

Endothelial nitric oxide synthase gene polymorphisms in patients with slow coronary flow

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(Received: March 16, 2017; Accepted: April 21, 2017)

Abstract: *Background and aims:* The aim of this study was to explore potential associations of the intron 4 variable number of tandem repeats (VNTR) and E298A polymorphisms of the endothelial nitric oxide synthase (eNOS) gene with slow coronary flow (SCF). The association between plasma nitrate and nitrite (NO_x) concentrations and eNOS gene polymorphisms was also assessed. *Materials and methods:* The intron 4 VNTR and E298A polymorphisms of the eNOS gene were evaluated in the isolated DNA blood samples obtained from the SCF patient group ($n = 30$) and healthy group consisted of age- and sex-matched controls ($n = 61$). *Results:* Plasma NO_x level was significantly lower in patients with SCF than in controls. In addition, patients with SCF have significantly lower nitric oxide levels than control subjects within each genotype variants. The allele and genotyped frequencies of the eNOS intron 4 VNTR and E298A polymorphisms were similar between patients with SCF and the controls. Plasma NO_x concentrations with respect to the relevant genotypes were found insignificant. *Discussion and conclusion:* Plasma NO_x is lower in patients with SCF than in healthy subjects. Our findings may suggest the lack of association between intron 4 VNTR and E298A polymorphisms of the eNOS gene and SCF.

Keywords: coronary disease, slow coronary flow, endothelial function, nitric oxide, endothelial nitric oxide synthase gene polymorphism

Introduction

Nitric oxide (NO) is one of the most important molecules that are responsible for the vasodilator tone required for the regulation of blood pressure [1]. NO is synthesized from L-arginine by a family of enzymes, the NO synthases (NOSs; EC 1.14.13.39), through the L-arginine/NO pathway [2]. There are at least three isoenzymes of NOS, namely inducible NOS, neuronal NOS, and endothelial NOS (eNOS) [3]. The gene encoding eNOS is located on chromosome 7q35–36 and comprises 26 exons spanning 21 kb [4]. The basal release of NO by endothelium inhibits platelet

aggregation [5], antagonizes vascular smooth muscle cell proliferation [6], and attenuates platelet and leukocyte adhesion [7–9]. All of these processes are important events during atherogenesis. Thus, the association of a subset of eNOS genotype with cardiovascular disease has extensively been screened. Among the reported polymorphisms of the eNOS gene, a significant association of intron 4 variable number of tandem repeats (VNTR) polymorphism of the eNOS gene with coronary artery disease has been reported [10]. In addition, E298A polymorphism in exon 7 of the eNOS gene was reported to be associated with coronary artery spasm [11]. The effects of aforementioned polymorphisms on *in vivo* NO

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generation cannot be directly measured because most endogenous NO rapidly oxidizes to nitrite (NO_2^-) and is eventually converted to nitrate (NO_3^-). Collectively, these inactive metabolites (nitrite and nitrate, termed as NO_x) have been used to reflect endogenous NO production.

Slow coronary flow (SCF), characterized by slow antegrade progression of a dye to the distal branch of a coronary artery in the absence of obstructive coronary disease, is not an infrequently detected finding during routine coronary arteriography [12].

Yet, the precise pathophysiological mechanisms of SCF remain uncertain. The imbalance between vasoconstrictor and vasodilator factors has previously been proposed as one of the possible mechanisms for SCF [13]. We also reported decreased NO levels in patients with SCF [14]. Thus, we planned this study in an attempt to delineate potential associations of the intron 4 VNTR and E298A polymorphisms of the eNOS gene and SCF.

Materials and Methods

Patients

The study population consisted of 30 patients with SCF and 61 control subjects with normal coronary flow. All participants were selected from among those who had undergone coronary arteriography. The majority of the subjects were suffering from intractable symptoms, such as angina and angina-like symptoms, shortness of breath, palpitations, and their symptoms could not be adequately clarified with non-invasive tests. All the patients who have SCF and do not have any other cardiac diseases such as coronary artery disease and heart failure were selected as patient group. Coronary arteriographies were performed by the same team and the definition of SCF was determined in accordance with the thrombolysis in myocardial infarction (TIMI) frame count (TFC) method as previously described [15].

Fasting blood samples were obtained 2 months after the coronary arteriography. From the blood samples, DNA was extracted by the phenol/chloroform method using the leukocyte fraction. For NO determination, plasma samples were stored frozen at -30°C . Biochemical parameters were determined by enzymatic methods (Modular DP, Roche Diagnostics, Mannheim, Germany). Laboratory tests were performed without knowing coronary arteriographic data. All of the participated patients were requested to sign the written informed consent form regarding the study protocol of this study that has been approved by the Ethics Committee of Başkent University, including the blood sample collection for DNA analysis.

Genotyping

After obtaining the blood samples, genomic DNA was prepared from leukocyte pellets by sodium dodecyl sulfate lysis, ammonium acetate extraction, and ethanol precipitation [16]. It was then used as a template for polymerase chain reaction (PCR) analysis, as previously described by Yoon et al. [17].

eNOS gene intron 4 VNTR genotyping

PCR products were separated by electrophoresis on a 2% agarose gel and identified by ethidium bromide staining. The products for the *b* and *a* alleles were 420 and 393 bp, respectively. Thus, each DNA sample revealed one of the three possible patterns at the end of electrophoresis: a 420-bp band (*bb* genotype), a 393-bp band (*aa* genotype), or both the 420-bp and the 393-bp bands (*ab* genotype) [18].

eNOS gene E298A genotyping

A 152-bp-amplified fragment was digested with BanII. The G allele consisted of 56 and 96 bp. Restriction site is lost in the case of G-to-T substitution [19].

NO analysis

Plasma nitrite concentration was accepted as an index of NO. For total nitrite detection, deproteinized plasma was treated with copperized cadmium granules to reduce NO^{-3} to NO^{-2} . Nitrite concentrations were quantified by a colorimetric assay based on the Griess reaction [20]. Briefly, a chromophore with strong absorbance at 545 nm is formed by the reaction of nitrite with a mixture of N-(1-naphthyl)ethylenediamine and sulfanilamide. A standard curve was established with a set of serial dilutions (10^{-8} – 10^{-3} mol/L) of sodium nitrite. The results are expressed as micromoles per liter of plasma ($\mu\text{mol/L}$).

Statistical analysis

Data analyses were performed using the Statistical Package for the Social Sciences, version 13.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean \pm standard error of the mean (SEM). Homogeneities of data set were controlled by Levene's test. Normality of distribution of variables was controlled by Shapiro–Wilk test. Continuous variables with normal distribution were analyzed by unpaired *t*-test. When parametric test assumptions were violated, the comparisons of the variables were carried out by Mann–Whitney *U* test or Kruskal–Wallis test. Kruskal–Wallis test was followed by Dunn's test for multiple comparisons. The relationships between categorical variables were statistically evaluated by χ^2 test, Fisher's exact test for 2×2 tables, and *G*-test. *G*-test was used because of the zero

values and small frequencies in the some cells of the contingency tables. *p* values less than 0.05 were considered statistically significant.

Results

Table I shows the clinical characteristics of the study population. There were no significant differences between patients with SCF and controls with respect to age, prevalence of hypertension and diabetes, smoking habits, and body mass index. The biochemical parameters were also similar between groups. But, patients with SCF had higher low-density lipoprotein cholesterol and lower NO_x blood levels than the controls.

The allele and genotyped frequencies of the eNOS intron 4 VNTR and E298A polymorphisms in patients with SCF and control subjects are shown in Table II.

The distributions of the eNOS gene were insignificant between patients with SCF and controls.

All subjects were genotyped for eNOS gene E298A; the frequencies of the G/G, G/T, and T/T genotypes were 0.367 (11/30), 0.433 (13/30), and 0.20 (6/30) in patients with SCF, and 0.311 (19/61), 0.41 (25/61), and 0.279 (17/61) in the controls, respectively. The genotype distribution for intron 4 VNTR revealed that among 30 patients with SCF, 24 had b/b (0.80), 6 had b/a (0.20), and none had a/a. Among 61 healthy subjects, 46 had b/b (0.754), 14 had b/a (0.23), and 1 had a/a genotype (0.016).

The plasma NO_x level was lower in patients with SCF than in control subjects (41.93 ± 11.5 vs. 49.81 ± 10.74, *p* < 0.001). NO_x concentrations with respect to the genotypes of the polymorphism are shown in Table III. For each genotype variants, patients with SCF had significantly lower NO levels than control subjects. Plasma

Table I Clinical and biochemical characteristics of the study subjects

	Control subjects (<i>n</i> = 61)	Patients with SCF (<i>n</i> = 30)	<i>p</i>
	Mean ± SEM Median (min–max)	Mean ± SEM Median (min–max)	
Age (years)	56.2 ± 1.06 55 (35–73)	54.27 ± 7.34 54 (41–74)	ns
NO (µmol/L)	49.81 ± 1.38 48.08 (33.3–98.79)	41.93 ± 2.1 40.52 (30.71–81.06)	<0.001
Glucose (mg/dL)	107.8 ± 5.81 97 (78–349)	100.93 ± 3.74 99.5 (70–197)	ns
BUN	14.69 ± 0.45 14 (8–24)	17.1 ± 1.29 16.5 (7–42)	ns
Cre	0.95 ± 0.03 0.92 (0.4–2.26)	1.11 ± 0.06 1.08 (0.65–2.26)	<0.01
Chol	175.02 ± 3.04 178 (117–226)	177.03 ± 5.22 171.5 (128–244)	ns
HDL-C	43.21 ± 0.92 43 (18–60)	47.57 ± 2.26 47.5 (30–99)	<0.05
LDL-C	98.05 ± 2.57 98 (22–149)	110.4 ± 4.27 109 (43–164)	<0.001
TG	133.39 ± 5.56 132 (46–311)	142.17 ± 11.46 140.5 (70–318)	ns
AST	18.21 ± 0.31 18 (12–27)	18.21 ± 0.85 18 (11–34)	ns
ALT	20.16 ± 0.64 20 (10–35)	20.97 ± 1.3 19.5 (10–40)	ns

SCF: slow coronary flow; NO: nitric oxide; BUN: blood urea nitrogen; Cre: creatine; Chol: total cholesterol; TG: triglycerides; HDL-C and LDL-C: high-density lipoprotein cholesterol and low-density lipoprotein cholesterol; AST: aspartate transaminase; ALT: alanine transaminase; ns: not significant

Table II | Frequencies of the alleles and genotypes of the Glu298Asp and the VNTR a/b in patients with SCF and control subjects

Variable	Control subjects (n = 61) n (%)	Patients with SCF (n = 30) n (%)	p
Alleles			
T	59/122 (48.4)	25/60 (41.7)	ns
G	63/122 (51.6)	35/60 (58.3)	ns
4b	107/122 (87.7)	53/60 (88.3)	ns
4a	15/122 (12.3)	7/60 (11.7)	ns
Genotypes			
T/T	17/61 (27.9)	6/30 (20.0)	ns
T/G	25/61 (41)	13/30 (43.3)	ns
G/G	19/61 (31.1)	11/30 (36.7)	ns
4b/b	46/61 (75.4)	24/30 (80.0)	ns
4b/a	14/61 (23.0)	6/30 (20.0)	ns
4a/a	1/61 (1.6)	0/30 (0.0)	ns

NO concentrations with respect to the genotypes of the polymorphisms were found not to be significantly different as shown in *Figs 1* and *2*.

Discussion

This study has examined the impact of intron 4 VNTR and E298A genotype of the eNOS gene in SCF. The

findings of this study may suggest that there exist no particular association between the relevant polymorphisms of eNOS gene and SCF in our population.

The mechanisms of the imbalance between vasoconstrictor and vasodilator factors have been proposed for slow coronary flow phenomenon (SCFP) [13, 14, 21]. Previous studies have highlighted the imbalance between endothelin-1 and NO release in patients with SCF as compared with controls with normal coronary flow [21]. Endothelin-1 and NO are the important molecules that modulate vasodilatory response to stress. There is also growing body of evidence indicating that a deficit of NO production causes endothelial dysfunction. Endothelial dysfunction may be regarded as an early contributor to atherogenesis and development of SCF as well [22]. A family-based study has demonstrated a significant linkage between the level of plasma metabolites of NO and markers located in the region of the eNOS gene, suggesting that variation in the eNOS gene sequence might affect NO production [23]. In line, Tsukada et al. [24] found a strong association between intron 4 VNTR polymorphism of the eNOS gene and plasma NO metabolite levels. Yoon et al. [17] also reported that the E298A polymorphism of the eNOS gene was associated with increased plasma NO_x levels. In addition, Veldman et al. [25] demonstrated that the presence of an Asp allele of the eNOS E298A polymorphism is associated with a reduced basal NO production. Given the importance of NO as a cardiovascular mediator, it is conceivable that polymorphisms of the eNOS gene could be associated with pathophysiological manifestations affecting cardiovascular system including SCFP.

Table III | Comparison of mean plasma NO_x levels between the genotypes of eNOS polymorphisms in patients with SCF and control subjects

	NO _x (μmol/L)		p
	Control subjects (n = 61) Mean ± SEM Median (min-max)	Patients with SCF (n = 30) Mean ± SEM Median (min-max)	
E298A			
T/T	47.86 ± 2.57 45.51 (33.3–72.15)	37.22 ± 2.77 36.63 (30.71–48.84)	<0.05
G/T	49.08 ± 2.33 47.36 (34.78–98.79)	41.45 ± 2.41 41.81 (30.71–53.28)	<0.05
G/G	52.49 ± 2.21 48.47 (39.96–81.4)	45.07 ± 4.72 41.36 (31.08–81.06)	<0.01
Intron 4			
4b/b	48.74 ± 1.47 47.72(33.3–98.79)	42.4 ± 2.27 41.26 (30.71–81.06)	<0.01
4b/a	52.54 ± 3.45 47.73 (35.15–81.4)	40.07 ± 5.7 35.15 (30.71–66.97)	<0.05

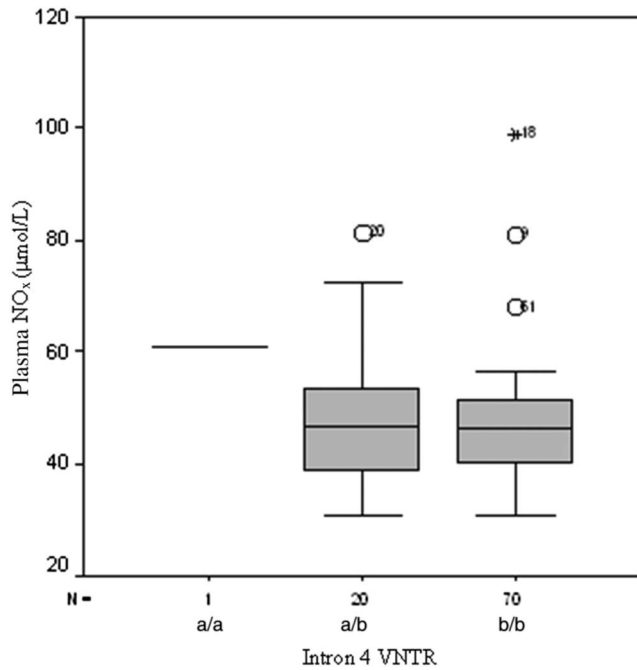


Fig. 1. The differences between the genotypes of eNOS polymorphism (intron 4 VNTR) with respect to plasma NO_x levels

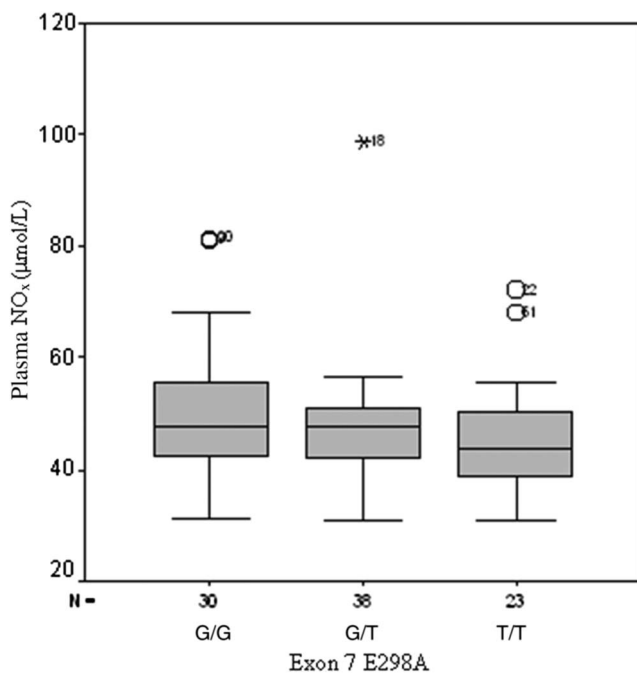


Fig. 2. The differences between the genotypes of eNOS polymorphism (exon 7 E298A) with respect to plasma NO_x levels

Up to date, eNOS single-nucleotide functional 786 T-C [26, 27], Glu298Asp [28], and intron 4a/b eNOS [29] have been evaluated in patients with SCF in Turkish population. Nurkalem et al. [26] reported an increased prevalence of the CC/CT genotypes (particularly the CT

heterozygote) in patients with the SCF and noted that the C allele was weakly correlated with the extent of slow flow as measured by the TFC. In contrast, in a recent study, Gazi et al. [27] found no relationship between T-786 T-C polymorphism and SCFP. Consistent with the results of this study, Caglayan et al. [28] showed that Glu298Asp polymorphism is not associated with SCF in Turkish population. In contrast to our results, Ekmekçi et al. [29] reported that in patients with SCF, the “a allele” of eNOS intron 4 VNTR is an independent predictor of the SCF (odds ratio 3.22, 95% confidence interval 1.28, 8.82) and is associated with an impaired coronary flow reserve. In this study, the distribution of genotypes calculated by direct counting of “a” and “b” alleles was insignificant between patients with SCF and controls. This study population included only a small number of patients, which could have been the cause of the different results.

Recently, Gupta et al. [30] examined the Glu298Asp and intron 4 VNTR NOS polymorphisms in patients with SCF in Indian population. In contrast to our and previous studies [27], the authors reported strong association between the Glu298Asp eNOS polymorphism and patients with SCF. Similar to this study, but in contrast to the previous study [29], they did not observe an association between the “a allele” and the SCF. However, somewhat surprisingly and in contrast to the previous studies [13, 14, 21], the plasma NO levels were elevated in patients with SCF compared with controls and increased with respective genotype. The authors explain the divergence in their results compared with previous studies as being attributable to (1) racial differences between Indian and Turkish patients and (2) the increased NO levels not necessarily reflecting differences in NO activity.

Finally, it should be appreciated that endothelial dysfunction is not the sole-proposed pathophysiological mechanism of SCF. There are several other hypotheses that have been suggested [31, 32].

Our findings are limited due to the fact that it was based on relatively few individuals who were all Caucasians. Larger sample sizes involving patients with different ethnicities are warranted.

Conclusion

In conclusion, our findings are suggestive of lack of association between intron 4 VNTR and E298A polymorphisms of the eNOS gene and SCF.

Funding sources: No financial support was received for this study.

Authors' contribution: NS and ATS contributed to conception and design. FBA and HV contributed to supervision and materials. NS, ATS, and AT contributed to data collection and/or processing. FBA, HV, and AT contributed to the analysis and/or interpretation. AT and ATS contributed to writing of this manuscript.

Conflict of interest: The authors declare no conflict of interest.

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