Salt Intake Affects the Relation Between Hypertension and the T-786C Polymorphism in the Endothelial Nitric Oxide Synthase Gene

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Background: Recent genetic studies have shown an association between the T-786C polymorphism in the endothelial NO synthase gene and coronary artery diseases, but any possible association with hypertension has been controversial. Other studies indicate the effect of restricting salt intake differ depending on individual salt-sensitivity, and the mixture of different sensitivity in study subjects may obscure the results. The objective of this study was to investigate the gene–environment interaction between the salt intake and this polymorphism.

Methods: We genotyped 281 healthy men after excluding 37 men on hypertensive therapy (mean age 44.8 ± 11.9 years) for the mutation, and evaluated their daily salt intake using a validated food frequency questionnaire.

Results: A quartile classification of salt intake revealed that the blood pressure of subjects with the mutation was significantly higher than that of subjects without the mutation, but only in the 4th quartile (the highest intake group). A multiple logistic regression analysis also showed that the presence of this mutation increased the risk of hypertension only in the 4th quartile (adjusted odds ratio = 6.38, P = .025).

Conclusions: The presence of this mutation alone does not significantly increase the risk of hypertension. However, high salt intake interacts with the mutation and leads to a significant increase in the risk of hypertension. The T-786C mutation warrants being considered a candidate for further study with the aim of tailoring hypertension prevention. Am J Hypertens 2005;18:1556–1562 © 2005 American Journal of Hypertension, Ltd.

Key Words: Hypertension, essential, nitric oxide synthase, polymorphism, sodium.

Hypertension, a complex disease, is a risk factor for cardiovascular diseases and is involved in multiple gene–environment associations. Although the pathogenic mechanisms of hypertension are not clear, accumulating evidence from clinical and animal studies strongly suggests an association between altered nitric oxide (NO) metabolism and hypertension.1,2 Hypertensive patients have low plasma and urinary NO levels compared with normotensive individuals,1 and animal models with impaired NO bioavailability develop hypertension.2

Nitric oxide, a potent vasodilator produced by endothelial cells, plays a prominent role in regulating blood pressure (BP), and many lines of evidence suggest correlations between various genetic polymorphisms of the endothelial nitric oxide synthase (eNOS) gene and cardiovascular diseases.3–17 The eNOS gene consists of 26 exons and has a total size of 21 kb;3 the various known genetic polymorphisms include a 27-base pair, tandem-repeat polymorphism in intron 4 (ecNOS4a/b), 3 linked point mutations in the 5′-flanking region (T-786C, A-922G, and T-1468A), and a missense mutation (Glu298Asp).4 According to multiple reports, the ecNOS4a/b polymorphism is associated with smoking-dependent coronary artery disease and myocardial infarction,5,6 the T-786C mutation is associated with coronary spasm,7 myocardial infarction,8 and internal carotid artery stenosis,9 and the missense Glu298Asp variant is associated with hypertension, myocardial infarction, coronary spasm, and other conditions.10–14 Furthermore, Yoshimura et al15 reported that ecNOS4a/b is in linkage
The mechanisms responsible for salt-sensitivity of an individual’s BP to a high-salt diet, but the renin–angiotensin system (RAS) certainly plays an important role. Many factors influence the response of an individual’s BP to a high-salt diet, but the renin–angiotensin system (RAS) certainly plays an important role.

The association between the T-786C mutation and hypertension was previously investigated, but salt intake was not taken into consideration. In this study we investigate whether salt intake affects the relationship between the T-786C point mutation and hypertension.

**Methods**

**Study Subjects**

We obtained written informed consent from genetically unrelated 318 healthy Japanese men for participation in the study and excluded 37 men who were receiving hypertensive therapy. The study design and the informed consent form were approved by the ethical committee of Keio University School of Medicine.

The mean (±SD) age and body mass index (BMI) of the enrolled 281 subjects were 44.8 ± 11.9 years and 23.3 ± 3.55 kg/m², respectively. These values are typical for healthy Japanese male workers.

Height, weight, waist circumference, systolic blood pressure (SBP), diastolic blood pressures (DBP), fasting plasma glucose level, and serum lipid levels were measured in all subjects. In addition information on age and smoking status of all subjects was obtained using a self-reported questionnaire.

Well-trained nurses measured the BP of each subject twice using a form PWV/ABI device (Nippon Colin, Aichi, Japan) while the subject rested in supine position. This device was approved by the United States Food and Drug Administration as VP-2000/1000.

We defined hypertension as SBP ≥140 mm Hg or DBP ≥90 mm Hg, according to the criteria of the World Health Organization.

**Estimation of Daily Nutrient Intake**

A food frequency questionnaire (FFQ) validated by Takahasi et al was used to calculate energy intake, salt intake, and protein:fat:carbohydrate (PFC) ratio. The FFQ used in this study contained questions about the consumption of food items in 29 food groups over the previous 1 or 2 months. The daily nutrient intake was calculated by multiplying the frequency at which each food was consumed by the nutrient content of the portion size and summing the products for all food items.

**Genotyping for eNOS Gene Polymorphisms**

A peripheral blood specimen was collected from each subject, and genotyping for the T-786C point mutation in the eNOS gene was performed using the polymerase chain reaction technique and a single nucleotide primer extension (SNuPE) assay with Ampdirect (Shimadzu Corporation, Kyoto, Japan).

A 393-bp fragment of the gene encompassing the mutation site was amplified by polymerase chain reaction using a 5’-primer (5’GAAAGAGGTCGGGGAGTCTA) and a 3’-primer (5’GATAGAGGCCCAGCAAGGAT) designed using DNASIS Pro Version 2.0 (Hitachi Software Engineering Co. Ltd., Tokyo, Japan). The SNuPE assay was based on the incorporation of a single fluorescent-labeled ddNTP, which was correctly paired with the template DNA to cause chain termination, to the 3’ terminus of a primer annealed next to the polymorphic site. The products were analyzed using an ABI 7700 (Amersham Biosciences Corp., Piscataway, NJ).

**Statistical Analysis**

A Student t test was used to compare normally distributed variables between groups. Variables that were not normally distributed were log transformed. When the log transformations of the variables were effective, a Student t test was used; when ineffective, a Mann-Whitney test was used. A χ² test was used to compare categorical variables.

Trend in quartiles of salt intake was analyzed by trend test with adjustment. To calculate the odds ratios (OR) and 95% confidence intervals (CI), a multiple logistic regression analysis was performed for hypertension, defined as SBP ≥140 mm Hg or DBP ≥90 mm Hg, with the presence of the T-786C mutation in the eNOS gene, age, BMI, and smoking status as variables.

Differences were assessed using two-sided tests, with an α level of 0.05. All statistical analyses were performed using the Statistical Package for the Social Sciences for Windows, Version 11, software (SPSS Inc., Chicago, IL).

**Results**

**Distribution of the T-786C Mutation in the Subject Population**

Genotyping for the T-786C mutation in the eNOS gene in the 318 healthy Japanese male subjects showed that 227 subjects were homozygous for the normal allele (T/T), 53 were heterozygous for the mutation (T/C), and one was homozygous for the mutation (C/C) (allelic frequency = 0.098). The frequencies of the genotypes agreed with those predicted by Hardy-Weinberg equilibrium.

**Clinical Characteristics of the Study Subjects, According to eNOS Genotype**

The main characteristics of the subjects are shown in Table 1. No significant differences in SBP, DBP, or the other parameters
Table 1. Clinical characteristics of the subjects according to eNOS T-786C genotype

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All subjects</th>
<th>Wild type (T/T)</th>
<th>Mutant carrier (T/C or C/C)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>281</td>
<td>227</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>44.8 ± 11.9</td>
<td>44.7 ± 11.9</td>
<td>45.1 ± 11.9</td>
<td>.830</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>169 ± 6.22</td>
<td>169 ± 6.00</td>
<td>168 ± 7.07</td>
<td>.294</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>66.7 ± 11.5</td>
<td>66.7 ± 11.5</td>
<td>66.6 ± 11.5</td>
<td>.936</td>
</tr>
<tr>
<td>Body mass index (kg/)</td>
<td>23.3 ± 3.55</td>
<td>23.2 ± 3.55</td>
<td>23.5 ± 3.57</td>
<td>.675</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>83.7 ± 9.71</td>
<td>83.4 ± 9.66</td>
<td>84.7 ± 9.72</td>
<td>.403</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>133 ± 17.3</td>
<td>132 ± 17.0</td>
<td>137 ± 17.6</td>
<td>.061</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>80.5 ± 12.6</td>
<td>79.9 ± 12.4</td>
<td>82.8 ± 12.9</td>
<td>.133</td>
</tr>
<tr>
<td>Fasting plasma glucose (mg/dL)*</td>
<td>96.0[76.2–121]</td>
<td>207 ± 38.0</td>
<td>206 ± 38.3</td>
<td>.831</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>207 ± 38.0</td>
<td>206 ± 38.3</td>
<td>208 ± 36.9</td>
<td>.648</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)*</td>
<td>113[65.0–196]</td>
<td>1836 ± 514</td>
<td>1870 ± 437</td>
<td>.654</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)*</td>
<td>54.1[42.3–69.3]</td>
<td>9.81 ± 3.33</td>
<td>9.67 ± 3.34</td>
<td>.171</td>
</tr>
<tr>
<td>PFC ratio (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (%)</td>
<td>13.9 ± 2.41</td>
<td>13.9 ± 2.52</td>
<td>13.6 ± 1.95</td>
<td>.388</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>27.8 ± 5.46</td>
<td>28.0 ± 5.50</td>
<td>27.1 ± 5.29</td>
<td>.295</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>58.4 ± 7.07</td>
<td>58.1 ± 7.21</td>
<td>59.3 ± 6.47</td>
<td>.269</td>
</tr>
<tr>
<td>Energy intake (kcal/day)</td>
<td>1843 ± 498</td>
<td>1836 ± 514</td>
<td>1870 ± 437</td>
<td>.654</td>
</tr>
<tr>
<td>Salt intake (g/day)</td>
<td>9.81 ± 3.33</td>
<td>9.67 ± 3.34</td>
<td>10.4 ± 3.25</td>
<td>.171</td>
</tr>
<tr>
<td>Smoker/nonsmoker†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DBP = diastolic blood pressure; PFC = protein:fat:carbohydrate; SBP = systolic blood pressure.

Subjects carrying T/C or C/C genotype were compared with subjects carrying T/T genotype.

Values are mean ± SD (Student t test).

* Mann-Whitney test (values are genometric mean and 95% confidence intervals).
† χ² test.

shown in Table 1 were seen between subjects with the T-786C mutation and subjects without the mutation (T/C or C/C versus T/T: SBP, 136.7 ± 17.6 vs 131.7 ± 17.0 mm Hg, P = .061; DBP, 82.8 ± 12.9 vs 79.9 ± 12.4 mm Hg, P = .133). These results were consistent with previously reported results for another Japanese population.16

The subjects were classified into quartiles according to salt intake based on their replies to the FFQ. The ranges of salt intake in each quartile are shown in Table 2 (1st quartile: <7.5 g/day, 2nd quartile: 7.5 to 9.2 g/day, 3rd quartile: 9.2 to 11.7 g/day, and 4th quartile: >11.7 g/day).

Table 2. Mean values of systolic blood pressure (SBP) and diastolic blood pressure (DBP) in each quartile according to salt intake

<table>
<thead>
<tr>
<th>Blood pressure</th>
<th>1st quartile (7.5 g/day)</th>
<th>2nd quartile (7.5–9.2 g/day)</th>
<th>3rd quartile (9.2–11.7 g/day)</th>
<th>4th quartile (11.7 g/day)</th>
<th>P value for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mm Hg)</td>
<td>130.6 ± 17.6</td>
<td>130.0 ± 17.3</td>
<td>132.4 ± 16.2</td>
<td>137.8 ± 18.2</td>
<td>.022†*</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>78.6 ± 12.0</td>
<td>79.2 ± 12.4</td>
<td>81.1 ± 11.9</td>
<td>83.1 ± 14.0</td>
<td>.192</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>Wild-type (T/T)</td>
<td>130.9 ± 18.3</td>
<td>130.5 ± 17.7</td>
<td>132.4 ± 17.2</td>
<td>.878</td>
</tr>
<tr>
<td></td>
<td>Mutant type (T/C or C/C)</td>
<td>129.3 ± 14.6</td>
<td>128.2 ± 15.8</td>
<td>132.5 ± 12.8</td>
<td>&lt;.001§</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>Wild-type (T/T)</td>
<td>78.7 ± 12.3</td>
<td>79.7 ± 12.2</td>
<td>80.8 ± 12.3</td>
<td>.676</td>
</tr>
<tr>
<td></td>
<td>Mutant type (T/C or C/C)</td>
<td>78.2 ± 10.8</td>
<td>77.2 ± 13.7</td>
<td>82.3 ± 10.4</td>
<td>.001§</td>
</tr>
</tbody>
</table>

Values are means ± SD. Student t test and trend test after adjustment using age, body mass index, and smoking.

* P < .05, Trend test.
† P < .01, Student t test.
‡ P < .01, Trend test.
§ P < .05, Student t test.
Table 3. Multiple logistic regression analysis on hypertension in 4th quartile

<table>
<thead>
<tr>
<th>Explanatory variable</th>
<th>Adjusted OR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of mutation in eNOS gene</td>
<td>6.38*</td>
<td>1.26–32.3</td>
<td>&lt;.025</td>
</tr>
<tr>
<td>Age</td>
<td>1.13†</td>
<td>1.04–1.23</td>
<td>&lt;.005</td>
</tr>
<tr>
<td>BMI</td>
<td>1.23*</td>
<td>1.02–1.49</td>
<td>&lt;.033</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.77</td>
<td>0.21–2.78</td>
<td>&lt;.686</td>
</tr>
</tbody>
</table>

CI = confidence interval; BMI = body mass index; OR = odds ratio.

Presence of mutation in eNOS gene, age, BMI, and smoking were considered as variables.
Hypertension was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg.
* P < .05; † P < .01

Association Between BP and eNOS Genotype, According to Salt Intake

The mean SBP and DBP values for each quartile are shown in Table 2. A trend test after adjustment using age, BMI, and smoking showed a positive linear trend between SBP and salt intake (SBP, P = .022).

We then divided each quartile into a subgroup with the T-786C mutation (T/C or C/C) and a subgroup without the mutation (T/T), and compared the BP values in the two subgroups for each quartile (Table 2). Both the SBP and DBP values of the subjects with the T-786C mutation were significantly higher than those in the subjects without the mutation, but only for subjects in the 4th quartile (T/C or C/C versus T/T: SBP, 149.9 ± 16.3 v 133.1 ± 16.9 mm Hg, P = .001; DBP, 90.3 ± 12.2 v 80.3 ± 13.7 mm Hg, P = .014). In addition, the trend test adjustment using age, BMI, and smoking showed a positive linear trend between BP and salt intake in the group with the T-786C mutation (SBP, P < .001; DBP, P = .001) but not in the group without the mutation (SBP, P = .878; DBP, P = .676).

Although an interaction between salt intake and the T-786C mutation was found to influence BP, no such interaction between smoking and the mutation was observed (data not shown).

Odds Ratio for Hypertension in the Presence of the T-786C Mutation

The results of the multiple logistic regression analysis for hypertension, defined as SBP ≥140 mm Hg or DBP ≥90 mm Hg, in the 4th quartile in subjects with the T-786C mutation, age, BMI, and smoking as variables are shown in Table 3. The presence of the T-786C mutation was strongly associated with an increased risk of hypertension (adjusted OR = 6.38, P = .025), compared with the other variables (age: adjusted OR = 1.13, P = .005; BMI: adjusted OR = 1.23, P = .033; smoking: adjusted OR = 0.767, P = .686). The results of multiple logistic regression analyses for hypertension using the same variables mentioned above demonstrated that the presence of the T-786C mutation was not associated with an increased risk of hypertension in the 1st, 2nd, or 3rd quartiles (data not shown).

Discussion

In this study, we demonstrated that the T-786C mutation in the 5'-flanking region of the eNOS gene certainly affected the positive linear trend between salt intake and BP. Furthermore, the presence of the T-786C mutation increased the risk of hypertension in subjects with a high salt intake (adjusted OR = 6.38, P = .025) (Table 3). However, when salt intake was not taken into consideration, no significant differences in SBP and DBP were seen between subjects with the T-786C mutation and those without the mutation (Table 1). Therefore the association between the T-786C mutation and BP might be veiled by the interaction effect of salt intake.

The relation between the T-786C mutation and hypertension remains controversial.16,17 Kajiyama et al16 showed that the T-786C mutation was not associated with hypertension. On the other hand, Hyndman et al17 reported that the T-786C mutation was associated with hypertension. Our results confirm those of Kajiyama et al16 for a Japanese population. The apparent discrepancies between the studies may be explained by the different allelic frequencies observed in ethnic groups. The frequencies of the C allele in populations of white ethnicity are much higher than those in the Japanese population.7–9,16,17 and homozygous subjects (C/C) can be separately analyzed in populations of white ethnicity. Therefore the findings of the present study might not be applicable to other ethnic groups, and further investigations comparing the situations in different populations are needed.

A positive linear trend between SBP and salt intake was seen in all subjects (Table 2). This finding is consistent with previous reports.18–21 When the subjects were divided into quartiles according to salt intake and then into subgroups according to the presence of the T-786C mutation, a positive linear trend between BP and salt intake was seen in the subgroup with the T-786C mutation but not in the group without the mutation (Table 2). The graphs of the trend between BP and salt intake according to the eNOS genotype are shown in Fig. 1. These data suggest that the overall positive linear trend between BP and salt intake may be partially attributed to a stronger association.
are important factors. When sodium is consumed, it is high-salt diet, renal dysfunctions in sodium excretion factors influence the response of an individual’s BP to a of eNOS in the kidney also exists. Although many and a positive relation between RAS and the expression between BP and salt intake among subjects with the T-786C mutation.

Among subjects in the 4th quartile, the subjects with the T-786C mutation had a significantly higher BP than those without the mutation. In other words, the subjects with the T-786C mutation had a significantly higher than in the group without the mutation (SBP, \( P = .001; \) DBP, \( P = .014 \)) and there is a positive linear trend between BP and salt intake in the group with the T-786C mutation (SBP, \( P < .001; \) DBP, \( P = .001 \)), whereas no positive linear trend was found between BP and salt intake in the group without the mutation (SBP, \( P = .878; \) DBP, \( P = .676 \)). Student \( t \) test and trend test were used after adjustment for age, body mass index, and smoking. \( * P < .05; \) \( \dagger P < .01 \) for Student \( t \) test.

In conclusion, the T-786C mutation in the eNOS gene, which has a high allelic frequency in the Japanese population, does not by itself increase the risk of hypertension, but a high salt intake interacts with the T-786C mutation, leading to a significant elevation in BP. These findings indicate that the proper control of salt intake may prevent the development of hypertension and its complications, even in subjects with the T-786C mutation. However the efficacy of salt intake control has not yet been demonstrated, and a standard for effective salt intake control for the prevention or treatment of hypertension has not yet been established in subjects with the T-786C mutation. To evaluate the effectiveness and feasibility of using the findings from this study, an intervention study is required. The T-786C point mutation warrants further study in individual approaches to the prevention of hypertension.

### Acknowledgments
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References


