Endothelial Nitric Oxide Synthase Exon 7 Polymorphism, Ischemic Cerebrovascular Disease, and Carotid Atheroma

Hugh S. Markus, DM; Ynte Ruigrok, BSc; Nadira Ali, MSc; John F. Powell, DPhil

Background and Purpose—The role of endothelial nitric oxide synthase (eNOS) in normal physiology suggests that it could be a potential candidate gene for stroke. Reduced eNOS activity could mediate an increased stroke risk through hypertension or independent of hypertension through abnormal vasomotor responses, promoting atherogenesis, or increased platelet adhesion and aggregation. Recently, a common polymorphism in exon 7 of the eNOS gene (894G→T) has been reported to be a strong risk factor for coronary artery disease. We determined whether it was also a risk factor for transient ischemic attack (TIA) and ischemic stroke and for carotid atheroma.

Methods—We studied 361 consecutive white patients presenting with ischemic stroke or TIA to a neurological cerebrovascular disease service and 236 normal white controls. In all patients CT and/or MR head imaging and high-resolution carotid duplex ultrasound were performed. The presence of the polymorphism (N/n) was determined by polymerase chain reaction and restriction with the enzyme BanII.

Results—There was no difference in the frequency of the NN genotype between patients and controls (13.0% versus 15.3%; P=0.44) or in N allele frequency (39% versus 37%; P=0.57). There was no association with genotype when only patients with stroke (excluding those with TIA) or when only individuals aged ≥65 years were considered. In contrast, there was a highly significant independent association between cerebrovascular disease and hypertension (odds ratio, 2.87; 95% CI, 2.0 to 4.15; P<0.00001), smoking (odds ratio, 2.58; 95% CI, 1.80 to 3.70; P<0.00001), and diabetes (odds ratio, 2.68; 95% CI, 1.38 to 5.24; P=0.004). There was no relationship between the polymorphism and any particular stroke subtype: large-vessel disease, for NN, 15 of 105 (14.3%); lacunar disease, 10 of 75 (13.3%); cardioembolic and unknown, 18 of 151 (11.9%); and tandem pathology, 4 of 30 (13.3%) (P=0.68, χ²). There was no difference in the mean degree of carotid stenosis between the 3 genotypes: NN, 31.1% (SD, 27.1); Nn, 30.1% (29.0); and nn, 31.2% (26.3) (P=0.9). There was no association between the NN genotype or the N allele and hypertension.

Conclusions—We failed to find a relationship between this exon 7 polymorphism and ischemic cerebrovascular disease. In particular, it was not associated with stroke and TIA secondary to large-vessel atherosclerosis or with the degree of carotid stenosis in patients with cerebrovascular disease. It is unlikely that this particular polymorphism or any closely linked polymorphism is a major risk factor in the majority of white patients with stroke. (Stroke. 1998;29:1908-1911.)

Key Words: atherosclerosis ■ cerebrovascular disorders ■ genetics ■ nitric oxide ■ risk factors

Genetic factors appear to be important in the pathogenesis of cerebrovascular disease, but the molecular basis of this genetic predisposition remains largely unknown. In a few patients with rare causes of stroke, often secondary to metabolic and coagulation disorders, an underlying genetic abnormality is known. However, in the majority of patients the genetic basis appears to be polygenic. In these individuals genetic influences may act independently or by predisposing to or modulating the effects of known risk factors such as hypertension.

A potential candidate gene is nitric oxide synthase (NOS). Nitric oxide (NO) is synthesized from the amino acid L-arginine by the enzyme NOS with the concomitant production of L-citrulline. At least 3 isoforms of NOS have been identified: 2 constitutive isoforms, neuronal and endothelial NOS (eNOS), and an inducible isoform. NO maintains basal cerebral blood flow in both animals and humans. Studies in knockout mice suggest that the endothelial isoform (eNOS) is most likely to synthesize the NO that is responsible for maintaining resting cerebral blood flow. In addition, NO reduces both platelet adhesion and aggregation and therefore may also have an antithromboembolic effect. During cerebral ischemia endothelial NO production appears to be protective, probably by increasing cerebral blood flow and possibly also as a result of its antiplatelet activity. Endothelial NO may also mediate cerebral vasodilatory responses and cerebral autoregulation. Impaired cerebral autoregulation has been reported in hypertension and may predispose toward stroke. Impaired NO-mediated vasodilatation has been reported in patients with a wide variety of...
cardiovascular risk factors, including hypertension,\textsuperscript{9} diabetes,\textsuperscript{10} and hypercholesterolemia.\textsuperscript{11}

eNOS is expressed in vascular endothelium, platelets, and the heart and is encoded for by a gene located on chromosome 7q35–36, compromising 26 exons that span 21 kb.\textsuperscript{12–14} Recently, a common polymorphism in exon 7 of the endothelial NOS gene (894G→T) has been reported to be a strong risk factor for coronary artery disease with homozygous genotype (NN) frequencies of 36% in cases versus 10% in controls.\textsuperscript{15} This polymorphism results in a Glu→Asp amino acid substitution. Since it is in an exon, this polymorphism may be functional, although no studies investigating this have been published. It might also be expected to be a risk factor for ischemic cerebrovascular disease, particularly in those patients in whom the pathogenesis is large-vessel atherosclerosis. There is a strong association between carotid atherosclerosis and ischemic heart disease.\textsuperscript{16} In this study, in an unselected group of patients presenting to a neurology stroke service, we determined whether this exon 7 polymorphism is a risk factor for ischemic stroke and transient ischemic attack (TIA) and also whether it is a risk factor for any particular stroke subtype, particularly large-vessel disease. In addition, in the patient group we determined whether there was any relationship between the polymorphism and the degree of carotid atheroma, determined ultrasonically.

Subjects and Methods
We studied 361 consecutive white patients with ischemic stroke or TIA presenting to a neurological cerebrovascular disease service and compared them with 236 normal white controls. Control subjects were recruited from consecutive spouses of the same patients when available (129 cases) and also from community controls randomly selected from health authority lists from the same family practices as the cases (107 cases). Because of the marked variation in common vascular candidate gene polymorphisms in different ethnic groups,\textsuperscript{17} only white case and control subjects were studied. Control subjects were included if they had vascular risk factors or a history of myocardial infarction or peripheral vascular disease but were excluded if they had cerebrovascular disease (3 spouses, 1 community control). All subjects gave informed consent, and the study was approved by the local ethics committee. In all patients, brain CT and/or MR head imaging was performed, as well as extracranial duplex ultrasonography and electrocardiography. Transcranial echocardiography was performed in approximately 40% of patients. On the basis of clinical features and the results of these investigations, patients were divided into 4 pathogenic subtypes: (1) large-vessel disease: internal carotid or vertebral artery stenosis >50%, diagnosed on carotid duplex for anterior circulation ischemia and carotid duplex and/or MR angiography for posterior circulation ischemia with symptoms in that arterial territory; (2) lacunar stroke: a clinical lacunar syndrome\textsuperscript{18} with an appropriate CT or MRI infarct or a typical clinical syndrome lasting >24 hours and a normal CT scan; (3) uncertain or probable cardiogenic source: these 2 categories were included together because not all patients had echocardiography, and transthoracic echocardiography does not detect all cardioembolic sources; and (4) tandem pathology: >1 cause of cerebral ischemia.

Hypertension was defined as systolic blood pressure >160 mm Hg or diastolic pressure >95 mm Hg or current treatment with antihypertensive drugs. A smoker was defined as a current smoker or ex-smoker. Carotid stenosis was determined from the internal and common carotid artery velocities for stenoses >50% and with the use of B-mode imaging for stenoses with no velocity increase. The mean internal carotid artery stenosis was determined for each case.

All molecular genetics studies were performed blinded to case-control status. DNA was extracted from leukocytes with a commercially available kit (Nucleon, Scotlab Ltd). The 894G→T substitution was identified by the use of the polymerase chain reaction and restriction enzyme digestion. The oligonucleotide primers were designed for a 129–base pair region, starting at base pair position 118 of exon 7 and ending at base pair 246 of exon 7. This region contained the polymorphism at base pair position 191. The sequences of the forward and reverse primers are GCATTCAACG-GCTGGA and GCTCCAGGGGACCTCAA, respectively. The polymerase chain reaction product was then incubated with the restriction enzyme BanII. This enzyme restricted the polymerase chain reaction product into two fragments of 76 and 53 base pairs when the nucleotide 894 was a G (n). If nucleotide 894 was a T (N), the 129–base pair fragment was not digested. The fragment size was determined by polyacrylamide gel electrophoresis and silver staining.

Differences between groups were examined with the use of the $\chi^2$ test or the unpaired Student’s $t$ test when appropriate. Logistic regression analysis was then used to determine the presence of any independent relationships between individual risk factors and cerebrovascular disease.

Results
Allele frequencies in both control and patient populations were in Hardy Weinberg equilibrium: in controls, estimated N:n frequency was 0.373:0.627 versus observed frequency of 0.370:0.630 ($P=0.97$); in cases, estimated N:n frequency was 0.389:0.611 versus observed frequency of 0.390:0.610 ($P=0.99$). There was no difference in the genotype distributions between controls obtained from the different sources: spouse controls, nn 53 (41.1%), Nn 58 (45.0%), NN 18 (14.0%); population controls, nn 43 (39.8%), Nn 47 (43.5%), NN 18 (16.7%) ($P=0.84$). There was no difference in the frequency of the NN genotype between patients and controls (13.0% versus 15.3%; $P=0.44$) or in N allele frequency (39% versus 37%; $P=0.57$) (Table 1). There was no association with genotype when only patients with stroke (excluding those with TIA) were considered (n=289; NN genotype frequency, 13.8%; $P=0.83$ versus controls) or when individuals aged ≥65 years were considered (NN cases, 16 of 134 (10.4%); controls, 19 of 122 (15.6%); $P=0.20$). In contrast, there was a highly significant association between hypertension ($P<0.00001$), smoking ($P<0.00001$), and diabetes.

<table>
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<th>TABLE 1. Distribution of Conventional Risk Factors and of the eNOS NN Genotype and N Allele in Patients and Controls</th>
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<td><strong>Patients</strong> (n=361)</td>
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<td>Mean (SD) age, y</td>
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<td>Male sex</td>
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<td>Hypertension</td>
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(P=0.003) and cerebrovascular risk (Table 1). Logistic regression was performed with entry of age, sex, hypertension, diabetes, smoking, and NOS genotype; hypertension (odds ratio, 2.87; 95% CI, 2.0 to 4.15; P<0.0001), smoking (odds ratio, 2.58; 95% CI, 1.80 to 3.70; P<0.0001), and diabetes (odds ratio, 2.68; 95% CI, 1.38 to 5.24; P=0.0038) were all independently associated with cerebrovascular risk. However, there was no association with either the N allele (odds ratio, 1.06; 95% CI, 0.82 to 1.38; P=0.66) or the NN genotype (odds ratio, 0.83; 95% CI, 0.51 to 1.37; P=0.47) and risk of cerebrovascular disease.

There was no relationship between the polymorphism and any particular stroke subtype: large-vessel disease, 15 of 105 (14.3%); lacunar disease, 10 of 75 (13.3%); cardioembolic and unknown, 18 of 151 (11.9%); and tandem pathology, 4 of 30 (13.3%) (P=0.68, χ²) (Table 2). There was no difference in the mean degree of carotid stenosis between patients with the 3 genotypes: NN, 31.1% (SD, 27.1%); Nn, 30.1% (29.0%); and nn, 31.2% (26.3%) (P=0.9). There was no difference between age at onset of stroke in patients with the 3 genotypes: NN, 66.7 (SD, 13.1) years; Nn, 65.8 (11.1) years; and nn, 65.4 (12.54) years (P=0.85, ANOVA). There was no association between the NN genotype or N allele and hypertension when we considered the population as a whole: normotensive, nn 113 (39.8%), Nn 133 (46.8%), and NN 38 (13.4%); hypertensive, nn 110 (35.1%), Nn 158 (50.5%), and NN 45 (14.4%) (P=0.5).

### Discussion

We failed to find a relationship between this exon 7 polymorphism and ischemic cerebrovascular disease. In particular, it was not associated with stroke and TIA secondary to large-vessel atherosclerosis or with the degree of carotid stenosis in patients with cerebrovascular disease. The gene frequency we found in both patients and controls is similar to that found in controls by Hingorani et al. It is possible that this genetic risk factor may only be important in younger patients with stroke, but we found that the relationship was unchanged when only younger patients and control subjects (aged ≈65 years) were considered, and there was no relationship between age at stroke onset and genotype. In contrast, we found strong independent associations with known major risk factors for stroke, namely, hypertension, smoking, and diabetes.

We are not aware of previous studies that have determined the relationship of mutations or polymorphisms in the eNOS gene and human cerebrovascular disease. Initial studies investigated whether eNOS could be a potential candidate gene in hypertension. Such an association would be consistent with a number of physiological and pathophysiological lines of evidence. First, NO inhibition results in a rise in arterial blood pressure in humans. Second, endothelium-mediated vasodilatation is impaired in patients with essential hypertension, although this change could either be the primary cause of hypertension or secondary to the hypertensive state itself. Third, transgenic mice lacking the whole eNOS gene are hypertensive. A number of studies in humans have determined the relationship between a highly polymorphic dinucleotide repeat of the CA/GT type located in intron 13 of the eNOS gene and essential hypertension. Two studies in white populations using the sib-pair method failed to find an association with hypertension. In addition, Bonnardeaux et al screened 8 exons of the eNOS gene using single-strand conformation polymorphism to find informative biallelic markers. They found 2 substitutions within intron 18 (A27-C) and intron 23 (G10-T), but there was no difference between hypertensive subjects and normotensive controls in their distribution. These results are consistent with our results showing no association between the exon 7 polymorphism and hypertension in either the control or patient groups. In contrast, Nakayama et al suggested an association between a subgroup of hypertensive subjects and the same intron 13 CA repeat in a relatively small case-control study in a Japanese population. The prevalence of this polymorphism differs in Japanese and white populations. While there was no significant difference in overall distribution of allele frequencies between patients with essential hypertension and normotensive controls, patients with essential hypertension without left ventricular hypertrophy had an increased number of repeats, with an odds ratio of 3.71. More recently, linkage
has been reported in familial pregnancy-induced hypertension.24 Again, no significant linkage was found with the intron 13 CA repeat, although a nearly significant result was found. In view of this result, the 2 nearest flanking markers D7S505 and D7S483, which map a 4-centimorgan region on chromosome 7q36, were used for further analysis. Affected sib-pair analysis showed linkage to both of these markers, with the most significant results for the marker D7S505 located =2 centimorgans from the eNOS gene. These results would be compatible with an association with the eNOS gene or an as yet unknown gene in close proximity.

Mutations of eNOS could be a risk factor for vascular disease independent of hypertension either through reduced antiplatelet activity or through impaired vasomotor responses, and either could potentially play a role in accelerated atherogenesis. In addition, NO inhibits smooth muscle proliferation and monocyte activation, actions that may be relevant to early-onset atherosclerosis.25 Wang et al26 reported that a rare polymorphism in a 27–base pair repeat in intron 4 was more frequent in patients with angiographically proven coronary artery disease, but this was only in a subgroup of patients, smokers and ex-smokers, and no association was found in nonsmokers. They argued that this may occur because of sensitivity of eNOS to smoking; it has been shown that NO-mediated vasodilatation is reduced in smokers.27 However, there was no association between the polymorphism and the number of vessels with angiographic stenoses, and the association in a subgroup only may reflect a chance finding and requires confirmation in other populations. The data of Hingorani et al15 suggested that the exon 7 polymorphism is an independent risk factor for ischemic heart disease in an unselected population from the United Kingdom. No direct correlation with the degree of atheroma was reported, but large-vessel atheroma accounts for the majority of coronary artery disease. We were unable to show an association of this polymorphism with stroke or with carotid atheroma. Our results do not exclude other polymorphisms or mutations in the eNOS gene playing a role in the pathogenesis of stroke. In particular, it would be worthwhile to test for any association between stroke and the intron 4 polymorphism reported by Wang et al,26 since this has recently been found to influence plasma nitrate levels.28 However, it appears unlikely that this particular exon 7 polymorphism or any closely linked polymorphism is a major risk factor in the majority of patients with stroke.

Acknowledgments

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References

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