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Evidence of Association of the ecNOS Gene Polymorphism with Plasma NO Metabolite Levels in Humans

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Nitric oxide (NO) synthesized by the vascular endothelium regulates mammalian blood vessels and other systems in humans. Recently, an endothelial nitric oxide synthase (ecNOS) gene polymorphism, the 27-bp repeat in intron 4 (ecNOS4), was reported to be related to the pathogenesis of coronary heart disease and terminal renal failure. We analyzed this polymorphism in a group of 413 healthy subjects, and measured their plasma nitrite and nitrate (NOx) levels. The frequency of the b allele was 89.8% , and the frequency of the a allele was 10.2%. The frequency of ecNOS4 b/b, ecNOS4 b/a, and ecNOS4 a/a in the healthy subjects in this study was 0.814 (n=336), 0.169 (n=70) and 0.017 (n=7), respectively. Using this polymorphism as a DNA marker, we found a strong association between the alleles of the ecNOS gene polymorphism and the plasma NOx levels. The basal NO metabolite levels were 28.8 μ mol/L in subjects with ecNOS4 a/a, 31.4 μ mol/L in those with ecNOS4 b/a, and 35.5 μ mol/L in those with ecNOS4 b/b. The mean plasma NOx level of the subjects who were homozygous for the a allele was nearly 20% lower than in the subjects with the b allele. The plasma NOx level of the subjects with the a allele was 31.2±2.00 μ mol/L, and significantly lower than the 35.5±0.93 μ mol/L in those without the a allele ($P < 0.05$). The results of this study indicate that the ecNOS4 gene locus may be responsible for variations in the genetic control of plasma NOx and that analysis of ecNOS4 gene polymorphism may be a useful tool for studying the relation between NO and diseases.

Key Words: **endothelial nitric oxide synthase (ecNOS); DNA polymorphism; plasma nitrite and nitrate (NOx).**

Nitroglycerin has been used for over a century to treat coronary heart disease, and it has long been hypothesized that humans synthesize oxides of nitrogen [1]. This observation has been brought into focus by the demonstration that endogenous nitric oxide (NO) regulates mammalian blood vessels and other systems (such as the cardiovascular- pulmonary system and peripheral nervous system) [2], meaning that virtually every mammalian cells are influenced by NO.

Obviously, gene polymorphism of the nitric oxide synthase (NOS) gene would be of some relevance to the pathogenesis of certain diseases [3]. Wang reported evidence of an association between higher susceptibility to coronary lesions and a particular polymorphic type of ecNOS locus in cigarette smokers [4]. Miyahara et al. reported cloning and structure characterization of the human ecNOS gene and suggested that a five tandem repeat in a 27-bp consensus sequence in intron 4 might serve as a genetic marker, [5] and Wang et al. explored two alleles: a common large allele and a smaller allele [4]. The larger allele has five tandem 27-bp repeats and was designated "b", and the smaller allele has four tandem 27-bp repeats and was designated "a". We have reported a higher frequency of type-a germ line polymorphism at that locus in patients with terminal renal failure [6]. Since both coronary lesions and renal failure are based on vascular disorders, angiopathy may be associated with DNA polymorphism of the ecNOS a allele.

To clarify the association between the ecNOS gene polymorphism and plasma NO metabolite levels in humans, we analyzed this polymorphism in a group of 413 healthy subjects, and measured their plasma NO metabolite levels.

METHODS

Subject population and blood samples. We selected 413 consecutive healthy subjects from among persons who participated in a com-

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TABLE 1

Demographic and Clinical Features of the Subjects

	Mean ± SEM	Range
Age (yr)	51.1 ± 0.44	27–79
Male/female ratio	280/133	
T.P. (g/dl)	7.34 ± 0.02	6.3–8.5
BUN (mg/dl)	14.9 ± 0.17	4.0–27
T.CHO (mg/dl)	203 ± 1.58	104–289
HDL-C (mg/ml)	50.7 ± 0.74	25–108
TG (mg/dl)	105 ± 2.75	32–299
AST (IU/L)	23.7 ± 0.29	13–48
HbA1c (%)	4.99 ± 0.02	4.2–5.9

prehensive annual health screening in the Health Screening Unit of Toranomon Hospital. We excluded persons with diabetes mellitus, renal disease, ischemic heart disease, and malignant neoplasm. We also excluded persons who did not reside in Tokyo. The demographic and clinical features of the subjects are shown in Table 1. All the subjects participated in the study after obtaining their informed consent.

A 2-mL peripheral venous blood sample was drawn into an EDTA specimen tube. The blood sample was centrifuged within 6 hours, and the plasma and cellular components were stored separately at −40°C until analyzed.

Determination of ecNOS genotypes. In accordance with the method already described elsewhere, we extracted genomic DNA from the cellular components of peripheral blood by the guanidine thiocyanate method. The extracted DNA was stored at 4°C until analyzed.

A genomic DNA fragment was amplified by the polymerase chain reaction (PCR) to determine the ecNOS genotype. Oligonucleotide primers that flank the 27-bp direct repeat region in intron 4 of the ecNOS gene were used [4]. The forward primer was 5′-AGGCCCTATGGTAGTGCCTTT-3′ (located at 5111 to 5130 bp), and the reverse primer was 5′-TCTCTTAGTGCTGTGGTCAC-3′ (located at 5530 to 5511 bp). A 50 -μ L volume was used for each PCR reaction, containing 100 pmol of each primer, 26 mmol of each dNTP, 1.2 mmol Mg2+, 5% DMSO, and 1 unit of Expand High Fidelity DNA polymerase and its reaction buffer (Boehringer Mannheim) together with 1 μ g genomic DNA. Each reaction mixture was heated at 94°C for 5 min for denaturation, followed by 35 cycles in a thermal cycler (TSR-300, Iwaki Glass, Japan) at 94°C for 1 min, 56°C for 1 min and 72°C for 2 min. The PCR products were separated by electrophoresis in 6% nondenaturing polyacrylamide gel. Fragments were detected with ethidium bromide. A 420-bp band indicated five repeats of the 27-bp (b allele), and a 393 bp band indicated four repeats (a allele) [Fig.1].

Determination of plasma NOx levels. Plasma NOx levels were measured as total nitrite concentration with Griess reagents [7] after incubation with nitrate reductase (EC 1.6.6.2) for enzymatic conversion as described by Schmidt et al [8]. Briefly, a 40 μ L plasma sample was applied to a microtiter plate well and reacted with 60 μ L of enzyme reagent. NADPH, FAD, and nitrate reductase were added to yield final concentrations of 0.35 mmol/L,15 μ mol/L, and 50 U/L, respectively. Samples were incubated for 60 min at room temperature, and this was followed by the addition of 100 μ L of Griess reagent (1 g/L sulfanilamide, 0.1 g/L N-1-naphthylendiamine, 25 g/L phosphoric acid). After 10 min of color development at room temperature, the absorbance was measured on a microplate reader (MPR-A4, Tosoh, Japan) at a wavelength of 540 nm and reference of 620 nm. Background values were determined, and calibration curves were plotted for potassium nitrate in distilled water (linear range L: 0 - 100 μ mol/L) [Fig.2].

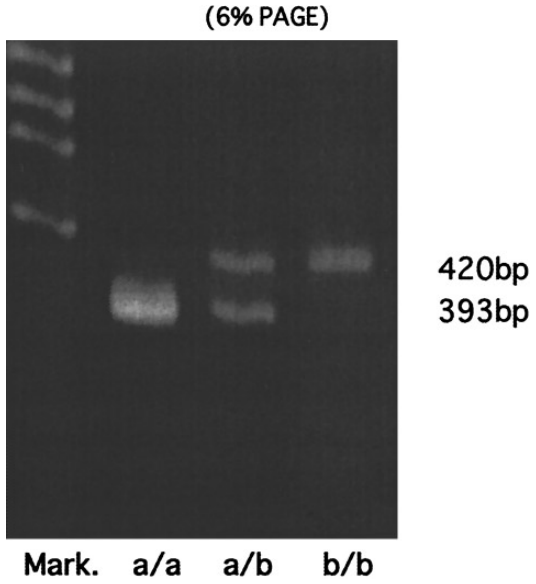

FIG. 1. Determination of ecNOS genotyping.

Statistical analyses. Values are expressed as mean ± standard error of the mean (SEM). The significance of difference was analyzed by using either the paired Student's *t*-test or the Wilcoxon signed-rank test. Because there were only seven subjects who were homozygous for ecNOS a, we considered both homo- and heterozygous subjects for ecNOS a compared to subjects having ecNOS a genotype. Differences were considered significant at $P < 0.05$.

RESULTS

The frequency of the b allele was 89.8%, and the frequency of the a allele was 10.2%. The frequency of ecNOS4 b/b, ecNOS4 b/a, and ecNOS4 a/a in the

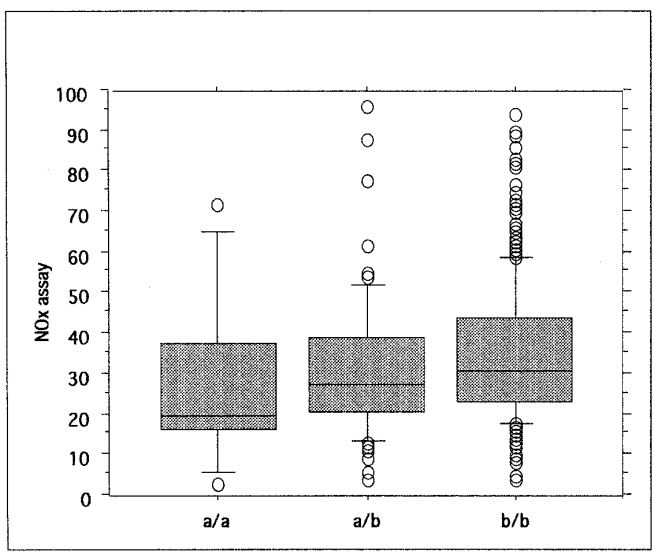

FIG. 2. The association between ecNOS gene polymorphism and plasma NOx level.

TABLE 2

Correlation Coefficient of the Plasma NOx Level in Subjects with a Allele and without a Allele

The plasma NOx level in subjects	N	Age	NOx (μmol/L)
With a allele (ecNOS4a/a and ecNOS4a/b)	77	49.7 ± 0.95	31.2 ± 2.00
Without a allele (ecNOS4b/b)	336	51.4 ± 0.49	35.5 ± 0.93
p value			P = 0.047

healthy subjects in this study was 0.814 (n=336), 0.169 (n=70) and 0.017 (n=7). Using this polymorphism as a DNA marker, we found a strong association between alleles of the ecNOS gene polymorphism and plasma NOx levels. The basal NO metabolite level was 35.5 μ mol/L in the subjects who were ecNOS4 b/b, 31.4 μ mol/L in those who were ecNOS4 b/a and 28.9 μ mol/L in those who were ecNOS4 a/a. The mean plasma NOx level in the subjects who were homozygous for the a allele was nearly 20% lower than that of the subjects with the b allele [Fig. 2]. The mean plasma NOx level in the subjects with the a allele (31.2±2.00 μ mol/L) was significantly lower than in those without the a allele (35.5±0.93 μ mol/L) (p=0.047) [Table 2].

Thus, the ecNOS gene locus itself may be responsible for variations in the genetic control of plasma NOx.

DISCUSSION

Nitric oxide (NO) synthesized by the vascular endothelium is important in regulating vascular tone and controlling blood pressure[9]. Previous studies have provided some evidence that impairment of NO production accounts for the abnormalities in vascular function that characterize many vascular diseases [10-12], including experimental hypertension in animals and human essential hypertension . Alteration of the activity of the L-arginine-NO pathway in these studies has been suggested by the response of regional blood flow and blood concentrations of L-citrulline, and cyclic guanosine monophosphate to agents that stimulate (acetylcholine) or inhibit (L-arginine analogs) NO production.

We analyzed this polymorphism in a group of 413 healthy subjects, and measured their plasma NOx levels. The frequency of the b allele was 89.8% and the frequency of the a allele was 10.2%. The frequency of ecNOS4 b/b, ecNOS4 b/a, and ecNOS4 a/a in the healthy subjects in this study was 0.814 (n=430), 0.169 (n=70), and 0.017% (n=7), respectively. Wang et al. used the same method to determine genotype frequencies in a Caucasian population, and reported that the frequencies of b/b, b/a, and a/a were 0.667 (n=102), 0327 (n=50), and 0.007 (n=1), respectively [4]. These figures are consistent with the frequencies observed in this study. Using this polymorphism as a DNA marker, we found a strong association between the alleles of the ecNOS gene polymorphism and plasma NOx levels. The mean plasma NOx level of the subjects who were homozygous for the a allele was nearly 20% lower than that in the subjects with the b allele. Thus, the ecNOS gene locus may be responsible for variations in the genetic control of plasma NOx.

Many studies have been published on the relation between NO and blood pressure; and SHR rats are known to have high NO values [13]. The endothelium-dependent vasodilatory response to Ach in the forearm is weaker in essential hypertension patients, but becomes similar to that of healthy subjects after administration of a nonselective NOS inhibitor (L-NMMA)[14]. Recently, it has just reported that plasma NO metabolite levels are low in those patients of essential hypertension[15]. It can therefore be concluded that NO release is decreased in patients with essential hypertension. The fact that plasma NO metabolite levels are different depending on ecNOS4 gene polymorphism suggests that ecNOS gene polymorphism is a useful parameter for studying the relations between NO and blood pressure. Bonnardeaux demonstrates that ecNOS CA repeat gene polymorphism is independent of blood pressure in linkage analysis [3]. Regarding study on blood pressure, ecNOS4 gene polymorphism might be a more useful factor than ecNOS CA repeat gene polymorphism.

It is disputable whether NO metabolite in blood is derived only from ecNOS in the endothelial cell of blood vessel. However it is the fact that plasma NO metabolite levels are different depending on ecNOS4 gene polymorphism. And there is a possibility that this fact suggests that ecNOS influences plasma NO metabolite level as far as it is not in a morbid situation.

In conclusion, we found a strong association between alleles of the ecNOS gene polymorphism and plasma NOx levels. The mean serum NOx level in subjects with the a allele was significantly lower than in those without the a allele. The results of this study indicate that the ecNOS gene locus may be responsible for variations in the genetic control of plasma NOx and a useful factor for studying the relations between NO and disease.

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