (forward) and hace3as 5′-GGATGGCTCTCCCCGCCTTGTCTC-3′ (reverse) was assayed for all samples. Thermocycling conditions consisted of denaturation at 94°C for 30 seconds, and annealing-extension at 72°C for 60 seconds, repeated for 30 cycles. PCR products were resolved by electrophoresis on a 2% agarose gel stained with ethidium bromide. D and I alleles resulted in 319-bp and 597-bp amplicons, respectively. Due to preferential amplification of the D allele in heterozygous samples, all DD samples were subjected to a second PCR reaction with insertion-specific primers hace5a 5′-TGGGACCACAGCGCCCGCCACTAC-3′ (forward) and hace5c 5′-TCGCCAGCCCTCCCATGCCCATAA-3′ (reverse), with identical thermocycling conditions as with primers hace3. A 335-bp amplicon was generated in the presence of an I allele but not in DD homozygous samples. All the reactives for PCR were supplied by Invitrogen (Carlsbad, Calif), and the endonuclease enzymes were from new England Biolabs (Beverly, Mass).

Statistical Methods

χ^2 analysis and unpaired *t* tests were used to compare genotype distributions and allele frequencies, as well as other ED risk factors, including age, cigarette smoking, diabetes mellitus, hy-

Table 1. *eNOS 894 G/T and ACE I/D genotype distribution and allele frequency in patients with erectile dysfunction and controls*

	n (%)		
	Patients (n = 53)	Controls (n = 62)	
eNOS 894 G/T genotype			
GT + TT	24 (45.28)	16 (25.80)	
GG	29 (54.71)	46 (74.19)	$\chi^2 = 4.97$, $P = .04$
T allele frequency	0.24	0.12	$\chi^2 = 4.42$, $P = .03$
ACE I/D genotype			
DD	5 (9.43)	13 (20.96)	
ID	26 (49.05)	31 (50.00)	
II	22 (41.50)	18 (29.03)	$\chi^2 = 3.71$, $P = .15$
D allele frequency	0.33	0.46	$\chi^2 = 2.94$, $P = .86$

pertension, and cardiac disease between the ED and the control groups. Hardy Weinberg equilibrium was tested by χ^2 analysis for the frequencies of the eNOS and ACE genotypes. Odds ratios were calculated as a measure of the association between the eNOS or ACE genotype and ED, with the effects of the T allele or the D allele, respectively, assumed to be dominant (TT + TG vs GG, and DD + ID vs II). To determine the independent risk factors for ED, we performed multiple logistic regression analysis for the effect of the 894 G/T eNOS allele and other risk factors for ED. For this analysis we used forward stepwise selection (Wald). A P value of $< .05$ was established as statistically significant. All statistical analyses were performed with SSPS software version 12.0 (SPSS, Chicago, Ill).

Results

A total of 53 patients with ED and 62 controls, all Mexican Mestizos, participated in this study. Genotype and allele frequencies of the eNOS 894 G/T and ACE I/D polymorphisms are shown in Table 1. We confirmed that the genotype frequency distributions were in Hardy Weinberg equilibrium in patients ($\chi^2 = 1.24$; $P = .87$) and controls ($\chi^2 = 1.10$; $P = .89$). For the analysis of the eNOS 894 G/T genotype we combined the homozygous TT and heterozygous TG because the TT genotype is absent in the control group and the prevalence was very low in the ED group (3.7%). The eNOS 894T allele was significantly more common in the ED group than in the control group ($\chi^2 = 4.42$; $P = .03$). It was associated with an increment of the probability of developing ED (odds ratio [OR], [TT + GT vs GG] = 2.37; 95% confidence interval [CI], 1.08 to 5.21; $P = .02$). No significant differences were observed between groups for the ACE I/D genotype ($\chi^2 = 3.71$; $P = .15$) and allele frequencies ($\chi^2 = 2.94$; $P = .86$). Therefore, there was no significant association between the ACE D allele and ED (OR, [DD + ID vs II] = 0.57; 95% CI, 0.26 to 1.25; $P = .16$).

Table 2. *Clinical characteristics of study subjects*

Variable	Control group (n = 62)	DE group (n = 53)	*P*
Age, (y)*	44.27 ± 7.54	46.94 ± 8.82	.083
Currently smoking (%)	19 (30.64)	20 (37.73)	.548
Diabetes (%)	5 (8.06)	20 (37.73)	<.001
Hypertension (%)	5 (8.06)	17 (32.07)	.002
Cardiac disease (%)	2 (3.22)	1 (1.88)	.887

* Mean ± SD.

We compared the ED and control groups for frequencies of ED risk factors, including age, diabetes mellitus, hypertension, cardiac disease, and cigarette smoking. As shown in Table 2, we found significant differences in the frequencies of diabetes mellitus ($P < .001$) and hypertension ($P = .002$) between the ED and control groups.

To define independent risk factors for ED, we performed multiple logistic regression analysis using forward stepwise selection (Wald). Table 3 shows that the eNOS 894T allele was an independent risk factor for ED ($P = .031$), as were hypertension ($P = .034$) and diabetes mellitus ($P = .001$).

Discussion

In the present study, we found that the eNOS 894T allele is associated with an increased risk for ED in a Mexican Mestizo population, along with other known risk factors such as diabetes mellitus and hypertension. Although the number of patients studied was rather small, the allele and genotype frequencies of the Glu298Asp eNOS polymorphism in controls resembled those that we reported previously (Rosas-Vargas et al, 2003).

After the production of eNO by eNOS, NO enhances the production of cGMP by activation of the guanylate cyclase. In smooth muscle of the blood vessels and the tissue of corpora cavernosa, cGMP has a vasodilating effect, which induces penile erection. The eNOS 298Asp polymorphism is associated with a more labile eNOS in acidic conditions, as Tesauro et al (2000) and Fairchild et al (2001) have demonstrated, respectively, either in vitro

Table 3. *Multiple logistic regression analysis: significant variables in equation using forward stepwise selection (Wald)*

Variable	Beta Coefficient	SE	*P**	OR† (95% CI)
Hypertension	1.267	.596	.034	3.55
Diabetes	1.880	.581	.001	6.55
Glu298Asp eNOS‡	0.960	.446	.031	2.61
Constant	−3.015	.790	<.001	

* One degree of freedom.
† Exp (beta).
‡ 894 G/T + T/T eNOS genotype.

or ex vivo. Although a definitive association between the eNOS 894T allele and ED remains to be elucidated, it might be related to down-regulation of NO levels, and a consequent altering of cavernosum smooth muscle relaxation. This has been previously suggested by Eisenhardt et al (2003), who demonstrated that in an ED cohort study, eNOS 894T allele carriers show a reduced sildenafil response in comparison to those who are eNOS 894G homozygous, and that this negative effect is enhanced in those who are eNOS 894T homozygous. In concordance, we detected only two eNOS 894T homozygous patients, and both individuals were in the ED group, suggesting a dosage-cumulative effect.

The eNOS 894T allele association with ED also has a practical implication that might be considered in the development of ED therapy protocols based on the stimulation of penile NO synthesis. To date, only laboratory studies with animal models are available, which consist of direct gene therapy (Bivalacqua et al, 2000, 2003; Gonzalez-Cadavid and Rajfer, 2000) or indirect treatment through the administration of an eNOS substrate, L-arginine (Klotz et al, 1999), with positive results; however, we might expect the development of clinical trials to test the effectiveness of the NO induction in patients with ED. Other eNOS polymorphisms have been described elsewhere, and it will be interesting to evaluate their effects and establish the association of haplotypes with ED.

We also analyzed the relationship between ACE I/D polymorphism and ED, but we found no difference in the genotypic and allelic frequencies between patients and controls. Our results coincided with the results of Kim et al (2001), who did not detect any association between the ACE I/D genotype and ED in a Korean population. However, we analyzed only the relation between one ACE polymorphism (ACE I/D) and ED, which does not exclude the possibility that some other polymorphism of the ACE gene or of any of the proteins that participate in the renin/angiotensin system might play an evident role as a risk factor for ED.

In conclusion, our findings indicate a higher risk for ED in carriers of the eNOS 894T allele in a Mexican population as part of genetic susceptibility factors. Further studies will be useful to reveal additional polymorphism associations with the disease, and these might be added to environmental factors to have a complete predictive scheme specific for each population.

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