ecNOS gene polymorphism is associated with endothelium-dependent vasodilation in Type 2 diabetes

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Komatsu, Miyoko, Takahiko Kawagishi, Masanori Emoto, Tetsuo Shoji, Atsuko Yamada, Kyoko Sato, Masayuki Hosoi, and Yoshiki Nishizawa. ecNOS gene polymorphism is associated with endothelium-dependent vasodilation in Type 2 diabetes. Am J Physiol Heart Circ Physiol 283: H557–H561, 2002. First published April 4, 2002; 10.1152/ajpheart.00653.2001.—The association between endothelial constitutive nitric oxide synthase (ecNOS) gene polymorphism and vascular endothelial function has not been clarified. We investigated the impact of ecNOS gene polymorphism on endothelial function in 95 patients with Type 2 diabetes (ecNOS genotype: 4b/b, n = 62; 4b/a, n = 30; 4a/a, n = 3). Flow-mediated (endothelium dependent, FMD) and nitroglycerin-induced (endothelium independent, NTG) vasodilations of the right brachial artery were studied using a phase-locked echotracking system. There were no significant differences in clinical characteristics among the ecNOS genotypes. The FMD was significantly lower in the patients with ecNOS4a allele than in those without ecNOS4a allele (P < 0.05). Multiple regression analysis showed that ecNOS4a allele and mean blood pressure were significant independent determinants for reduced FMD in all patients (R² = 0.122, P = 0.0025). The ecNOS4a allele was an independent determinant for reduced FMD in smokers but not in nonsmokers. These results suggest that ecNOS4a allele is a genetic risk factor for endothelial dysfunction in diabetic patients, especially in smokers.

endothelial constitutive nitric oxide synthase gene; diabetes mellitus; flow-mediated vasodilation; smoking

VASCULAR COMPLICATIONS are the main causes of morbidity and mortality in patients with diabetes. Several lines of evidence suggest that endothelial damage could play a key role in the development of both micro- and macroangiopathy in diabetes. Recently, the role of the L-arginine/nitric oxide (NO) pathway in the regulation of vascular smooth muscle tone has attracted increasing interest. Endothelium-derived NO is a potent endogenous vasodilator (17). In addition to regulating vascular tone, endothelium-derived NO suppresses vascular smooth muscle proliferation (9), inhibits platelet adhesion and aggregation (20), and interferes with leukocyte-endothelial cell interaction (15), which lead to the development of atherosclerosis.

Despite the overwhelming evidence of impaired endothelium-dependent vasodilation in diabetes mellitus (21, 24), there are sporadic reports of preserved endothelium-dependent vasodilation (2). Possible explanations for discrepancies between studies include differences in several risk factors for endothelium-dependent vasodilation: hyperlipidemia (4), smoking (6), diabetic patients with and without complications (16), hypertension with diabetes with coronary heart disease (19), and a family history of coronary artery disease (7). In addition, several lines of evidence suggest that genetic factors contribute to the impairment of vascular endothelial function (3). Among previously reported polymorphisms in the endothelial constitutive NO synthase (ecNOS) gene, a 27-base pair repeat polymorphism in intron 4 of the ecNOS gene (4a/a genotype) was found to be associated with a smoking-dependent risk of coronary artery disease (27). This genotype was also associated with a history of myocardial infarction. These observations raise the possibility that this polymorphism is associated with endothelial dysfunction in patients with Type 2 diabetes, which is a potent risk factor for the development of atherosclerosis and coronary artery disease.

The aim of this study was to evaluate the possible relationships between ecNOS gene polymorphism, endothelium-dependent vasodilation, and endothelium-independent vasodilation in patients with Type 2 diabetes. In addition, because strong evidence for gene-environment interaction between smoking and the ecNOS gene has been obtained (27), we also investigated how cigarette smoking affects the relationship between ecNOS gene polymorphism and endothelial dysfunction.

METHODS

Subjects. A total of 95 patients with Type 2 diabetes were studied. The patients were selected from 120 consecutive patients admitted to Osaka City University Hospital for educational programs on diabetes. The diagnosis of diabetes was made in accordance with diagnostic criteria of the American Diabetes Association (21), and all patients were on diet therapy. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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was based on a previous history of diabetes or on the criteria
given in the Report of the Expert Committee on the Diagnosis
and Classification of Diabetes Mellitus (23). Patients with a
history of cardiovascular diseases, those with advanced dia-
betic complications (macroalbuminuria or proliferative reti-
opathy), and those treated with antioxidative vitamins, an-
giotensin-converting enzyme inhibitors, or insulin were
excluded from the present study. Each subject gave informed
consent to participate in this study. The Ethics Committee on
Clinical Investigation of the Osaka City University Medical
School approved the study methodology.

Blood pressure was measured as previously described (14),
and 30 patients had hypertension defined as follows: systolic
blood pressure ≥160 mmHg or diastolic blood pressure ≥95
mmHg. The lifelong exposure to smoking was expressed as
cigarettes per day multiplied by exposure year (termed as
cigarette years).

Thirty-eight patients were treated with diet alone (25 to 30
kcal per ideal body weight), and 57 patients were treated with
sulfonylureas. The patients were seen at least at 14-day inter-
vals before the study. Sulfonylureas were discontinued 24 h
before the study. After an overnight fast, endothelium-depen-
dent and -independent vasodilations were studied, and blood
sampling was performed before the vascular study in each
patient. Fifty-two patients had dyslipidemia defined as the
following: serum levels of total cholesterol ≥5.69 mmol/l, trigly-
ceride ≥1.69 mmol/l, or high-density lipoprotein (HDL) cholesterol
≥1.03 mmol/l. Twenty-eight patients were treated with statins.

Endothelium-dependent and -independent vasodilations of
the right brachial artery. Endothelium-dependent (flow-me-
diated, FMD) and -independent (nitroglycerin-induced, NTG)
vasodilations of the right brachial artery were measured by the
same examiner. The measurements were performed ac-
cording to the method described by Celermajer et al. (5, 6) in
a temperature-controlled (22°C) room. The arterial diameter
was measured using an ultrasonic phase-locked echo-track-
ing system, which was equipped with a high-resolution, real-
time 7.5-MHz linear scanner in B-mode (SSD 610; Aloka,
Tokyo). The first ultrasound examination was performed with
subjects in the supine position after they had rested for
at least 15 min. A longitudinal section of the right brachial
artery 2–12 cm above the elbow was scanned. The same
observer, who was unaware of clinical details and the stage of
the experiment, measured vessel diameter. The arterial di-
ameter was measured from the anterior to the posterior
interface between the media and adventitia (“m” line (14) at
a fixed distance from an anatomic marker (6). The mean
diameter was calculated from four cardiac cycles synchro-
nized with the R wave peaks on the electrocardiogram (ECG).
All measurements were made at end diastole. Blood flow was
then increased by inflating a pneumatic tourniquet to a
pressure of 250 mmHg for 5 min. The second scan was taken
for 30 s before and 90 s after cuff deflation; additional scans
were recorded 3, 5, and 10 min later. Another resting scan
was obtained 15 min later to confirm the vessel recovery. Scans
were then obtained 3, 5, and 10 min after administra-
tion of a sublingual spray of nitroglycerin (300 µg/spray). The
ECG was monitored continuously, and blood pressure was
measured in the left arm during the ultrasound study. The
maximal diameter changes caused by percent FMD (%FMD)
and percent NTG (%NTG) were expressed as the percent change
relative to that at the initial resting scan. In addition, the
intima-media thickness of the right brachial arterial
posterior wall was measured 2 cm proximal to the elbow joint
as previously reported (12).

Reproducibility of the ultrasound study. Ten diabetic pa-
tients were examined on two different occasions 7 days apart
to estimate the intraobserver variability of the values of
%FMD and %NTG of the brachial arterial diameter by the
same examiner, who was unaware of the values from the first
examination. The coefficient of variation was 4.3% for %FMD
and 3.8% for %NTG.

DNA study. Polymorphism of the ecNOS gene in intron 4
was assessed by the polymerase chain reaction (PCR) method
from peripheral leukocytes as previously reported (27). The
PCR products were separated by electrophoresis in 2% agar-
ose gel. We identified two alleles as ecNOS4a for four tan-
dem 27-bp repeats (393 bp) and ecNOS4b for tandem repeats
(420 bp).

Biochemical analysis. For each diabetic patient, the 24-h
urinary albumin excretion was the mean value obtained from
3 consecutive days. Normoalbuminuria was defined as a
urinary albumin excretion <20 µg/min and microalbumin-
uria as a urinary albumin excretion ≥20 µg/min and ≤200
µg/min. The plasma glucose and HbA1c levels were mea-
sured as previously described (13). Serum total cholesterol,
triglyceride, HDL cholesterol, and creatinine levels were
measured by an autoanalyzer. Urinary albumin was mea-
sured by immunoturbidimetry (TIA MicroAlb Kit; Nittobo,
Tokyo).

Statistical analysis. Values are expressed as means ± SE
unless otherwise indicated. Differences in variables among
the groups were analyzed by unpaired t-test or χ²-test. Be-
cause of the low prevalence of the ecNOS4a/a genotype, mul-
tiple regression analyses were performed to assess the
magnitude of individual effects on vascular endothelial func-
tion. The following factors were considered as independent
variables: age, gender (male = 1, female = 0), body mass
index (BMI), cigarette years, duration of diabetes, mean
blood pressure, fasting plasma glucose, HbA1c, low-density
lipoprotein (LDL) cholesterol, triglyceride, HDL cholesterol,
and presence of the ecNOS4a allele (4b/b, 0; 4b/a or
4a/a = 1) (model 1); and age, gender (male = 1, female = 0), BMI,
duration of diabetes, mean blood pressure, fasting plasma
glucose, HbA1c, LDL cholesterol, triglyceride, HDL choles-
terol, and presence of the ecNOS4a allele (4b/b = 0, 4b/a or
4a/a = 1) (model 2). Because the smoking-related risk for
 coronary disease has been demonstrated for subjects with
ecNOS4a/a genotype, the association between gene polymor-
phism and endothelial function was separately analyzed in
smokers and nonsmokers. The %FMD and %NTG were com-
pared among the four groups (smokers with and without
ecNOS4a allele and nonsmokers with and without ecNOS4a allele).
These procedures were performed on a Macintosh computer using the StatView V Statistical System. Values of
P < 0.05 were considered statistically significant.

RESULTS

The frequencies of ecNOS genotypes in our diabetic
patients (4a/a, n = 3, 3%; 4b/a, n = 30, 32%; and 4b/b,
4b/b, n = 62, 65%) were consistent with those reported by
Wang et al. (27) (1%, 32%, and 67%, respectively). Clinical characteristics of the patients are shown in
Table 1. There was no signi

ificant difference in the percentage of patients treated with statins between the
patients with and without ecNOS4a allele (4a/a or 4b/a,
n = 9, 27%; and 4b/b, n = 19, 31%).

ecNOS gene polymorphism and endothelial function,
%FMD and %NTG. The %FMD was significantly lower in the patients with ecNOS4a allele (5.10 ± 0.67%)
than in those without ecNOS4a allele (7.07 ± 0.40%) (P < 0.05) (Fig. 1A). On the other hand, there were no

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significant differences in %NTG between the patients with and without ecNOS4a allele (4a/a or 4b/a, 12.58 ± 0.61%, respectively) (Fig. 1B). Because of the small number of patients with ecNOS4a/a genotype, multiple regression analysis was performed to determine the impact of ecNOS4a allele on %FMD and %NTG in all patients. The presence of ecNOS4a allele and mean blood pressure were independent determinants for reduced %FMD ($R^2 = 0.122$, $P = 0.0025$), and the duration of diabetes was an independent determinant for reduced %NTG ($R^2 = 0.076$, $P = 0.0069$) in all patients (model 1) (Table 2).

**DISCUSSION**

This is the first study demonstrating that 4a/b polymorphism of the ecNOS gene is associated with endothelium-dependent vasodilation but not with endothelium-independent vasodilation in patients with Type 2 diabetes. Our findings that the ecNOS4a allele has a significant effect on endothelium-dependent vasodilation but not on endothelium-independent vasodilation suggest that this is a specific effect limited to NO production and/or release. We previously reported (12) a similar finding for patients with Type 2 diabetes, in a study in which endothelium-dependent vasodilation was significantly lower in Type 2 diabetic patients than in control subjects. We also found a significant association of the ecNOS4a allele with impairment of endothelium-dependent vasodilation in smokers but not in nonsmokers. These results indicate that the ecNOS4a allele has a detrimental effect on endothelium-dependent vasodilation but not on endothelium-independent vasodilation.

**Table 1. Characteristics of diabetic patients segregated by the presence of ecNOS4a allele**

<table>
<thead>
<tr>
<th>ecNOS4a/a:b Genotype</th>
<th>4a/a + 4b/a</th>
<th>4b/b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>58 ± 11</td>
<td>55 ± 10</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>24/9</td>
<td>48/14</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22 ± 3</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>Current smoker (+/−)</td>
<td>15/18</td>
<td>28/34</td>
</tr>
<tr>
<td>Duration of diabetes, yr</td>
<td>14 ± 10</td>
<td>10 ± 10</td>
</tr>
<tr>
<td>Fasting plasma glucose, mM/l</td>
<td>8.6 ± 2.8</td>
<td>9.0 ± 2.6</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>8.6 ± 1.5</td>
<td>8.6 ± 2.2</td>
</tr>
<tr>
<td>Total cholesterol, mM/l</td>
<td>5.1 ± 0.8</td>
<td>5.4 ± 1.2</td>
</tr>
<tr>
<td>HDL cholesterol, mM/l</td>
<td>1.4 ± 0.5</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>Triglyceride, mM/l</td>
<td>1.3 ± 0.9</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>126 ± 22</td>
<td>125 ± 20</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>74 ± 11</td>
<td>74 ± 11</td>
</tr>
<tr>
<td>Microalbuminuria (+/−)</td>
<td>21/12</td>
<td>40/22</td>
</tr>
<tr>
<td>Retinopathy (+/−)</td>
<td>15/18</td>
<td>36/26</td>
</tr>
<tr>
<td>Hypertension (+/−)</td>
<td>25/8</td>
<td>40/22</td>
</tr>
<tr>
<td>Dyslipidemia (+/−)</td>
<td>16/17</td>
<td>27/35</td>
</tr>
<tr>
<td>Diabetes therapy (diet/SU)</td>
<td>10/23</td>
<td>29/34</td>
</tr>
<tr>
<td>Baseline vessel diameter, mm*</td>
<td>3.8 ± 0.7</td>
<td>4.1 ± 0.7</td>
</tr>
<tr>
<td>IMT of brachial artery</td>
<td>0.44 ± 0.12</td>
<td>0.46 ± 0.11</td>
</tr>
</tbody>
</table>

Values are means ± SD. ecNOS, endothelial constitutive nitric oxide synthase; BMI, body mass index; HDL, high-density lipoprotein; SU, sulfonylurea; IMT, intima-media thickness. *Baseline diameter of the right brachial artery.

Fig. 1. A: percent flow-mediated (endothelium-dependent) vasodilation (%FMD) among patients with and without endothelium constitutive nitric oxide (ecNOS) 4a allele. Data are expressed as medians, interquartiles (open boxes), and ranges (10th and 90th percentile levels). %FMD was significantly lower in patients with ecNOS4a allele than in those without ecNOS4a allele. *$P < 0.05$ vs. patients without ecNOS4a allele. B: percent nitroglycerin-mediated (endothelium-independent) vasodilation (%NTG) between patients with and without ecNOS4a allele. There was no significant difference in %NTG between patients with and without ecNOS4a allele.

**Table 2. Factors associated with %FMD and %NTG in all patients**

<table>
<thead>
<tr>
<th>Independent</th>
<th>β</th>
<th>$F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>%FMD</td>
<td>ecNOS4a allele</td>
<td>−0.256</td>
</tr>
<tr>
<td>Mean blood pressure</td>
<td>−0.232</td>
<td>5.660</td>
</tr>
<tr>
<td>%NTG</td>
<td>Duration of diabetes</td>
<td>−0.276</td>
</tr>
</tbody>
</table>

Independent variables included presence of ecNOS4a allele, age, gender, BMI, cigarette smoking, duration of diabetes, mean blood pressure, fasting plasma glucose, HbA1c, low density lipoprotein (LDL) cholesterol, triglyceride, and HDL cholesterol for percent flow-mediated vasodilation (%FMD) or percent nitroglycerin-mediated vasodilation (%NTG). The $F$ value was set at 4.0 at each step. $β$ is the standard regression coefficient; $R^2$ is the multiple coefficient of determination.
long-term smokers (5). Our findings demonstrate asso-
ciations between the 4a/b polymorphism of the ecNOS gene and smoking, which is an established risk factor for impaired endothelial function. Although our study does not identify any mechanism involved in the contribution of the ecNOS gene to endothelial dysfunction, we hypothesize that polymorphism of the ecNOS gene could affect NO production-induced reactive hyperemia. NO contributes to all phases of reactive hyperemia in the human peripheral vasculature (8). Evidence for a relationship between ecNOS gene polymorphism and impaired function of ecNOS was obtained in a previous study of NO metabolite (NOx) levels in patients with different ecNOS genotypes. Homozygotes for the ecNOS4a allele had a nearly 20% lower mean plasma NOx level, and carriers of the ecNOS4a allele had a significantly lower NOx level than did noncarriers (25). Thus, because in vivo and in vitro studies have demonstrated that the endothelium, by reduced NO synthesis and/or release, blunts endothelium-dependent vasodilation (1, 26), there may be less production of NO in patients with the ecNOS4a allele. Change in endothelial function may have important clinical implications for the pathogenesis of cardiovascular diseases. As suggested by the present study, functional alterations of the endothelium-derived NO pathway, especially those involved in the pathogenesis of diabetic vascular complications, may be due to decreased endothelial release of NO related to the presence of the ecNOS4a allele. Among few reported polymorphic markers in ecNOS gene, associations between these polymorphisms and endothelial function are controversial. The G894T polymorphism of the ecNOS gene was shown to be associated with an enhanced vascular responsiveness to phenylephrine (18). On the other hand, the Glu298Asp polymorphism of the ecNOS gene was reported to have no association with endothelial function (22). The present study showed the first evidence for an association between the 4a/b polymorphism of the ecNOS gene and endothelium-dependent vasodilation in Type 2 diabetes.

Blunting of endothelium-dependent vasodilation by smoking was observed in patients with the ecNOS4a allele but not in those without it in the present study. Furthermore, multiple regression analysis demonstrated that this allele was an independent determinant for reduced endothelium-dependent vasodilation in smokers but not in nonsmokers. Previously, Wang et al. (27) presented strong evidence for a gene-environ-
ment interaction between smoking and the ecNOS gene. The association between ecNOS gene polymorphism and ecNOS activity was reported to be modifiable by smoking (28). Smoking-induced vascular damage is considered a consequence of enhanced degradation of NO secondary to formation of oxygen-derived free radicals (11) as well as thrombogenic effects (10). The antioxidant vitamin C has been reported to improve endothelial dys-
function in smokers (11). Taken together, these findings suggest that smoking could be risk factor for cardiovascular disease by enhancing degradation of NO by free radicals. In the subjects with the 4a allele of the ecNOS gene, NO might be susceptible to degradation by the free radicals derived from smoking.

In conclusion, we have found a modest association between ecNOS polymorphism of the NO synthase gene and endothelium-dependent vasodilation and have identified increased risk for impaired endothelium-dependent vasodilation in diabetic patients with the ecNOS4a allele associated with cigarette smoking.

Table 3. Factors associated with %FMD in smokers

<table>
<thead>
<tr>
<th>Dependent</th>
<th>Independent</th>
<th>β</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>%FMD</td>
<td>ecNOS4a allele</td>
<td>-0.341</td>
<td>7.579</td>
</tr>
<tr>
<td></td>
<td>Triglyceride</td>
<td>-0.312</td>
<td>6.320</td>
</tr>
</tbody>
</table>

Independent variables included presence of ecNOS4a allele, age, gender, BMI, duration of diabetes, mean blood pressure, fasting plasma glucose, HbA1c, LDL cholesterol, triglyceride, and HDL cholesterol for %FMD. The F value was set at 4.0 at each step.
REFERENCES


