Endothelial Nitric Oxide Synthase Gene Polymorphism and Maternal Vascular Adaptation to Pregnancy

Makrina D. Savvidou, Patrick J.T. Vallance, Kypros H. Nicolaides, Aroon D. Hingorani

Abstract—A common polymorphism of the endothelial NO synthase gene that predicts a Glu298Asp amino acid substitution in the mature protein has been associated with cardiovascular disorders in which NO bioactivity is impaired. However, the influence of this polymorphism on endothelial function is unknown. Healthy pregnancy is associated with enhanced endothelium-dependent, flow-mediated dilation (FMD) of the brachial artery, a response mediated by NO. In this study, we investigated the effect of the endothelial NO synthase Glu298Asp polymorphism on endothelium-dependent vasodilation in early pregnancy, making the hypothesis that any genotype-dependent differences in NO generation would be more marked during pregnancy, when the production of NO is upregulated. FMD of the brachial artery was recorded during the first trimester in 139 healthy women with normal singleton pregnancies genotyped for the Glu298Asp variant of endothelial NO synthase. Maternal FMD exhibited a codominant inverse relation with the number of Asp298 alleles \( (r = -0.21, P = 0.01) \). Among homozygotes for endothelial NO synthase Asp298, FMD (7.99 ± 1.46%) was significantly lower than that observed among individuals homozygous for endothelial NO synthase Glu298 (10.12 ± 3.44) \( (P = 0.002) \). In a backward stepwise multiple regression analysis, vessel size \( (P < 0.0001) \) and Glu298Asp polymorphism \( (P = 0.01) \) were significantly and independently correlated with FMD. Our findings indicate that the endothelial NO synthase Glu298Asp polymorphism is associated with differences in endothelium-dependent dilation at 12-week gestation and are the first to implicate genetic factors in the normal vascular adaptation to pregnancy. They also provide a potential mechanism linking the endothelial NO synthase polymorphism with the development of cardiovascular disorders and have implications for understanding the genetic basis of preeclampsia. (Hypertension. 2001;38:1289-1293.)

Key Words: nitric oxide synthase • endothelium • pregnancy

NO is an endothelial vasodilator with additional anti-thrombotic and atheroprotective properties,1–5 and its deficiency has been implicated in the pathogenesis of hypertension, atherosclerosis, and preeclampsia.6–11 Recently, a common Glu298Asp polymorphism of the endothelial NO synthase (eNOS; the enzyme that synthesizes NO in the endothelium) has been associated with the development of these disorders, in which endothelium-dependent vasodilation and NO bioactivity are impaired.12–17 The Asp298 variant has also been shown to be susceptible to enhanced proteolytic cleavage and this might contribute to abnormally low NO generation in carriers of this allele.18 However, the effects of this variant on endothelial function in human beings are unknown.

Normal pregnancy is associated with an increase in blood volume and cardiac output and a fall of blood pressure (BP) in the first half of pregnancy caused by systemic arteriolar vasodilation.19,20 It has been proposed that the enhanced endothelium synthesis of the NO is responsible for this vasodilation,21–25 and we and others have shown that flow-mediated vasodilation (FMD) of the brachial artery (an NO-dependent response) is enhanced from early gestation.26,27 In this study, we have examined the relation between endothelium-dependent FMD and eNOS genotype in 139 pregnant white women during the first trimester of pregnancy, making the hypothesis that any genotype-dependent differences in NO production would be more marked during this period, when the synthesis of NO is upregulated.

Methods

Local ethics committee approval was obtained, and 139 unrelated white women with normal singleton pregnancies recruited from the antenatal clinic gave written informed consent for study. Maternal age, height, weight at the time of the study, smoking status, gestational age, parity, and heart rate were recorded. BP was measured by an ambulatory BP monitor (SpaceLabs Medical) with the subject seated. Three measurements were taken and averaged.

Vascular Study

An ultrasound scan of the right brachial artery was performed using a 7-MHz linear array transducer and an Aspen Acuson system.9 End-diastolic images of the artery were acquired every 3 seconds and...
stored in digital format. Arterial diameter was determined for each image with a semiautomatic edge detection algorithm. Baseline vessel diameter was calculated as the mean of all the measurements during the first minute of recording. FMD of the brachial artery was defined as the maximum percentage increase in vessel diameter during reactive hyperemia. All the scans were performed from the same experienced operator, who was blinded to the genotype data.

In our laboratory, the interobserver variability for FMD is 1.02% ± 0.6% for FMD (95% limits of agreement, 1.7% to 2.4%). Typically, increments in diameter of >200 μm (0.2 mm) are measured, which is far above the resolution limits of the system. Outside the setting of pregnancy, endothelium-independent dilation to sublingual glyceryl trinitrate (GTN) is commonly used as a control, but in the current study, GTN use was avoided at this early stage of gestation. However, a previous study has shown that GTN-induced dilation does not alter as a result of pregnancy.

DNA Extraction and Genotyping

DNA was extracted by means of the QIAamp blood minikit. The 894 bp Glu/T polymorphism in exon 7 of the eNOS gene, which predicts a Glu298Asp amino acid substitution in the mature protein, was genotyped by polymerase chain reaction (PCR) with primer pairs 5’-CCCCCTCATTCCACCCGCCAGTCAAC-3’ and 5’-AGGAAACGCGTGCTGCTGCTGCTG-3’ and allele-specific restriction enzyme digestion. PCR was performed for 35 cycles in a volume of 30 μL. Denaturation was at 95°C, annealing at 63°C, and a final extension at 72°C, all for 45 seconds. Ten microliters of each PCR product (151 bp) was then subjected to restriction digestion with 2 U Dpn II, which cleaves the PCR product (into fragments of 49 and 101 bp) only in the presence of the T allele (corresponding to Asp298). Digested samples were resolved by electrophoresis. For quality control, 40 (29%) samples were subject to repeat PCR and genotyping, and no discrepancies were detected.

Estradiol (E2), progesterone, and estriol (E3) were measured in cord blood by sensitive radioimmunoassay. An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Results

Baseline Characteristics of Study Participants

Recordings of FMD were successfully obtained from all women, and all tolerated the studies well. For the whole cohort, the maternal age was 33 (30 to 35) years, body mass index was 22.94 (21.11 to 25.21), 24% were cigarette smokers, and 48% were primigravidae. The gestational age was 12.4 (12.3 to 12.9) weeks. The mean heart rate was 75.9 ± 2.4%. The mean mean arterial pressure was 107.1 ± 5.8 mm Hg. The baseline characteristics of the subjects, divided according to eNOS genotype, are given in the Table. No significant differences in these baseline parameters were detected in subjects of different genotypes. The distribution of eNOS genotypes did not differ significantly from that expected under Hardy-Weinberg equilibrium (P = 0.8), and the frequency of Asp298 alleles (63.3%) and Asp298 homozygotes (7.1%) was similar to that reported previously.

Maternal FMD in Early Gestation

The mean FMD, baseline vessel size, baseline flow, and reactive hyperemia for all the subjects were 9.42 ± 3.17%, 2.92 ± 0.33 mm, 103.33 (65.7 to 163.9) mL/min, and 691.66% (453.6 to 956.9%), respectively. FMD was inversely correlated with the baseline vessel size (FMD = −3.52 vessel size + 19.72, r = −0.37, P < 0.0001), in line with several previous reports, but was not correlated with maternal age, smoking status, parity, heart rate, MAP, baseline flow, reactive hyperemia, E2, E3, progesterone, glucose, or cholesterol levels (data not shown).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glu/Glu (n = 61)</th>
<th>Glu/Asp (n = 68)</th>
<th>Asp/Asp (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>33 (30–35)</td>
<td>32 (28.2–35.7)</td>
<td>31 (30–34.5)</td>
</tr>
<tr>
<td>Smokers</td>
<td>15 (24.6%)</td>
<td>16 (23.5%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>115.7 ± 11</td>
<td>115.5 ± 9.5</td>
<td>113.14 ± 10</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>68.7 ± 10.8</td>
<td>68.8 ± 10.42</td>
<td>69.6 ± 7</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>75.4 ± 8.14</td>
<td>75.41 ± 9.75</td>
<td>76.1 ± 7.8</td>
</tr>
<tr>
<td>FMD, %</td>
<td>10.12 ± 3.44</td>
<td>9.01 ± 2.97</td>
<td>7.99 ± 1.46</td>
</tr>
<tr>
<td>Vessel size, mm</td>
<td>2.92 ± 0.35</td>
<td>2.9 ± 0.32</td>
<td>2.99 ± 0.27</td>
</tr>
<tr>
<td>Baseline flow, mL/min</td>
<td>94.18 (67.76–154.5)</td>
<td>106.95 (62.8–159.9)</td>
<td>130.63 (55.2–256.5)</td>
</tr>
<tr>
<td>Reactive hyperemia, %</td>
<td>692.3 (514.41–951.8)</td>
<td>672.36 (439.8–1032.1)</td>
<td>564.85 (344.5–1036.4)</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>4.4 (3.85–4.9)</td>
<td>4.25 (3.8–4.6)</td>
<td>4.3 (4.05–4.95)</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>5.1 ± 0.75</td>
<td>5.09 ± 0.84</td>
<td>5.25 ± 0.87</td>
</tr>
</tbody>
</table>

There was no significant difference for any baseline characteristics apart from FMD.
Maternal FMD and the eNOS Glu298Asp Polymorphism

Maternal FMD exhibited a codominant inverse relation with the number of Asp298 alleles ($r = 0.21$, $P = 0.01$). Among homozygotes for eNOS Asp298, FMD (7.99 ± 1.46%) was significantly lower than that observed among individuals homozygous for eNOS Glu298 (10.12 ± 3.44%) ($t = 3.32$, $df = 29$, $P = 0.002$, Figure 1). Mean FMD in heterozygotes was of an intermediate value of 9.01 ± 2.97%. Mean FMD in the 78 Asp carriers (68 heterozygotes + 10 homozygotes combined) was 8.88 ± 0.32%, which was also significantly lower than that in Glu/Glu homozygotes (mean difference, 1.24 ± 0.53%, $P = 0.02$). In a backward stepwise multiple regression analysis, the vessel size ($P = 0.0001$) and the Glu298Asp polymorphism ($P = 0.01$) were the only parameters significantly and independently correlated with FMD. Further analysis revealed that MAP was inversely correlated with FMD, but only among individuals homozygous for Asp298 (FMD $= -0.15$ MAP + 20.39, $r = -0.69$, $P = 0.026$, Figure 2). Furthermore, the inverse correlation between vessel size and FMD observed in the cohort as a whole was not detected among Asp298 homozygotes ($r = 0.05$, $P = 0.89$).

Discussion

The study has shown that in early pregnancy, the magnitude of endothelium-dependent FMD of the brachial artery is determined in part by carriage of the Asp298 variant of eNOS. This observation endorses the view that the NO pathway is activated during normal pregnancy and points to an important role for genetic factors in determining the magnitude of NO-dependent effects. It also suggests, for the first time, that genetic factors influence maternal vascular adaptation in pregnancy. These findings lend insight into the physiology of normal pregnancy but also have implications for understanding the underlying causes of preeclampsia and cardiovascular disorders.

Mean brachial artery FMD at 12-week gestation was ≈40% higher than that observed previously among healthy nonpregnant women, and we have previously shown that the increase in FMD that occurs during pregnancy is maintained until at least 30 weeks. The enhanced FMD of early pregnancy may be due to an increase in circulating estrogens because FMD and other measures of NO-dependent responses are augmented during healthy pregnancy and during the follicular phase of the menstrual cycle. Although in the current study, at the very high levels of estrogens observed during gestation, there was no correlation between the absolute level of E2 or E3 and FMD, our observations would be consistent with the observations that estrogens cause transcriptional activation of the eNOS gene and up-regulation of NO synthesis. These changes are thought to be an important physiological adaptation that accommodates the increased circulating blood volume and cardiac output during pregnancy.

The magnitude of the enhanced FMD in individual subjects during pregnancy, as indicated by our findings, is related to eNOS genotype. There was an inverse and highly statistically significant relation between brachial artery FMD and the
number of maternal eNOS Asp298 alleles. Among women homozygous for the eNOS Asp298 allele, FMD was 21% lower than that in Glu298 homozygotes and 11.3% lower than that in heterozygotes. We have demonstrated previously that the Glu298Asp polymorphism is not associated with differences in FMD outside the setting of pregnancy.29 This suggests that it is the magnitude of the adaptive vascular response that is influenced by the Glu298Asp polymorphism, with Asp298 homozygotes having a more limited enhancement of endothelium-dependent FMD in early pregnancy. These differences in FMD by genotype could not be explained by baseline differences in BP, the concentration of estrogens, cholesterol, and glucose, or other factors that are known to affect endothelial function and FMD.9,28,30 Moreover, the magnitude of the differences in FMD by genotype are of the order of those observed in the presence of cardiovascular risk factors such as smoking and hypercholesterolemia, which are known to impair endothelial NO generation.9

The eNOS Glu298Asp polymorphism has been implicated in the development of several vascular disorders in which NO bioactivity is reduced. These include essential hypertension, myocardial infarction, angiographic coronary artery disease, coronary spasm, and preeclampsia.12–17 This suggests that possession of the Asp298 allele is associated with diminished NO bioactivity. In keeping with this, the pressor response to intravenous phenylephrine is correlated with the number of Asp298 alleles.31 Although the Michaelis constant (Km) and maximal activity (Vmax) of isolated recombinant eNOS Glu298 and eNOS Asp298 are no different (Alderton W, Hingorani AD and Knowles R, unpublished data, 1999), a recent report has shown that eNOS Asp298 is subject to selective proteolytic cleavage in endothelial cells and platelets.18 Therefore, enhanced degradation of eNOS leading to reduced NO synthesis could account for the association between eNOS Asp298 and cardiovascular disease or the vascular response to pregnancy reported here. It is noteworthy that only in women homozygous for eNOS Asp298 did BP relate inversely with FMD. One interpretation of these findings is that homozygosity for Asp298 attenuates endothelial NO generation to such a degree that the endothelium in these individuals is rendered more susceptible to BP-induced endothelial dysfunction.

Although our study has examined vascular responses in healthy pregnancy, it also has implications for understanding the pathogenesis of preeclampsia. Although the cause of preeclampsia is unknown, it is widely believed that a primary defect of placentation causes the release of unknown factors from the placenta that trigger the development of endothelial dysfunction that might be caused by a reduction in NO availability.10,11,32 This leads to an increase in systemic vascular resistance and sensitivity to pressor agents.32–34 In support of this model, experimental inhibition of NO synthesis in pregnant rats results in the development of sustained hypertension, proteinuria, thrombocytopenia, and intrauterine growth restriction mimicking the human disorder.35,36 Preeclampsia is known to be heritable, and although the nature and number of predisposing genes are unknown, linkage studies of affected sibling pairs have implicated the eNOS gene locus on chromosome 7q35 to 36.37–40 Furthermore, the presence of the Glu298Asp variant has recently been associated with severe preeclampsia.17 In our study, carriage of the eNOS Asp298 allele was associated with more limited enhancement of endothelium-dependent vasodilation in pregnancy, and we suggest that as a result, carriers of this allele might be more susceptible to the endothelial dysfunction that could be triggered by factors released from and abnormal placenta and compounded by the presence of preexisting hypertension or diabetes. If this were the case, carriage of eNOS Asp298 would be neither necessary nor sufficient for the development of preeclampsia but would instead lower the threshold for the development of preeclamptic maternal endothelial dysfunction after abnormal placentation. Further prospective studies of high-risk pregnancies would be required to test this hypothesis directly and to determine whether high-risk mothers who carry the Asp298 allele should be targeted for therapy.

In summary, we have shown that a carriage common Glu298Asp polymorphism in eNOS has functional consequences and that during normal pregnancy, the magnitude of the enhanced vasodilatory response to a flow stimulus is related to this polymorphism. These findings implicate an important role for genetic factors in determining the vascular adaptation to pregnancy and provide further support for a role for the eNOS gene in determining susceptibility to preeclampsia.


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Hypertension. 2001;38:1289-1293
doi: 10.1161/hy1201.097305

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