

984G>T/Glu298Asp **E**ndothelial nitric oxide synthase gene polymorphism and its association with the bioavailability of nitric oxide and hypertension in Venezuela

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Abstract

Objective: Detection of the 984G>T endothelial nitric oxide synthase (eNOS) genotype and the association with essential hypertension and Nitric Oxide (NO) serum level in the Venezuelan population. **Methods:** Samples from 192 patients with hypertension and 192 control subjects were analyzed for genotyping 984G>T eNOS polymorphism. A subset of 78 samples underwent the determination of serum NO concentration (μM). Results: The frequency for the GT genotype was 31.77 % in hypertensive individuals vs 33.85 % in controls individuals; and 6.25 % and 1.56 % respectively, for TT genotype; corresponding to T allelic frequency of 0.23 in hypertensive individuals. The OR for hypertensive individuals carriers for the T allele was 1.12 (IC 95%: 0.72-1.72, $p>0.05$). In contrast, the Odds ratio (OR), considering only the GG vs TT genotypes, we found a statistically significant 4.17 fold increased risk for hypertension (OR= 4.17, 95% CI: 1.06-19.11, $p<0.05$). Additionally, we found a significant decrease in serum concentrations of nitric oxide (46.47%; $p<0.05$) in the subgroup of carriers of the risk allele T ($13.80\pm 4.85 \mu\text{M}$, $n=35$), compared with the subgroup GG ($25.78\pm 16.75 \mu\text{M}$, $n=43$). **Conclusions:** These results suggest that the T risk allele of the polymorphism 984G>T in the eNOS gene, the main enzyme responsible for NO synthesis in endothelial level, may be associated with decreased serum levels of NO as intermediate phenotype, and this in turn be associated with the development of hypertension and clinical phenotype.

Key Words: 984 G>T polymorphism, eNOS gene, nitric oxide, hypertension

Introduction

The healthy vascular endothelium exerts atheroprotective actions through vasoactive mediators such as nitric oxide (NO), which is one of the major molecules responsible for vascular regulation. NO is produced in endothelial cells by endothelial NO synthase (eNOS), which catalyzes the conversion of L-arginine to L-citrulline. Endothelial NO is involved in the regulation of vascular tone and inhibition of platelet activities, leukocyte adhesion and vascular smooth muscle proliferation and plays a role as an oxygen radical scavenger¹⁻⁴. Disruption of eNOS gene leads to hypertension in mice⁵ and inhibition of nitric oxide (NO) production to increased blood pressure in healthy humans⁶. Furthermore, it has been shown that whole-body NO production in patients with essential hypertension is diminished under basal conditions⁷.

Because of these important evidences of NO and eNOS involvement in the blood pressure regulation, the eNOS gene has therefore been studied as a putative candidate gene for essential hypertension. However, the results of most association studies were inconsistent among different ethnicities, even among different study populations from the same ethnicity. A single nucleotide polymorphism (SNP) 984G>T within exon 7, resulting in a replacement of glutamic acid by aspartic acid at codon 298 (Glu-298Asp), is the most highlighted and controversial variant with regard to its association with hypertension. Lacolley et al⁸ reported that the 984G allele was associated with increased risk of hypertension in Caucasians, and on the other hand Miyamoto et al⁹ and Shoji et al¹⁰ found that the 984T allele was associated with increased risk of hypertension in Japanese. However, two other studies did not find any association between the 984G>T polymorphism and hypertension in Caucasians and Japanese, respectively^{11,12}. Despite these controversies, Tesauro et al.¹³ found that this polymorphism has a functional effect on

the eNOS protein, since this protein with an aspartate instead of a glutamate at position 298 is cleaved, likely because increases the susceptibility to protease activity. This was confirmed later by Persu et al.¹⁴.

In the present study, we investigated the association between the occurrence of essential hypertension and the 984G>T polymorphism in the Venezuelan population, where the prevalence rates of hypertension (25%) is similar to that reported worldwide (26%)¹⁵. We further analyzed the relationship between this polymorphism and the serum NO levels.

Subjects

The complete sample comprises 384 subjects that were classified in two groups: 192 patients with hypertension (defined as patients using antihypertensive drugs or having a systolic blood pressure of at least 140 mm Hg and/ or a diastolic blood pressure of at least 90 mm Hg)¹⁶ and 192 control subjects randomly selected, unrelated and apparently healthy without hypertension. Peripheral blood was collected from all subjects between January 2009 and January 2010, after a signed consent was obtained. A standard pro-forma was filled up with special emphasis on age, gender, smoking (current smokers or non-smokers), presence of diabetes mellitus (defined by a blood glucose level of at least 6.93 mmol/L)¹⁷ for all subjects. Blood from hypertensive patients was provided by the "Servicio de Endocrinología y Cardiología del Hospital Militar Dr. Carlos Arvelo" (Caracas, Venezuela).

Genotyping of the 984 G>T eNOS gene polymorphism

Genomic DNA was extracted from total peripheral blood as described by Bowen and Keenney¹⁸. eNOS genotyping for the 984G>T polymorphism was performed by Polymerase Chain Reaction (PCR) amplification of exon 7 and followed by Mbol restriction enzyme digestion for 16 h at 37°C¹⁹. The PCR was performed using the following primers eNOSup: 5'-CATGAGGCTCAGCCCCAGAAC-3' and eNOSdw: 5' AGTCAATCCCTTTGGTGCTCAC-3', yielding a fragment of 206 bp. Following enzymatic digestion in the presence of a T at nucleotide 984, which corresponds to Asp298, the 206-bp PCR product is cleaved into two fragments of 119 and 87 bp. The PCR was performed using 60 ng of genomic DNA in a 20 µL PCR reaction containing 0.025 U/µL of Taq DNA polymerase, 1.0 pmol/µL of each primer, 0.2 mM deoxynucleotide triphosphates (dNTPs), 1.5 mM MgCl₂, and 1X Taq polymerase buffer (10 mM Tris-HCl pH 8.3 and 50 mM KCl). Thirty five cycles were performed following one denaturation step at 95°C for 10 min. Each cycle consisted of incubations at 95°C for 1 minute, 58 °C for 1 minute and 72 °C for 1 minute. A final extension step was carried out at 72°C for 5 min. The products of PCR were analyzed by electrophoresis on a 2.5% agarose gel and visualized by 1X Sybr Safe staining. On the other hand, the products of the digestion process were separated by electrophoresis on a 8% polyacrylamide gel and visualized by silver nitrate staining²⁰.

Gel images were documented by using a digital camera (Fotodyne) equipped with ultraviolet filters, and the intensities of electrophoretic signals were estimated by the Foto Analyst PC Image program.

Biochemical Measurements

Blood samples are collected in tubes containing EDTA. The samples are centrifuged at 2000 g for 15 minutes. The plasma extracted was stored at -20°C until biochemical determination. Serum concentration of NO was detected by colorimetric non-enzymatic detection by reduction of NO₃ to NO₂ catalyzed by cadmium, followed by quantitation of nitrite using the Griess Reagent in a Nitric Oxide Assay kit obtained from Oxford Biomedical Research.

Statistical Analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS), version 9.0. Values of continuous variables were expressed as means ± standard deviations (SD). The frequencies of the alleles and genotypes among the case patients and controls were counted and were compared by the chi-square test with the values ($\chi^2_{HW} = \sum(O-E)^2/E$, one degree of freedom). Odds Ratio (OR) was calculated as a measure of the association of the eNOS genotype with the hypertensive phenotype. Multivariable logistic curve regression analyses were used to evaluate the risk to develop hypertension under various conditions: genotype, age, gender, smoking and diabetes mellitus. The regression coefficients that were obtained represent the probability to suffer the disease as a consequence of the presence of the T allele and the others variables studied. Statistical significance was set to a p value ≤ 0.05.

Results

General characteristics

The general demographic and clinical characteristics of the hypertensive patients and the control groups are shown in Table 1. The hypertensive group has the highest mean age (58.52± 12.05) and the highest percentages of males, smokers, subjects with diabetes mellitus (68, 54 and 30%, respectively). Additionally, multivariable logistic regression analysis was performed to determine the effect of conventional risk factors on hypertension. We found a positive correlation between hypertension and the following variables: age, diabetes and smoking (p<0.05).

Table 1. Demographic and clinical characteristics of the study population

Characteristic	Hypertensive patients (n=192)	Controls (n=192)	P value
Mean (SE) age, years	58.52±12.05	41.91±14.64	p<0.05
Male sex, n (%)	67.88	56.63	p>0.05
Diabetes, n (%)	30.23	3.53	p<0.05
Smokers, n (%)	54.17	34.32	p<0.05

Abbreviations: SD: Standard Deviation.

Determination of the eNOS gene 984 G>T polymorphism

First we determined the principle of Hardy-Weinberg equilibrium in the control group, in order to determine which frequencies should be observed in the population

for each genotype as a function of allele frequencies. In this sense, the χ^2 calculated was 3.09; hence, there is a probability between 5 and 10% of the differences between observed and expected are randomly, so it is accepted that this population is consistent with the Hardy-Weinberg equilibrium.

The 984G>T genotypes were determined by PCR-RFLP as described in the materials and methods section. The digestion products were separated 8% polyacrylamide gel electrophoresis and the presence of the T allele was detected as 119 and 87 bp DNA fragments, the G allele as a 206 bp DNA fragment and three products in heterozygous genotypes (GT) (Figure 1).

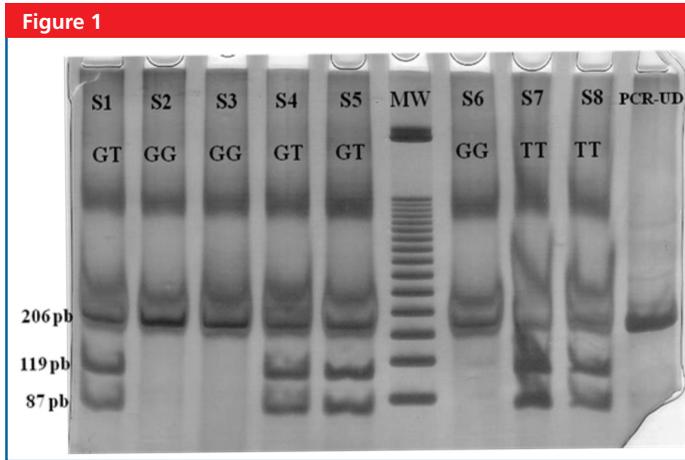


Figure 1. Polyacrylamide gel electrophoresis (8%) for detection of eNOS 984G>T polymorphism by PCR-RFLP. Samples: 2,3 and 6, homozygous genotype (GG); samples 1,4 and 5, heterozygous genotype (GT); samples 7 and 8 homozygous genotype (TT); PCR-UD: PCR fragment undigested and 50 bp molecular weight marker (MW).

The distribution of genotypes and allelic frequencies of the 984G>T polymorphism in both groups are shown in Table 2. It was determined that the frequency for the heterozygous (GT) was 31.77% (HT group) vs 33.85% (control group) and 6.25% vs 1.56% for homozygous individuals for the T allele (TT), respectively. Furthermore, the T allelic frequency was very similar for both populations with values of 0.23 for the HT patients compared to 0.18 for the controls individuals (Table 2).

The Odds Ratio (OR) for hypertension in carriers for the T risk allele (GT and TT genotypes) was 1.12 (95% CI: 0.72- 1.72, $p>0.05$) (Table 2). In contrast, when we estimated the OR, considering only the GG vs TT genotypes between hypertensive and control group, we found a statistically significant 4.17 fold increased risk of hypertension (OR=4.17, 95% CI: 1.06-19.11, $p<0.05$) (Table 2).

Table 2. Genotype, allele frequencies and OR for hypertension among carriers of the eNOS 984 G>T polymorphism				
eNOS 984G>T Genotype	Hypertensive patients n (%)	Controls n (%)	OR _{HT/Control} (CI 95 %) GG vs GT +TT	OR _{HT/Control} (CI 95 %) GG vs TT
GG	119 (61.98)	123 (64.58)	1.12 (0.72- 1.72) $p> 0.05$	4.17 (1.06-19.11) $p < 0.05^*$
GT	61 (31.77)	65 (33.85)		
TT	12 (6.25)	3 (1.56)		
T Allelic frequency (95 %CI)	0.23 (0.226-0.234)	0.18 (0.176-0.184)		

Abbreviations: eNOS, endothelial nitric oxide synthase; T, thymine; G, guanine; HT, Hypertension; OR, Odds Ratio; CI, Confidence Interval; *, statistically significant.

Discussion

eNOS gene 984G>T polymorphism and nitric oxide (NO)

A subset of 78 samples underwent the NO serum concentration (μM), by the colorimetric detection of total nitrate (NO_2 , NO_3), in order to determine the existence of any possible association of the T allele and decreased levels of this biochemical parameter. In this regard, we observed that individuals carrying the T allele showed the lowest levels of NO compared with the homozygous individuals GG ($13.80\pm 4.85 \mu\text{M}$ GT genotype vs $25.78\pm 16.75 \mu\text{M}$ GG genotype; Table 3), corresponding to a significant decrease of 46.47% ($p < 0.05$) in NO serum concentrations.

Hypertension is a multifactorial disease involving both environmental and genetic components. Clinical and experimental studies suggest that an alteration in NO metabolism may be a contributing factor in the pathogenesis of hypertension, in which specific polymorphisms of eNOS could be involved. This study investigated the relationship between the eNOS 984G>T polymorphism and the presence of hypertension in Venezuelan subjects. We did not find a significant association of the T allele with hypertension (OR 1.12; $p>0.05$) (Table 3); however, this association was significant when the T allele was in homozygous condition (4.17; $p<0.05$) (Table 2). In a previous study made in Venezuela, no association between this polymorphism and hypertension was found²¹, although the frequency of the T allele detected (30%) was bigger than that found in this study (18-23%; Table 2). The difference in these frequencies may be due to the ethnic origin of the Venezuelan population, which carries an important influence from Spain (58.8%) and also from other Europeans, Africans and native Indians. All these racial groups gradually intermixed and as a consequence the Venezuelan population is highly heterogeneous, being less mixed in some groups of natives^{22,23}. Official statistical data²⁴ shows that the Venezuelan population is composed of 67% mestizos, 21% whites, 10% blacks and 2% aboriginals. In agreement with this idea, the value of the allele T frequency here found lies between that reported for African American and Caucasians (10.5–16% and 30–37%, respectively) [25-29]. The T allele of the eNOS 984 G>T polymorphism is less common in Japanese (5–10.2%) and in this population the T allele has been associated with the pathology^{9,10}.

The relationship between 984G>T genotype polymorphism and hypertension has been controversial and it is not clearly elucidated how this polymorphism affects the gene expression and/or eNOS activity in the cells. Studies dealing with this problem using different methodological approaches have shown that the polymorphism does not affect the catalytic activity of the protein³⁰⁻³². Because glutamate and aspartate are conservative substitutions, it has been postulated that the polymorphism serves as a marker for a functional effect elsewhere in the eNOS gene or in its vicinity. In contrast to these conclusions, two previous

studies^{13,14} reported that the eNOS gene product with an aspartate, but not a glutamate, at position 298 is subject to proteolytic cleavage in primary human endothelial cells overexpressing eNOS and in arterial renal tissues. The effect of the polymorphism on the protein supports the results obtained in this study; we found that NO level in serum of individuals carrying the T allele was statistically significantly lower than in those who were homozygous GG (Table 3).

Table 3. Relationship between 984G>T genotype and NO serum concentrations (μM)

Study population	n	Nitric Oxide (μM) X \pm SD
Homozygous individuals GG	43	25.78 \pm 16.75
Individuals carrying T allele (GT+TT)	35	13.80 \pm 4.85

↓ **46.47% NO ($p < 0.05$)* in individuals heterozygous GT**

Abbreviations: NO, nitric oxide; X, mean; SD, Standard Deviation; T, thymine; G, guanine; *, statistically significant.

Thus, the lack of a strong association between the T allele of the eNOS 984G>T polymorphism and the hypertension, except when it was in homozygous condition, could be explained by the multifactorial nature of this pathology. We found in patients with hypertension the presence of multiple risk factors including diabetes mellitus, age and smoking ($p < 0.05$ for all of the variables). The presence of the T allele under any condition, which is associated with a diminished NO level, suggests that this effect could contribute, but not be determinant in the development of hypertension. The results obtained in our study allowed us to suggest the importance of genetic testing in the detection of an additional risk factor to be considered in the treatment and prevention of hypertension.

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