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Polymorphisms of the *NOS3* gene in Southern Chilean subjects with coronary artery disease and controls

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ABSTRACT

Background: Nitric oxide (NO) from the endothelium, produced by oxidation of L-arginine to L-citruline for the action at the endothelial nitric oxide synthase (eNOS) is considered an important atheroprotective factor. The 894G>T, −786T>C and 4a/4b polymorphic variants of the *NOS3* gene have been implicated in the development of coronary artery disease (CAD). We investigated the association between occurrence of CAD documented by angiography and the 894G>T, −786T>C and 4a/4b polymorphisms of the *NOS3* gene in Southern Chilean individuals.
Methods: A total of 112 unrelated patients with diagnosis of CAD confirmed by angiography and 112 controls were included in this study. The 894G>T and −786T>C single nucleotide polymorphisms were analyzed by PCR-RFLP, and 4a/4b polymorphism just for PCR.
Results: The genotype distribution and the relative allelic frequencies for the 3 variants investigated were not significantly different between CAD and control subjects (p = NS). Moreover, the odds ratio for CAD associated with the 894T (OR = 1.22, 95% CI 0.76–1.95), −786C (OR = 1.16, 95% CI 0.75–1.80) and 4a (OR = 0.97, 95% CI 0.48–1.95) variants failed to reach statistical significance.
Conclusion: These findings suggest that the 894G>T, −786T>C and 4a/4b polymorphisms of the *NOS3* were not associated with CAD in the studied subjects.

1. Introduction

The endothelial dysfunction is a common characteristic of diverse pathologies that include atherosclerosis. Atherosclerosis is a complex and progressive disease that develops in specific areas of the arterial wall. Endothelial dysfunction, manifested by changes in permeability and expression of the new adhesion molecules, is an early event in the evolution of atherosclerosis, preceding plaque formation and clinical disease [1].

The vascular endothelium plays an important role in the atherosclerotic process through the release of mediators such as nitric oxide (NO). Nitric oxide is an important factor in endothelial homeostasis and inhibits platelet aggregation, leukocyte adhesion, smooth muscle cell migration and proliferation [2] and oxidation of atherogenic low-density lipoprotein [3]. Several evidences suggest that alterations in the NO pathway might be involved in endothelial dysfunction and atherosclerosis [1,4].

Three distinct isoforms of nitric oxide synthase are responsible for NO biosynthesis in various tissues. The endothelial nitric oxide synthase (eNOS) catalyzes nitric oxide biosynthesis, from amino acid L-arginine in endothelial cells [5,6]. Several studies have examined the possibility that genetic variants in the gene encoding this enzyme could influence the expression and functional activity of the enzyme increasing predisposition to cardiovascular disease [7,8]. Human eNOS is determined by 26-exon gene (*NOS3*) on chromosome 7, that encode a 135-KDa protein containing 1203 amino acids [9].

The most investigated polymorphism in the eNOS gene, a polymorphism located in exon 7, consisting of a mutation of guanine to thymine at nucleotide 894 (894G>T, also called Glu298Asp, rs1799983), which leads to an amino acid change from Glu to Asp in the 298 site [10] modifying the primary structure of the protein might alter one or more functional properties of the enzyme [11]. This variant has been associated with endothelial dysfunction and subsequently identified as a risk factor for coronary artery disease (CAD) in several populations [1,12,13]. Nevertheless, controversial results were obtained in other studies [14–16].

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Another genetic variant, a single nucleotide polymorphism (SNP), resulting from a thymine being replaced by a cytosine at nucleotide −786, has been identified in the promoter region of the eNOS gene (−786T>C) and speculated to influence mRNA transcription and reduce eNOS gene expression. The −786T>C variant has been associated with an increased risk for the predisposition to coronary spasm in the Japanese population and was shown to reduce eNOS promoter activity by 50% approximately [17]. Rios et al. [18] discovered that the −786T>C variant may be the most relevant eNOS polymorphism for the development of cardiovascular disease in the Brazilian population. These observations strongly suggest that this polymorphism could be responsible for decreased eNOS production, however, similar to the 894G>T variant, inconsistent associations of the −786T>C polymorphism with CAD, were also shown [19–21].

The eNOS gene have as well a variable number of tandem repeat (VNTR) polymorphisms, with 27-base pair tandem repeat, in intron 4 called eNOS 4a4b [1,22]. Given the intronic location, this polymorphism may be less likely functional. The role of *NOS3* intron 4 variation is unclear. Because the intron sequences will be excised during RNA processing, subsequently the normal and variant alleles will produce eNOS mRNAs, and therefore eNOS proteins, that are identical. However, because mutations within introns could affect rates of eNOS transcription and/or processing of the primary transcript such mutations could ultimately affect eNOS enzyme levels [23] .

The 4a4b eNOS polymorphism was discovered and associated with atherosclerosis in the Australian population [24]. Likewise, Yoon et al., observed that this genetic variation was significantly associated with physiology variation of the plasmatic concentrations of nitric oxide [25]. Also this variant has been associated with CAD in the Turkish population [26].

2. Materials and methods

2.1. Subjects

The *NOS3* polymorphisms were analyzed in 112 unrelated Chilean patients (83 men and 29 women), ages 33 to 74 y, with diagnosis of CAD documented by angiography (coronary artery stenosis >70%), admitted to the Cardiology Service of the Hernán Henríquez Hospital of Temuco city, Chile. For the control group, consisting of 112 unrelated individuals (66 men and 46 women), ages 30 to 68 y, from Temuco city, we used a structured questionnaire to identify disease free controls and to exclude subjects who were suspected of having any form of vascular disease. Controls with a familial history of CAD, determined by interviewing, were excluded from the study. Demographic data and history of hypertension, diabetes mellitus, cigarette smoking, and hypercholesterolemia were assessed in each subject. In both groups there was no preselection of serum lipid levels. Subjects with a history of diabetes or basal glycemia ≥126 mg/dl were defined as diabetic. The study protocol was approved by the Ethics Committee of the University of La Frontera, and all subjects gave written informed consents.

2.2. Serum measurements

Biochemical measurements were determined from blood sample collected by venipuncture after an overnight (>12 h) fast. Triglycerides were determined by enzymatic assay [27], and total cholesterol was assayed by the esterase-oxidase method [28]. High-density lipoprotein cholesterol (HDL-C) concentrations were measured by enzymatic assay after phosphotungstic acid and magnesium precipitation [29]. Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation when the triglyceride concentrations did not exceed 4.8 mmol/l [30]. Serum glucose and uric acid levels were determined by enzymatic methods [31,32].

2.3. DNA analysis

Detection of the eNOS polymorphisms were analyzed after genomic DNA was extracted from peripheral blood leukocytes using a salting out procedure optimized by Salazar et al. [33]. The 894G>T polymorphism localized in the exon 7 of the *NOS3* gene were determined by polymerase chain reaction (PCR) according to conditions described by Hirata et al. [34]. A 340-bp fragment was amplified by PCR in a final volume of 50 µl containing 50 ng of genomic DNA, 10 pmol of each primer: 5′-agccccagaaccccctctgg-3′ (sense) and 5′-cccggcagtgtccaacatgc-3′ (antisense), 200 µl of each dNTP, 1 U of Taq DNA polymerase and PCR buffer (50 mmol/l KCl, 2 mmol/l $MgCl_2$, 20 mmol/l $(NH_4)_2SO_4$, 75 mmol/l Tris–HCl, pH 9.0). After initial denaturation at 98 °C for 3 min, the amplification was performed in 30 cycles consisting of 1 min at 95 °C, 1 min at 59 °C and 1 min at 72 °C. A final extension of 10 min at 72 °C completed the reaction. PCR products were submitted to MboI cleavage (5 U, Fermentas, Lithuania) in a total reaction volume of 20 µl. Enzymatic digestions were carried out at 37 °C for 4 h. The fragments were separated on 3% agarose gel for about 45 min at 100 V and stained with 0.5 mg/dl of ethidium bromide, and visualized on a UV transilluminator.

The −786T>C polymorphism in the 5-flanking region of *NOS3* gene was detected by PCR according to conditions described by Colombo et al. [35]. A 236-bp fragment was amplified by PCR in a final volume of 50 µl containing 50 ng of genomic DNA, 10 ρmol of each primer: 5′-atgctcccaccagggcatca-3′ (sense) and 5′-gtccttgagtct gacattaggg-3′ (antisense), 200 µl of each dNTP, 1 U of *Taq* DNA polymerase and PCR buffer (50 mmol/l KCl, 2 mmol/l $MgCl_2$, 20 mmol/l $(NH_4)_2SO_4$, 75 mmol/l Tris–HCl, pH 9.0). After initial denaturation at 98 °C for 3 min, the amplification was performed in 30 cycles consisting of 1 min at 94 °C, 1 min at 59 °C and 2 min at 72 °C. A final extension of 10 min at 72 °C completed the reaction. PCR products were submitted to PdiI cleavage (5 U, Fermentas) in a total reaction volume of 20 µl. Enzymatic digestions were carried out at 37 °C for 16 h. The wild-type allele (T) has no PdiI cleavage site, whereas the PCR product is cleaved into 2 fragments of 203 and 33 bp in the presence of the C allele. The fragments were separated on 3% agarose gel for about 45 min at 100 V and stained with 0.5 mg/dl of ethidium bromide, and visualized on a UV transilluminator.

The correct assessment of genotypes for 894G>T and T-786C polymorphisms at the *NOS3* gene were evaluated using a homozygous sample for restriction site as a positive control. In addition, all gels were reread blindly by 2 persons without any change, and 20% of the analyses was repeated randomly.

The presence of VNTR polymorphism in intron 4 was determined by PCR amplification according to conditions described by Marroni et al. [36]. PCR reaction was performed using primers: 5′-aggcccctatggtagtgccttt-3′ (sense) and 5′-tctcttagtgct gtggtcac-3′ (antisense) and DNA was amplified in a final volume of 50 µl. The amplification was realized in 30 cycles consisting of 1 min at 94 °C, 1 min at 59 °C and 2 min at 72 °C. A final extension of 10 min at 72 °C completed the reaction. The PCR products of 420 and 393 bp were separated on 4% agarose gel for about 60 min at 100 V and stained with 0.5 mg/dl of ethidium bromide, and visualized on a UV transilluminator.

2.4. Statistical analysis

Statistical analysis was carried out using the Sigma Stat Software, Ver. 3.5 (Jandel Sci., San Rafael, CA). Data are presented as mean ± SD. Differences between the means of the 2 continuous variables were evaluated by Student *t*-test. The allelic frequencies and genotype distribution were estimated by gene counting. Differences between noncontinuous variables, genotype distribution, allele frequency, and Hardy–Weinberg equilibrium were tested by χ^2 analysis. The odds ratio (OR) for CAD and their 95% confidence interval (CI) associated