Endothelial nitric oxide synthase gene haplotypes associated with circulating concentrations of nitric oxide products in healthy men

Ingrid F. Metzger\textsuperscript{a}, Debora C. Souza-Costa\textsuperscript{a}, Aline S. Marroni\textsuperscript{b}, Sabrina Nagassaki\textsuperscript{a}, Zeruesenay Desta\textsuperscript{c}, David A. Flockhart\textsuperscript{c} and Jose E. Tanus-Santos\textsuperscript{a}

Objectives Controversy exists regarding the effects of polymorphisms in the endothelial nitric oxide synthase (eNOS) gene on nitrites/nitrates (NO\textsubscript{x}) plasma concentrations. In this study we compared the distribution of haplotypes involving three relevant eNOS polymorphisms (T–786C in the promoter region; b/a in intron 4, and Glu298Asp in exon 7) in healthy subjects with low and high circulating NO\textsubscript{x} levels.

Methods We studied 154 healthy subjects (fasting, white males, who were non-smokers, 18–60 years of age, and not taking any medication). Genomic DNA was isolated from blood samples and genotypes were determined by PCR and restriction fragment length digestion. Circulating NO\textsubscript{x} was determined by chemiluminescence.

Results Haplotype frequencies were compared in two groups of subjects: those with the 30 lowest NO\textsubscript{x} levels (group L) and those with the 30 highest NO\textsubscript{x} levels (group H). NO\textsubscript{x} levels in group L and H were 24.2 ± 4.5 lM and 80.9 ± 8.9 lM, respectively. Genotype frequencies for the three polymorphisms were not different when the two groups were compared (all \(P\) > 0.05, chi-squared test). However, the haplotype including the alleles C (promoter), 4b (intron 4), and Glu (exon 7) was significantly more common in group L (16%) than in group H (4%) (\(P=0.0047\)). The frequencies of the remaining haplotypes were not different among group L and H.

Conclusions While eNOS genotypes are not significantly associated with changes in the circulating NO\textsubscript{x} concentrations, the specific eNOS haplotype that includes the ‘C’, ‘4b’, and ‘Glu’ alleles is associated with lower circulating NO\textsubscript{x} concentrations. Pharmacogenetics and Genomics 15:565–570 © 2005 Lippincott Williams & Wilkins.

Keywords: endothelial nitric oxide synthase, genotypes, haplotypes, nitric oxide, polymorphisms

*Department of Pharmacology, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Ribeirao Preto, Brazil, \textsuperscript{b}Department of Pharmacology, State University of Campinas, Campinas, SP, Brazil and \textsuperscript{c}Division of Clinical Pharmacology, Indiana University School of Medicine, Indianapolis, Indiana, USA.

Sponsorship: Fundac¸a˜o de Amparo a Pesquisa do Estado de Sa ˜o Paulo (FAPESP-Brazil), Conselho Nacional de Desenvolvimento Cientı´fico e Tecnolı´gico (CNPq-Brazil).

Correspondence and requests for reprints to Jose Eduardo Tanus-Santos, MD, PhD, Department of Pharmacology, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Av. Bandeirantes, 3900, 14049-900 Ribeirao Preto, SP, Brazil. Tel: +55 16 602 3163; fax: +55 16 633 2301; e-mail: tanus@fmrp.usp.br

Received 23 March 2005 Accepted 14 April 2005

Introduction

Nitric oxide (NO) is widely acknowledged as a major regulator of the cardiovascular system [1,2]. This highly reactive molecule [3] is produced in endothelial cells and platelets by endothelial NO synthase (eNOS), and maintains basal vasodilator tone, inhibits platelet aggregation, attenuates leukocyte adhesion to the endothelium, and modulates smooth muscle proliferation [4]. Therefore a number of studies have been carried out to examine whether polymorphisms in the eNOS gene are associated with cardiovascular diseases [5]. Three polymorphisms in the eNOS gene have been widely studied: a single nucleotide polymorphism (SNP) in the promoter region (T–786C), a SNP in exon 7, and a variable number of tandem repeats (VNTR) in intron 4 [5,6]. However, few studies have attempted to determine the mechanisms by which these genetic variations might affect eNOS enzyme activity. To date, there is limited evidence for impaired NO production as a result of the polymorphism in exon 7 [7] and no evidence for changes associated with the VNTR in intron 4 [5]. Conversely, the T–786C polymorphism reduces the promoter activity by approximately 50% [8,9], thereby lending experimental support to a physiologic role for this SNP.

NO is rapidly oxidized to nitrite and nitrate (NO\textsubscript{x}) in vivo and in vitro. Therefore, measurement of NO\textsubscript{x} in plasma from blood collected after an overnight fast reflects endogenously produced NO [10–13]. While few studies have examined whether eNOS polymorphisms affect the
circulating concentrations of NO$_x$, many studies have not taken into consideration the influence of confounding factors that alter plasma NO$_x$ such as diet, sex differences, ethnicity, clinical conditions, medications, smoking, and environmental chemicals [14]. In addition, most of these studies have focused upon the effects of only a single nucleotide polymorphism on the circulating NO$_x$ concentrations [12,15–17], and this may lead to conclusions that are limited by the lack of power to detect modest effects. As a result of these limitations in previous studies, there is much controversy regarding the possible effects of eNOS polymorphisms on the circulating concentrations of NO$_x$. For example, lower [9], similar [18,19], or higher [20] NO$_x$ concentrations have been reported in carriers of the ‘C’ allele for the polymorphism in the promoter region of eNOS gene. Moreover, lower [21], similar [22,23], or higher [12] NO$_x$ concentrations have been reported in carriers of the ‘4a’ allele for the polymorphism in intron 4. Finally, similar [15] or higher [23] NO$_x$ concentrations were reported in carriers of the ‘Asp’ allele for the polymorphism in exon 7.

In the present study, we simultaneously addressed the effects of the above mentioned eNOS polymorphisms on plasma NO$_x$ in a relatively homogeneous group of 154 healthy subjects that were controlled for all known major confounding factors affecting plasma NO$_x$ concentrations. We also compared the distribution of eNOS haplotypes involving these three relevant eNOS polymorphisms in subjects with low and high circulating NO$_x$ levels.

Materials and methods

Subjects

Approval for use of human subjects was obtained from the Institutional Review Board at the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil. Healthy male white volunteers (N = 154; age range: 18–60 years) who were non-smokers, were recruited from the local population to give blood after informed consent had been obtained. Arterial blood pressure and heart rate were measured three times after at least 15 min of rest. Venous blood samples were collected after overnight (> 12 h) fasting and plasma was separated to measure plasma total cholesterol, triglycerides, and nitrite/nitrate concentrations. Genomic DNA was extracted from the cellular component of 1 ml of whole blood by a salting-out method and stored at −20°C until analyzed.

Genotype determination

T−876C polymorphism in the 5′-flanking region of eNOS

Genotypes for the T−876C polymorphism in the 5′-flanking region of eNOS were determined by PCR amplification using the primers 5′-TGG AGA GTG CTG GTG TAG CCC A-3′ (sense) and 5′-GCC TCC ACC CCC ACC CTG TC-3′ (antisense), and PCR conditions as previously described [6,24,25]. The amplified products were digested with MSPI for at least 3h, at 37°C, producing fragments of 140 bp and 40 bp for the wild-type allele (allele “T”), or 90 bp, 50 bp, and 40 bp in the case of a polymorphic variant (allele ‘C’). Fragments were separated by electrophoresis in 12% polyacrylamide gels and visualized by silver staining.

Glu298Asp polymorphism in exon 7

For the detection of the Glu298Asp polymorphisms in exon 7, the primers 5′-AAG GCA GGA GAC AGT GGA TGG A-3′ (sense) and 5′-CCC AGT CAA TCC CTT TGG TGC TCA-3′ (antisense), and the previously described PCR conditions were used [6,24,25]. The resulting 258 bp fragment was digested with the enzyme BanI for 6 h, at 37°C, producing 163 bp and 85 bp fragments (wild-type) or no digestion (variant allele) that were analyzed by gel electrophoresis in 12% polyacrylamide gels and visualized by silver staining.

VNTR (27 bp-repeat) polymorphism in intron 4

Genotypes for the polymorphic VNTR in intron 4 were determined by PCR using the primers 5′-AGG CCC TTA GTG AGT GCC TTT-3′ (sense) and 5′-TCT CTT AGT GCT GTG GTC AC-3′ (antisense), and the previously described PCR conditions [6,24,25]. Fragments were separated by electrophoresis in 8% polyacrylamide gels and visualized by silver staining.

Measurement of plasma nitrite/nitrate (NO$_x$) concentrations

Venous blood samples were collected in tubes containing ethylenediaminetetraacetic acid and immediately centrifuged at 1000 g for 4 min. Plasma aliquots were then immediately removed and stored at −70°C until analyzed in duplicate for their nitrate content using an ozone-based chemiluminescence assay as previously described [26,27]. This methodology for the measurement of NO$_x$ concentrations has intra-assay and interassay coefficients of variation less than 2.5%, and less than 4.0%, respectively (data not shown).

Statistical analysis and estimation of haplotype frequencies

NO$_x$ concentrations were represented as the mean ± SD. We compared the distribution of genotypes, alleles, and the estimated haplotypes frequencies in subjects with the 30 lowest (L group) NO$_x$ concentrations to those in subjects with the 30 highest (H group) NO$_x$ concentrations measured among the volunteers included in our study. These two groups of subjects were defined with basis on previous studies showing that eNOS polymorphisms may reduce eNOS expression [8,9] or NO release [25] by approximately 40–50%. Therefore, subjects with NO$_x$ concentrations lower than approximately 50% of the general mean NO$_x$ concentration were included in L group. Correspondingly, subjects with NO$_x$ concentrations greater than 150% of the general mean NO$_x$
concentration were included in H group. Differences between the two groups in the genotypes and in the allele frequencies for each polymorphism were assessed using chi-squared tests (StatView for Windows, Cary, North Carolina, USA). The distribution of genotypes for each polymorphism was also assessed for deviation from the Hardy–Weinberg equilibrium by using chi-squared tests.

The Estimating Haplotypes (EH) software program (ftp://linkage.rockefeller.edu/software/eh; assessed on March 19, 2005) was used to estimate the haplotype frequencies in the two groups of subjects. Differences between the two groups in haplotype frequencies were assessed using chi-squared tests. Because we compared the frequencies of eight different haplotypes between the two groups, a corrected P value < 0.0063 (= 0.05/8, which would allow eight simultaneous comparisons) was considered significant. Otherwise, a P value < 0.05 was considered to be statistically significant.

Results
Table 1 summarizes the characteristics of the 154 subjects enrolled in the study, and the characteristics of subjects in groups L and H. No significant differences in age, body mass index, systolic and diastolic arterial blood pressure, or heart rate were found when subjects in group L were compared with subjects in group H, except for NO\textsubscript{x} levels (Table 1, P < 0.001).

The distribution of genotypes for the three polymorphisms studied here showed no deviation from Hardy–Weinberg equilibrium (all P > 0.05). Importantly, we found no significant effects of genotypes for the three polymorphisms on the circulating concentrations of NO\textsubscript{x} (Fig. 1; all P > 0.05). In addition, we found no significant differences in the distribution of genotypes or in allele frequencies for the three polymorphisms studied here when subjects in the L group were compared with subjects in the H group (Table 2; all P > 0.05). However, while the frequencies of seven out of eight possible haplotypes were not significantly different in the L group compared with H group (Table 3; P > 0.05), the haplotype including the ‘C’, ‘4b’, and ‘Glu’ alleles for the polymorphisms in the promoter region, in intron 4, and in exon 7, respectively, was significantly more common in the L group (16%) than in the H group (4%; Table 3; P = 0.0047).

Discussion
Plasma NO\textsubscript{x} are stable end products of NO and most circulating NO\textsubscript{x} derives from NOS-produced NO in fasted volunteers [28,29]. Although we did not control dietary intake of nitrates for a few days before the study, an overnight fasting (> 12 h) reduces NO\textsubscript{x} in plasma by about 50% and reflects endogenously produced NO [10–13]. Importantly, we studied NO\textsubscript{x} in plasma from a relatively homogeneous group of healthy volunteers. Indeed, all subjects included in the present study were white, healthy males, between 18 and 60 years of age, who were non-smokers and not taking any medication. Therefore, factors such as sex differences, effects of the menstrual cycle, age, clinical conditions, medications, and smoking [14] could not affect our findings. This is very important, since men have been reported to have twice as much circulating NO\textsubscript{x} as women [30]. Moreover, circulating NO\textsubscript{x} increases with follicular development in adult women [10]; therefore studies including women should probably take effects of the menstrual cycle into consideration. In contrast with previous reports, the present study was carefully designed to specifically address this issue. Although we chose these strict inclusion criteria in order to maximize the chances of finding an effect of genotype on circulating NO\textsubscript{x}, we found no significant differences in plasma NO\textsubscript{x} in subjects with different genotypes for the three polymorphisms addressed in the present study.

While these findings confirm previous studies showing no effects of the eNOS genotypes on NO\textsubscript{x} [15,18,19,22,23], we found that the haplotype including the ‘C’, ‘4b’, and ‘Glu’ alleles for the polymorphisms in the promoter region, in intron 4, and in exon 7, respectively, was significantly more common in subjects with low circulating

---

### Table 1 Demographic characteristics of study participants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total</th>
<th>L group</th>
<th>H group</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>154</td>
<td>30</td>
<td>30</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td>31.3 ± 10.3</td>
<td>30.5 ± 8.4</td>
<td>34.1 ± 9.7</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m\textsuperscript{2})</td>
<td>25.7 ± 4.1</td>
<td>25.9 ± 4.9</td>
<td>26.8 ± 5.7</td>
<td>NS</td>
</tr>
<tr>
<td>SAP (mmHg)</td>
<td>123.6 ± 12.1</td>
<td>117.5 ± 10.4</td>
<td>132.3 ± 22.0</td>
<td>NS</td>
</tr>
<tr>
<td>DAP (mmHg)</td>
<td>72.3 ± 9.9</td>
<td>66.7 ± 14.2</td>
<td>76.7 ± 13.9</td>
<td>NS</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>66.1 ± 10.1</td>
<td>66.0 ± 15.4</td>
<td>68.3 ± 14.2</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>172.0 ± 33.6</td>
<td>178.5 ± 34.2</td>
<td>177.5 ± 36.5</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>112.8 ± 68.1</td>
<td>90.8 ± 53.4</td>
<td>101.7 ± 59.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NO\textsubscript{x} (μmol/l)</td>
<td>49.7 ± 19.9</td>
<td>24.2 ± 4.5</td>
<td>80.9 ± 8.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are the mean ± S.D.

n = number of subjects.

BMI, body mass index; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; HR, heart rate; NS, not significant.

*P values for L group compared with H group.
Plasma concentrations of nitrite/nitrate (NO\textsubscript{x}) in 154 healthy subjects grouped by polymorphism and genotype: (a) T\textsuperscript{−786}C: n = 54, 78, and 22 for genotypes ‘TT’, ‘TC’, and ‘CC’ respectively; (b) Intron 4: ‘4b/4b’ n = 106, ‘4a/4b’ n = 42 and ‘4a/4a’ n = 6; and (c) Glu298Asp: ‘GG’ n = 65, ‘GT’ n = 76 and ‘TT’ n = 13. The bars indicate the mean ± SD.

Table 2 Genotypes and allele frequency in the L and H groups

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total (n = 154)</th>
<th>L group (n = 30)</th>
<th>H group (n = 30)</th>
<th>Chi-square</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>T\textsuperscript{−786}C</td>
<td>TT</td>
<td>0.351 (54)</td>
<td>0.367 (11)</td>
<td>0.400 (12)</td>
<td>0.5082</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>0.506 (78)</td>
<td>0.500 (15)</td>
<td>0.500 (15)</td>
<td>0.5082</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>0.143 (22)</td>
<td>0.133 (4)</td>
<td>0.100 (3)</td>
<td>0.5082</td>
</tr>
<tr>
<td>Intron 4</td>
<td>4b, 4b</td>
<td>0.688 (106)</td>
<td>0.633 (19)</td>
<td>0.600 (18)</td>
<td>3.073</td>
</tr>
<tr>
<td></td>
<td>4a, 4b</td>
<td>0.273 (42)</td>
<td>0.367 (11)</td>
<td>0.367 (11)</td>
<td>3.073</td>
</tr>
<tr>
<td></td>
<td>4a, 4a</td>
<td>0.039 (6)</td>
<td>0.000 (0)</td>
<td>0.033 (1)</td>
<td>3.073</td>
</tr>
<tr>
<td>Glu298Asp</td>
<td>Glu, Glu</td>
<td>0.422 (65)</td>
<td>0.367 (11)</td>
<td>0.467 (14)</td>
<td>4.365</td>
</tr>
<tr>
<td></td>
<td>Glu, Asp</td>
<td>0.494 (76)</td>
<td>0.600 (18)</td>
<td>0.467 (14)</td>
<td>4.365</td>
</tr>
<tr>
<td></td>
<td>Asp, Asp</td>
<td>0.084 (13)</td>
<td>0.033 (1)</td>
<td>0.067 (2)</td>
<td>4.365</td>
</tr>
<tr>
<td>Alleles</td>
<td>Total (308)</td>
<td>L group (n = 60)</td>
<td>H group (n = 60)</td>
<td>Chi-square</td>
<td>P</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>------------</td>
<td>-------</td>
</tr>
<tr>
<td>T\textsuperscript{−786}C</td>
<td>T</td>
<td>0.604 (186)</td>
<td>0.617 (37)</td>
<td>0.650 (39)</td>
<td>0.1942</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.396 (122)</td>
<td>0.383 (23)</td>
<td>0.350 (21)</td>
<td>0.1942</td>
</tr>
<tr>
<td>Intron 4</td>
<td>4b</td>
<td>0.825 (254)</td>
<td>0.817 (49)</td>
<td>0.783 (47)</td>
<td>0.5000</td>
</tr>
<tr>
<td></td>
<td>4a</td>
<td>0.175 (54)</td>
<td>0.183 (11)</td>
<td>0.217 (13)</td>
<td>0.5000</td>
</tr>
<tr>
<td>Glu298Asp</td>
<td>Glu</td>
<td>0.669 (206)</td>
<td>0.667 (40)</td>
<td>0.70 (42)</td>
<td>0.2086</td>
</tr>
<tr>
<td></td>
<td>Asp</td>
<td>0.331 (102)</td>
<td>0.333 (20)</td>
<td>0.300 (18)</td>
<td>0.2086</td>
</tr>
</tbody>
</table>

Table 3 Estimated haplotype frequencies in the L and H groups

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>L group</th>
<th>H group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>T\textsuperscript{−786}C Intron 4 Glu298Asp</td>
<td>T 4b Glu 0.35 0.46 0.1131</td>
<td>T 4b Asp 0.19 0.12 0.1714</td>
<td>T 4a Glu 0.04 0.05 0.7330</td>
</tr>
<tr>
<td></td>
<td>T 4a Asp 0.03 0.02 0.6506</td>
<td>C 4b Glu 0.16 0.04 0.0047</td>
<td>C 4b Asp 0.12 0.18 0.3008</td>
</tr>
<tr>
<td></td>
<td>C 4a Glu 0.11 0.15 0.4003</td>
<td>C 4a Asp 0.00 0.00 –</td>
<td>C 4a Asp 0.00 0.00 –</td>
</tr>
</tbody>
</table>

P values were considered significant only when <0.0063 (=0.05/8, which would allow eight simultaneous comparisons); chi-squared test.

NO\textsubscript{x} compared with subjects with high circulating NO\textsubscript{x} concentrations, even when correction for multiple comparisons was carried out. Interestingly, this specific haplotype includes the less common genetic variant (‘C’ allele) only for the eNOS polymorphism in the promoter region. These data suggest that the occurrence of the rarer ‘C’ allele in the promoter region decreases the endogenous production of NO. These findings are supported by experimental evidence showing that the occurrence of the ‘C’ allele in the promoter region of the eNOS gene reduces the promoter activity by approximately 50% [8,9]. Indeed, we have previously studied healthy volunteers that were genotyped only for the genetic T\textsuperscript{−786}C polymorphism in the promoter region [16]. Although we found no significant effects of the T\textsuperscript{−786}C polymorphism on plasma NO\textsubscript{x}, a finding confirmed in the present study, plasma NO\textsubscript{x} tended to decrease with increasing number of ‘C’ alleles. However, when only the T\textsuperscript{−786}C polymorphism is taken into consideration, other genetic variants may preclude the detection of significant effects of the polymorphism in the promoter region.
Our findings are consistent with a major contribution of eNOS haplotypes to variation in NO\textsubscript{x} concentrations, which is obscured when specific eNOS genotypes alone are considered [20]. Interestingly, a previous in-vitro functional study showed that the polymorphism in the promoter region and in intron 4 had an haplotype-dependent effect on transcription efficiency [31]. Although the polymorphism in exon 7 was not taken into consideration in that study, it supports the suggestion that haplotype-based association studies are more appropriate than single polymorphism-based studies [31].

Some limitations of our study should be taken into consideration. First, the relatively small number of volunteers (N=154) may have limited the power of our study to detect an effect of individual genotypes on the circulating concentrations of NO\textsubscript{x}. In addition, all the volunteers enrolled in this study were white men. The effects of marked interethnic differences in genotype and haplotype frequency distribution [6,24] were not assessed in the present study. For example, the low frequency with which the allele ‘a’ is found in white subjects [6,24] may have decreased the power of this study to detect an effect for this polymorphism on circulating concentrations of NO\textsubscript{x}. Further studies aimed at identifying the specific eNOS haplotypes affecting the concentrations of NO\textsubscript{x} in other ethnic groups and in women are needed. Finally, some subjects included in the present study have a body mass index over 25. Therefore, we should consider them healthy with some caution.

In conclusion, we found that, while eNOS genotypes are not significantly associated with changes in the circulating NO\textsubscript{x} concentrations in healthy subjects, the specific eNOS haplotype that includes the ‘C’, ‘4b’, and ‘Glu’ alleles for the polymorphisms in the promoter region, in intron 4, and in exon 7, respectively, is associated with lower circulating NO\textsubscript{x} concentrations in men.

Acknowledgements
This study was funded by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP-Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil) and Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Brazil).

References

