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Plasma Nitric Oxide Concentrations and Nitric Oxide Synthase Gene Polymorphisms in Coronary Artery Disease

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Background: **Plasma NOx (nitrate and nitrite) is a stable end product of the vasodilator NO. Several polymorphisms in the endothelial constitutive NO synthase (ecNOS) gene have been reported, including the 4a/4b VNTR polymorphism in intron 4, the E298D mutation in exon 7, and the G10-T polymorphism in intron 23. The aims of this study were to examine plasma NOx in patients with coronary artery disease (CAD) and to assess the association between plasma NOx concentrations and the three ecNOS gene polymorphisms.**

Methods: **Plasma NOx was measured in samples from 128 healthy controls and from 110 CAD patients at least 2 months after myocardial infarction. Three genetic polymorphisms that are known or have been suggested to be associated with plasma NOx concentration were also analyzed by PCR-restriction fragment length polymorphism.**

Results: **Median plasma NOx was significantly higher ($P < 0.001$) in CAD patients (95.9 μmol/L) than in controls (73.8 μmol/L). Furthermore, the median plasma NOx was significantly higher ($P < 0.001$) in hypertensive CAD patients (116.0 μmol/L) than in controls and normotensive CAD patients (86.0 μmol/L). The G-allele frequency of the G10-T polymorphism in intron 23 was significantly higher in CAD patients than in controls. Other polymorphisms showed no differences in allelic frequencies among the control and CAD groups. In controls, individuals with the E298D mutation in exon 7 (136.1 μmol/L) showed significantly higher ($P = 0.001$) median plasma NOx than those without this mutation (64.5 μmol/L).**

Conclusions: **Plasma NOx was higher in hypertensive CAD patients than in normotensive CAD patients and controls. The E298D polymorphism of the ecNOS gene was associated with increased plasma NOx. Further study is needed to understand the gene expression and enzyme activity of ecNOS and their association with genotypes.**

NO is synthesized from the amino acid L-arginine by a family of enzymes, the nitric oxide synthases (NOS),[1] through a metabolic route, namely, the L-arginine-nitric oxide pathway *(1)*. The synthesis of NO by vascular endothelium is responsible for the vasodilator tone that is essential for the regulation of blood pressure *(2)*. NO also contributes to the control of platelet aggregation and the regulation of cardiac contractility *(3)*. These actions are all mediated by the activation of soluble guanylate cyclase, and the consequent increase in the concentration of cyclic GMP in target cells *(4)*. Emerging evidence suggests that coronary artery disease (CAD) is related to defects in the generation or action of NO. NO released from cells rapidly autooxidizes to yield NO_2^-, which interacts with hemoglobin to yield NO_3^- *(5)*. Because NO_2^- plus NO_3^- (termed NOx) is relatively stable in blood, the concentration of NOx in blood may be an indicator of the endogenous formation of NO *(6)*.

There are at least three isoenzymes of NOS: inducible NOS, constitutive neuronal NOS, and constitutive endothelial NOS (ecNOS) *(7)*, which constitute a "gene family" located on different chromosomes and expressed in different cell lines. The gene encoding ecNOS is located on chromosome 7q35-36, which comprises 26 exons spanning 21 kb *(8)*. Moreover, a significant association has been found between the 27-bp repeat polymorphism in intron 4 of the ecNOS gene (intron 4 VNTR) and CAD *(9)*. In addition, the $Glu_{298} \rightarrow Asp$ polymorphism in exon 7 of the

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[1] Nonstandard abbreviations: NOS, nitric oxide synthase; CAD, coronary artery disease; NOx, NO_2^- plus NO_3^-; ecNOS, constitutive endothelial NOS; and VNTR, variable number of tandem repeats.

ecNOS gene (E298D) has been reported to be a strong risk factor for CAD, with homozygous genotype (T/T) frequencies of 36% in CAD cases vs 10% in controls *(10)*. No significant association between the G10-T polymorphism in intron 23 (G10-T) and arterial hypertension has been found *(11)*.

Studies have rarely been performed on the specific association between the genetic polymorphisms of the ecNOS gene and plasma NOx concentrations in CAD patients. Moreover, because gene pools, life-styles, and gene-environment interactions differ among populations, ethnic differences in the allelic frequencies of ecNOS polymorphisms and in the genetic associations between disease and plasma NOx concentration may exist *(12)*. The purpose of this study was to examine the concentrations of NO_2^- and NO_3^- in plasma of CAD patients and to assess the association between these factors and three polymorphisms of ecNOS in the Korean population.

Materials and Methods

STUDY SUBJECTS AND SAMPLES

Patients with CAD (n = 110; 70 males and 40 females) who had undergone coronary angiography because of recent myocardial infarction or angina were selected at Seoul National University Hospital, Seoul, Korea. None of the selected CAD patients was on therapy to lower lipids at the time of sampling. In myocardial infarction patients, blood samples were obtained 2 months after the occurrence of myocardial infarction. Hypertension was defined as a diastolic blood pressure >90 mmHg. The control group consisted of 128 individuals (70 males and 58 females), within the same age range as the patients, who were selected by health-screening at the same hospital to screen out those who had a history of chest pain, diabetes, hypertension, and general illness. Clinical details of these groups are summarized in Table 1. Blood samples were obtained from all subjects after fasting for 12 h to exclude the influence of dietary NO_3^- on plasma NOx *(13)*. Samples were placed in EDTA tubes and stored at −70 °C until the time of assay.

LIPID AND APOLIPOPROTEIN ANALYSIS

Plasma cholesterol and triglycerides were measured by enzymatic methods (Roche Diagnostics). HDL-cholesterol was measured directly with the HDL-C diagnostic method (Kyowa Medex) using either a Hitachi 747 or 7170 automatic chemistry analyzer. LDL-cholesterol was calculated using the formula of Friedewald et al. *(14)*, and apolipoproteins A-I and B were measured by immunonephelometric assay (Bering Nephelometer; Beringwerke). Lipoprotein(a) was measured using a commercially available ELISA method (IMMUNO). The within- and between-run CVs were <3% for total cholesterol, triglycerides, and HDL-cholesterol, and <5% for apolipoproteins A-I and B, and lipoprotein(a). Body mass index was calculated by dividing weight by height squared (kg/m^2).

Table 1. Clinical characteristics of the study subjects.

	Control (n = 128)	CAD without HT[a] (n = 52)	CAD with HT (n = 58)
Age, years	59.3 ± 7.0	61.3 ± 9.1	60.5 ± 8.1
Sex			
M	70	38	32
F	58	14	26
Smoking			
Yes	27	20	19
No	101	32	39
BMI,[b] kg/m^2	23.6 ± 2.7	24.7 ± 2.6	25.5 ± 2.5
Chol,[b] mmol/L	5.33 ± 0.92	5.38 ± 0.91	4.92 ± 1.16
TG, mmol/L	1.28 ± 0.54	1.48 ± 0.85	1.52 ± 0.86
HDL-C,[b] mmol/L	1.52 ± 0.38	1.13 ± 0.27	1.06 ± 0.34
LDL-C,[b] mmol/L	3.22 ± 0.83	3.57 ± 0.81	3.15 ± 1.04
ApoAI,[b] g/L	1.39 ± 0.28	1.12 ± 0.24	1.10 ± 0.28
ApoB, g/L	1.09 ± 0.26	1.13 ± 0.26	1.04 ± 0.31
Lp(a), g/L	0.23 ± 0.17	0.30 ± 0.24	0.29 ± 0.21

[a] HT, hypertension; BMI, body mass index; Chol, total cholesterol; TG, triglycerides; HDL-C and LDL-C, HDL- and LDL-cholesterol; Apo, apolipoprotein; Lp(a), lipoprotein(a).

[b] Significant differences among controls, CAD patients without hypertension, and CAD patients with hypertension ($P < 0.05$, Kruskal–Wallis test).

DETERMINATION OF PLASMA NO_2^- AND NO_3^-

Plasma NO_3^- plus NO_2^- (NOx) was measured as NO_2^- after enzymatic conversion of NO_3^- to NO_2^- by nitrate reductase, as described by Schmidt et al. *(15)* using a commercial method (R&D Systems). Briefly, plasma samples were diluted twofold with reaction buffer and ultrafiltered through a M_r 10 000 cutoff filter to eliminate protein. After centrifugation at 14 000*g* for 30 min at room temperature, 50 μL of each deproteinized plasma sample was incubated with 25 μL each of 100 U/L nitrate reductase and 0.35 mmol/L NADH. After a 30-min incubation at 37 °C, Griess reagents I and II (50 μL of each) were added. The mixture was incubated at room temperature for 10 min, and the absorbance was measured on a microplate reader at a wavelength of 540 nm. The plasma NOx concentration was determined relative to a calibration curve prepared with NO_3^- calibrators.

DNA ANALYSIS

Total genomic DNA was prepared from the leukocytes of 10 mL of blood after lysis of the red blood cells *(16)*. The intron 4 VNTR polymorphism of the ecNOS gene was detected by the method of Wang et al. *(9)* with minor modification. Briefly, the DNA samples were amplified by PCR using primer pairs that flanked the region of the 27-bp direct repeat in intron 4 of the ecNOS gene. Primer pairs for PCR were as follows: sense, 5′-AGGCCCTATGGTAGTGCCTTT-3′; antisense, 5′-TCTCTTAGTGCTGTGGTCAC-3′. The amplified DNAs were separated on 2% agarose gels and visualized by ethidium bromide staining.

For detection of the E298A polymorphism in exon 7 of the ecNOS gene, a primer pair was used to amplify a part

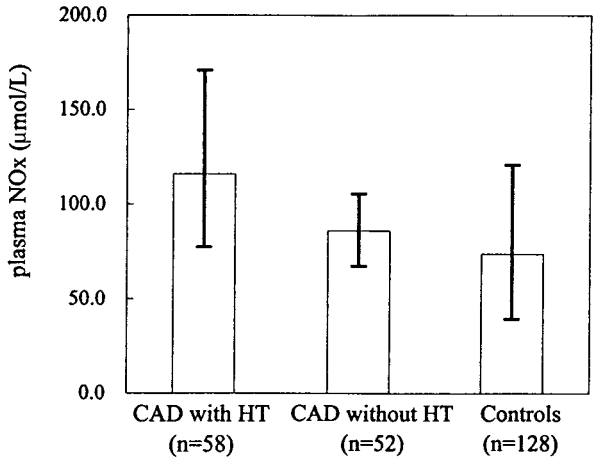

Fig. 1. Significant differences (P <0.001, by Kruskal–Wallis test) between median plasma NOx (NO_2^- + NO_3^-) concentrations in CAD patients with hypertension (116.0 μmol/L; interquartile range, 77.4–170.7 μmol/L) and CAD patients without hypertension (86.0 μmol/L; interquartile range, 67.3–105.6 μmol/L) or controls (73.8 μmol/L; interquartile range, 39.4–120.9 μmol/L).

Values given the medians (*columns*) and interquartile ranges (*bars*), and *n* represents the number of samples. *HT*, hypertension.

of the ecNOS gene containing exon 7 by PCR. The primer pair for PCR was as follows: sense, 5′-CCCCCTCTGGCCCACT-3′; antisense, 5′-AYACZTCCCTTTGGTGCTCAC-3′. The resulting 152-bp amplification product was incubated at 37 °C for 2 h with 10 U of the restriction enzyme *Ban*II (Roche Diagnostics). The amplified DNAs were digested by *Ban*II into smaller fragments (56 and 96 bp). In the case of a G-to-T substitution at position 894 in exon 7 of the ecNOS gene, a *Ban*II restriction site is lost.

For the G10-T polymorphism in intron 23 of the ecNOS gene, the primer pair for PCR was as follows: sense, 5′-CCCCTGAGTCATCTAAGTATTC-3′; antisense, 5′-AGCTCTGGCACAGTCAAG-3′. The resulting 676-bp amplification products were incubated at 37 °C for 2 h with 10 U of the restriction enzyme *Hin*dII (New England Biolabs). The amplified DNAs were then digested by *Hin*dII into fragments (577 and 99 bp). In the case of a G-to-T substitution at position 10 of intron 23 of the ecNOS gene, an additional *Hin*dII restriction site was produced and the amplified fragments were digested into smaller fragments (374, 203, and 99 bp).

STATISTICAL ANALYSIS

Statistical analyses were performed with the Statistical Analysis System package (SAS Institute) and SPSS, Ver. 9.01 (SPSS). Variables in two or three groups were compared using the Mann–Whitney *U*-test or the Kruskal–Wallis test. To test for independent relationships between variables, the χ^2 test and the Fisher exact test were performed. Statistical significance was accepted at P <0.05. Genotypic and allelic frequencies were determined using the gene counting method, and the χ^2 test was used to ensure that data complied with the Hardy-Weinberg equilibrium

Results

The precision of the within- and between-run NOx measurements was relatively good, with CVs <7.3%. The body mass index, cholesterol, HDL-cholesterol, LDL-cholesterol, and apolipoprotein AI showed significant differences among CAD patients with hypertension, CAD patients without hypertension, and controls. The median plasma NOx in CAD patients (95.9 μmol/L; interquartile range, 68.7–132.1 μmol/L) was significantly higher (P <0.001) than in controls (73.8 μmol/L; interquartile range, 39.4–120.9 μmol/L; n = 128). However, when the CAD group was categorized in terms of hypertension, the difference between plasma NOx in controls and in normotensive CAD patients (median, 86.0 μmol/L; interquartile range, 67.3–105.6 μmol/L; n = 52) disappeared. The median plasma NOx was significantly higher in hypertensive CAD patients (116.0 μmol/L; interquartile range, 77.4–170.7 μmol/L; n = 58) than in normotensive CAD patients or in controls (P <0.001, Kruskal–Wallis test; Fig. 1).

Table 2. Genotypic and allelic frequencies in controls (n = 128) and CAD patients (n = 110) of various ecNOS polymorphisms.

		Genotypic frequency, n (%)			Allelic frequency		
Polymorphism	**Group**	**4a/4a**	**4a/4b**	**4b/4b**	**4a**	**4b**	***P*[a]**
VNTR in intron 4	Control	9 (7.0)	23 (18.0)	96 (75.0)	0.160	0.840	
	CAD	7 (6.4)	23 (20.9)	80 (72.7)	0.168	0.832	NS[b]
		GG	**GT**	**TT**	**G**	**T**	
E298D mutation in exon 7	Control	110 (85.9)	18 (14.1)	0 (0.0)	0.930	0.070	
	CAD	94 (85.5)	15 (13.6)	1 (0.9)	0.923	0.077	NS
		GG	**GT**	**TT**	**G**	**T**	
G10-T polymorphism in intron 23	Control	112 (87.5)	16 (12.5)	0 (0.0)	0.938	0.063	
	CAD	108 (98.2)	2 (1.8)	0 (0.0)	0.991	0.009	0.002

[a] P value of χ^2 test or Fisher exact test between the allelic frequencies in controls and CAD patients.
[b] NS, not significant.

A comparison of allelic frequencies of the E298D, G10-T, and intron 4 VNTR polymorphisms between CAD patients and controls is shown in Table 2. The G-allele frequency of the G10-T polymorphism in intron 23 of the ecNOS gene was higher in CAD patients than in controls. However, there were no significant differences between the allelic frequencies of the E298D and intron 4 VNTR polymorphisms in CAD patients and in controls. Plasma NOx concentrations according to the genotypes of the polymorphisms are shown in Table 3. In controls, plasma NOx showed significant differences according to genotype ($P = 0.001$); the median plasma NOx increased in those individuals with an E298D mutation in exon 7 (136.1 μmol/L) compared with those without the mutation (64.5 μmol/L). In hypertensive CAD patients, the median plasma NOx showed an increasing tendency in the genotype with the 4a allele (127.9 μmol/L) compared with the genotypes without the 4a allele (107.7 μmol/L), although it was not statistically significant ($P = 0.083$).

Discussion

NOx is a stable end product of NO metabolism. In fasted individuals, as much as 90% of the circulating NO_2^- is derived from the L-arginine-nitric oxide pathway, and NO_2^- is a valid indicator of NO production *(6)*. The subjects in our study had fasted for 12 h; therefore, dietary sources can be excluded as a possible source of the increased NO_2^- concentrations observed. Thus, our finding of increased plasma NOx in patients with CAD is suggestive of increased cumulative NO synthesis. Our study demonstrated increased plasma NOx in CAD patients with hypertension. However, plasma NOx in CAD patients without hypertension did not differ significantly from that of the controls. These findings suggest that an increase of plasma NOx in hypertensive CAD patients may play a compensatory role because of an increased superoxide anion concentration, which is a characteristic feature of experimental hypertension models *(17)*, and of increased shear stress in blood vessels *(18)*, which may stimulate NO production. Xiao and Pang *(19)* showed that NO synthesis in vascular smooth muscle cells increases during development in spontaneously hypertensive rats. However, it remains unknown whether the increase in plasma NOx is caused by or the result of the impairment of endothelial function.

CAD is a multifactorial disease that may differ in each race or ethnic group; for example, the prevalence of CAD differs widely among different populations, and the frequencies of ecNOS gene polymorphisms have been reported to vary among ethnic groups *(12)*. Several polymorphisms of the ecNOS gene associated with CAD have been identified. Wang et al. *(9)* detected an association between homozygosity for the 4a allele in the intron 4 VNTR polymorphism of the ecNOS gene and an increased risk of CAD only in current and ex-smokers in the Australian population.

In the present study, the association between CAD and three polymorphisms of the ecNOS gene was investigated. Our results showed that the G-allele frequency of the ecNOS gene polymorphism G10-T in intron 23 was significantly higher in the CAD group than in the controls. However, no significant differences were found between patients and controls in terms of the allelic frequencies of the intron 4 VNTR or the E298D polymorphism. This result differs from previous reports on the associations between these polymorphisms and Caucasian CAD patients *(9–11)*.

The E298D polymorphism in exon 7 of the ecNOS gene has been reported to be a strong risk factor for CAD *(10)*, and Wang et al. *(20)* detected an association between homozygosity for the 4a allele in the intron 4 VNTR polymorphism of the ecNOS gene and increased plasma NOx in healthy Caucasians. However, studies on the association between genetic polymorphisms of ecNOS and the plasma NOx concentration have been few. The present study shows that only in the control group was

Table 3. Comparison of median plasma NOx (interquartile range) between the genotypes in various polymorphisms.

		Control (n = 28)		CAD with HT[a] (n = 58)		CAD without HT (n = 52)	
Polymorphism	Genotype	Plasma NOx, μmol/L	n	Plasma NOx, μmol/L	n	Plasma NOx, μmol/L	n
VNTR in intron 4	4a/4a+4a/4b	74.6 (40.0–129.5)	32	127.9 (60.9–294.5)	17	87.3 (69.4–109.4)	13
	4b/4b	73.8 (38.8–106.5)	96	107.7 (78.4–152.1)	41	81.8 (63.6–103.8)	39
P[b]		NS[c]		NS (0.083)[d]		NS	
E298D mutation in exon 7	GT+TT	136.1 (76.8–170.7)	18	107.7 (74.3–204.9)	9	63.5 (44.8–125.7)	7
	GG	64.5 (35.9–95.2)	110	116.3 (77.6–171.5)	49	86.8 (68.7–105.3)	45
P		0.001		NS		NS	
G10-T polymorphism in intron 23	GT	85.5 (42.2–122.0)	16	116.2	1	58.9	1
	GG	71.3 (37.7–120.9)	112	115.7 (77.0–171.5)	57	86.8 (67.8–105.9)	51
P		NS		NS		NS	

[a] HT, hypertension.
[b] *P* value of Mann–Whitney *U*-test, plasma NOx between the genotypes in various polymorphisms.
[c] NS, not significant.
[d] In hypertensive CAD patients, the median plasma NOx was increased in the genotype with the 4a allele, but the increase was not statistically significant ($P = 0.083$).

plasma NOx significantly dependent on the genotypes of the E298D polymorphism; plasma NOx was increased in those individuals having the E298D mutation. In CAD patients, however, this relationship was not observed. The lack of correlation between controls and patients might be explained as follows. In CAD patients with pathogenic conditions such as atherogenesis, angina, and hypertension, which probably influence plasma NOx, the plasma NOx regulation mechanism could be changed, and therefore the genetic influences seen in healthy subjects might be masked. Otherwise, the significance observed only in healthy subjects might be invalid because of a statistical flaw in the multiple comparison factors. To determine whether the genetic polymorphism of the ecNOS gene, especially the E298D and intron 4 VNTR mutations, may influence plasma NOx, the study of gene expression and protein production is mandatory. We are now studying the relationship between these polymorphisms and mRNA concentrations or the enzyme activity of ecNOS, using human umbilical vein cell culture.

In conclusion, plasma NOx is higher in CAD patients with hypertension than in CAD patients without hypertension and in controls, which might be attributable to the compensatory phenomenon developed in hypertension. A dependency between increased plasma NOx and the polymorphisms of the ecNOS gene is observed in controls with the E298D mutation. To evaluate the exact causal relationships between the ecNOS polymorphisms and plasma NOx, more studies of gene expression and the enzyme activity of ecNOS, and the nature of their dependence on genotypes are required.

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