Endothelial Nitric Oxide Gene Haplotypes and Risk of Cerebral Small-Vessel Disease

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***Background and Purpose*—**Genetic influences are important in multifactorial cerebral small-vessel disease (SVD) and may act via endothelial dysfunction. Nitric oxide (NO) synthesized by endothelial nitric oxide synthase (eNOS) is a key mediator of endothelial function. We determined the role of 3 potentially functional eNOS polymorphisms (T-786C, intron 4ab, G894T) located toward the 5′ flanking end of the gene as risk factors for SVD and different SVD subtypes: isolated lacunar infarction (n=137) and ischemic leukoaraiosis (n=160).

***Methods*—**Three hundred patients with SVD and 600 community controls were studied. Genotypes were determined through polymerase chain reaction with or without restriction fragment digestion. Nitrate (NO_x) levels were determined in a subgroup by use of a Griess method. Polymorphisms were tested individually and in combination with haplotype analysis.

***Results*—**The intron 4a variant was protective against SVD. This effect was confined to isolated lacunar infarction (odds ratio, 0.55; 95% confidence interval, 0.35 to 0.86; $P=0.01$). Haplotypes encountered were significantly different in this subtype compared with controls ($P=0.001$), with the -786C promoter/intron 4a combination particularly underrepresented. NO_x levels were associated with the T-786C locus ($P=0.03$) but only in the presence of the intron 4a allele ($P=0.07$ for interaction).

***Conclusions*—**The intron 4ab insertion/deletion genotype was associated with isolated lacunar infarction. Haplotype and functional studies suggested that the protective effect of the 4a variant could be mediated through changes in eNOS promoter activity and increased NO levels. The specific association with isolated symptomatic lacunar infarction and not ischemic leukoaraiosis may reflect different etiopathogeneses of the 2 subtypes. Lack of NO could predispose to localized microatheroma in proximal arterioles rather than diffuse arteriosclerosis affecting distal perforating vessels. **(*Stroke*. 2004;35:654-659.)**

Key Words: endothelium ■ genetics ■ nitric oxide ■ nitric oxide synthase ■ small-vessel disease ■ stroke

Genetic influences are important in polygenic stroke and may be particularly relevant in certain well-defined phenotypes, eg, cerebral small-vessel disease (SVD).[1,2] Therefore a logical approach would be to focus on this phenotype for studies of genetic risk factors. In SVD, genetic factors could act by modulating endothelial dysfunction, which is an important feature.[3] Several key genes are concerned with the endothelial system, including endothelial nitric oxide synthase (eNOS), the enzyme catalyzing formation of soluble nitric oxide (NO) from L-arginine. Endothelium-derived NO has a number of roles, including maintaining basal cerebral blood flow,[4] cerebral vasodilation, and autoregulation[5,6]; maintaining vascular integrity[7]; and inhibiting smooth muscle proliferation.[8] A lack of endothelium-derived NO would be expected to lead to several features characteristic of SVD, including cerebral hypoperfusion,[9] impaired cerebral autoregulation,[10,11] endothelial damage with breakdown of the blood-brain barrier, and vessel remodeling.[12,13]

The eNOS gene is located on chromosome 7q35-36 and comprises 26 exons spanning 21 kb. Toward the 5′ flanking region (Figure 1), a promoter (T-786C) and a 27-bp deletion (a)/insertion (b) polymorphism within intron 4 have been associated with alterations in promoter activity.[14] A variant located in exon 7 (G894T) encodes an amino acid change from Glu→Asp that is believed to render the enzyme more susceptible to proteolytic cleavage.[15] The effects of these polymorphisms on in vivo NO generation cannot be measured directly because most endogenous NO rapidly oxidizes to nitrite (NO_2^-) and is eventually converted to nitrate (NO_3^-), the predominant stable form of NO. Collectively, these inactive metabolites (NO_x) have been used to reflect endog-

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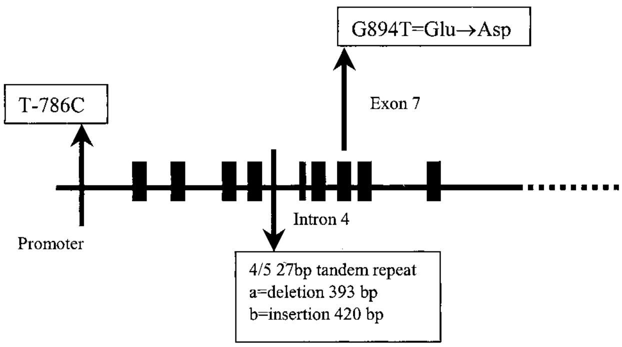

	D'
T-786C/ intron4	0.86
T-786C/G894T	0.42
G894T/intron 4	0.77

Figure 1. Organization of 5′ flanking end of eNOS gene. Polymorphisms studied are highlighted, with estimated linkage disequilibrium coefficients (D').

enous NO production,[16] and in turn, levels appear to be associated with eNOS polymorphisms.[17,18]

Despite biological plausibility, few studies have examined the influence of eNOS variants in SVD. In most cases, a small number of lacunar stroke patients were studied as part of a larger investigation of genetic risk factors in all ischemic stroke phenotypes.[19–22] Findings from these studies have been contradictory, possibly reflecting small sample size, study of different populations, and testing of polymorphisms in isolation, which can be misleading. Selection of phenotype could also be an issue because SVD is a heterogeneous entity. Lacunar infarcts can occur in isolation or can be associated with diffuse white-matter change, an appearance referred to as "ischemic" leukoaraiosis.[3] It is suggested that different mechanisms are involved in the pathogenesis of the 2 subtypes, with microatheroma playing a major role in isolated lacunar infarcts and a diffuse arteriopathy being more important in ischemic leukoaraiosis.[3,23,24]

Testing of multilocus haplotypes overcomes some of the problems encountered with using single polymorphisms in genetic association studies, particularly when interactions between polymorphisms on the same chromosome are more important in determining disease risk.[25] Because determination of individual haplotypes is time consuming and requires either isolation of individual chromosomes for sequencing or genotyping of family members, statistical methods have been developed that allow estimation of haplotype frequencies from unphased genotype data and can be used for case-control analysis.[26]

In the present study, we determined the role of the eNOS gene in a large, well-phenotyped series of patients with SVD. Variants were tested individually and as haplotypes in SVD and SVD subtypes.

Subjects and Methods

Study Population

Three hundred consecutive white patients with SVD attending participating stroke services were recruited. Cerebral SVD was defined as a clinical lacunar syndrome with a compatible lesion on MRI or CT. All patients had standard stroke investigation, including brain imaging and imaging of the carotid arteries with duplex or MR angiography. Exclusion criteria included subcortical infarction ≥1.5 cm in diameter, cortical infarction of any size, a potential cardiac source of embolism, and large-vessel cerebrovascular disease defined as carotid, vertebral, or basilar intracranial artery stenosis ≥50%.

Six hundred white community controls free of symptomatic cerebrovascular disease were also recruited by sampling family doctor lists from the same geographic regions as the patients. Sampling was stratified to provide a distribution of age and sex similar to that in the patient group. The study protocol was approved by local research ethics committees, and informed consent was obtained from all participants.

Assays

All assays were performed by researchers blinded to patient details. Genotyping was based on published[20,27] or in-house protocols (Table I, available online at http://stroke.ahajournals.org). In a combined subgroup of 68 individuals (39 cases, 29 controls), fasting plasma samples were obtained after a period of controlled nitrate intake. This entailed a 12-hour fast during which patients were allowed to drink only nitrate-free water. Plasma NO_x levels were measured by use of a modified Griess reaction.[28]

SVD Subtyping

Leukoaraiosis was graded by scoring periventricular changes on MRI (n=202; 67.3%) or CT (n=70; 23.3%) using a semiquantitative scale. On the basis of leukoaraiosis grade, patients were subtyped into 2 groups: isolated lacunar infarction (at least 1 focal lesion and

TABLE 1. Clinical Characteristics of Study Groups

Characteristic	Controls (n=600)	All Cerebral SVD (n=300)	Isolated Lacunar Infarction (n=137)	Ischemic Leukoaraiosis (n=160)
Age	66.85 (8.15)	67.10 (10.26)	63.52 (10.31)*	70.21 (9.04)*§
Male sex	387 (64.5)	198 (66.0)	93 (67.9)	103 (64.4)
Hypertension	254 (42.6)	223 (74.3)*	93 (67.9)*	127 (79.4)*‖
Ever smoked	361 (60.3)	213 (71.5)†	100 (73.5)†	112 (70.4)‡
Current smokers	93 (15.5)	174 (19.4)*	39 (28.7)*	42 (26.4)†
Myocardial infarction	37 (6.2)	17 (5.7)	7 (5.1)	10 (6.3)
Diabetes mellitus	23 (3.8)	29 (9.7)*	17 (12.4)*	12 (7.5)‡

Values in parentheses denote SD for continuous data and % for categorical data.
*$P<0.0005$, †$P<0.005$, ‡$P<0.05$ vs controls; §$P<0.0005$, ‖$P<0.05$ vs lacunar infarction.

TABLE 2. Genotype Distribution of eNOS Polymorphisms

		Genotype Distribution, n (%)						
Locus	Genotype	Controls	All SVD	*P**	Lacunar Infarction	*P**	Ischemic Leukoaraiosis	*P**
T-786C	TT	220 (36.7)	115 (38.6)		55 (40.4)		59 (37.1)	
	CT	283 (47.2)	143 (48.0)		65 (47.8)		77 (48.4)	
	CC	96 (16.0)	40 (13.4)	0.58	16 (11.8)	0.42	23 (14.5)	0.88
eNOS 4ab	bb	433 (72.8)	231 (78.3)		110 (83.3)		119 (74.4)	
	ab	145 (24.4)	58 (19.7)		21 (15.9)		36 (22.5)	
	aa	17 (2.9)	6 (2.0)	0.20	1 (0.8)	0.03	5 (3.1)	0.88
G894T	GG	242 (40.5)	117 (39.4)		48 (35.3)		68 (43.0)	
	GT	294 (49.2)	138 (46.5)		69 (50.7)		68 (43.0)	
	TT	62 (10.4)	42 (14.1)	0.25	19 (14.0)	0.35	22 (13.9)	0.27

*χ^2 test vs control group.

absent or mild leukoaraiosis) or ischemic leukoaraiosis (at least 1 focal lesion and moderate or severe leukoaraiosis). A complete description and validation of this subtyping method have been published.[3] In 28 patients (9.3%), original hard copies of brain imaging could not be retrieved; in 25 of these, subtyping was possible through formal radiological reports.

Statistical Analysis

Univariate comparisons of categorical variables were performed with χ^2 statistics. Continuous variables were compared by use of Student's *t* test for 2 groups or analysis of variance (ANOVA) for 3 groups. Multivariate logistic regression was used to determine the influence of eNOS polymorphisms on disease risk, controlling for vascular risk factors. To assess different models at each locus, genotypes at each position were coded: 1 (-786TT, intron 4bb, 894GG), 2 (-786CT, intron 4ab, 894GT), and 3 (-786CC, intron 4aa, 894TT). An additive model compared genotype 3 versus 2 versus 1. A dominant model compared genotypes 3 and 2 versus 1. The recessive model compared genotype 3 versus 1 and 2. These analyses were performed with SPSS for Windows, version 10.0 (SPSS Inc). Haplotype analysis was performed with FASTEHPLUS,[26] a program based on EH (estimating haplotypes).[29] It uses unphased marker genotypes from a group of unrelated individuals or groups of cases and controls and a gene-counting algorithm to estimate haplotype frequencies. It also allows testing of association between a disease locus and groups of markers by providing asymptotic and permutation test statistics. Linkage disequilibrium coefficients (D') were calculated with the 2LD program. Software downloads are available at http://www.iop.kcl.ac.uk/iop/Departments/PsychMed/GEpiBSt/software.shtml.

Results

Subject Characteristics

SVD cases and controls were matched for age and sex, but typical differences for other conventional cerebrovascular risk factors were observed (Table 1). One hundred thirty-seven cases (45.8%) were subtyped as isolated lacunar infarction; 160 (53.3%) were classified as ischemic leukoaraiosis.

Genotype Distributions

For each polymorphism, genotyping was successful in at least 98.9% cases. Genotype frequencies at all loci were in Hardy-Weinberg equilibrium for cases and controls. All 3 loci were in linkage disequilibrium with each other ($P<0.0005$). D' values are provided in Figure 1. A significant difference was observed in the distribution of the intron 4ab genotype (Table 2), which was confined to the isolated lacunar infarction group ($P=0.03$). The aa/ab genotypes were underrepresented compared with controls. The univariate odds ratios (ORs) associated with the -786C, intron 4a, and 894T alleles are shown in Table 3. On multivariate analysis

TABLE 3. ORs Associated With eNOS Polymorphisms for SVD and Different SVD Subtypes

		Univariate OR (95% CI)		
Allele	Model	All SVD	Lacunar Infarction	Ischemic Leukoaraiosis
−786C	Additive	0.91 (0.74–1.11)	0.84 (0.64–1.11)	0.96 (0.75–1.24)
	Dominant	0.96 (0.83–1.11)	0.93 (0.76–1.12)	0.99 (0.83–1.19)
	Recessive	0.90 (0.74–1.11)	0.84 (0.63–1.11)	0.94 (0.74–1.20)
Intron 4a	Additive	0.77 (0.58–1.03)*	0.55 (0.35–0.86)**	0.95 (0.68–1.34)
	Dominant	0.74 (0.53–1.03)*	0.53 (0.33–0.87)**	0.92 (0.62–1.37)
894T	Additive	1.02 (0.89–1.18)	1.12 (0.92–1.36)	0.95 (0.79–1.13)
	Dominant	1.18 (0.91–1.38)	1.02 (0.78–1.33)	1.23 (0.92–1.63)
	Recessive	1.19 (0.97–1.47)	1.19 (0.9–1.56)	1.18 (0.91–1.54)

*p=0.08, **p=0.01.

TABLE 4. Haplotype Frequencies among the Different Groups

	Frequency, n (proportion)			
Haplotype	Controls (n=1182)	Cases (n=582)	Lacunar Infarction (n=266)	Ischemic Leukoaraiosis (n=314)
t-b-g	0.45	0.48	0.50	0.48
t-b-t	0.13	0.13	0.12	0.13
t-a-g	0.01	0.01	0.01	0.02
t-a-t	0.00	0.01	0.01	0.01
c-b-g	0.05	0.05	0.04	0.04
c-b-t	0.21	0.24	0.25	0.23
c-a-g	0.13	0.10	0.07	0.12
c-a-t	0.02	0.00	0.00	0.00

P=0.01, SVD vs controls; P=0.001, isolated lacunar infarction vs. control group, P based on ≥1000 permutations (n=number of haplotypes).

controlling for age, smoking, hypertension, diabetes mellitus, and myocardial infarction, the 4a allele remained independently associated with lacunar infarction (dominant model: OR, 0.58; 95% confidence interval [CI], 0.35 to 0.97; P=0.04; additive model: OR, 0.59; 95% CI, 0.37 to 0.95; P=0.03). The low frequency of the intron 4aa genotype precluded analysis of this locus according to a recessive model. We found no significant interaction between intron 4a genotype and smoking on disease risk.

Haplotype Analysis

All 8 potential haplotypes were represented (Table 4), denoted by the allele at positions -786, intron 4, and 894. Compared with controls, there was a difference in haplotype distribution among all SVDs (P=0.01) and isolated lacunar infarction (P=0.001), with the c-a-t and c-a-g haplotypes underrepresented in disease. There was no association with the ischemic leukoaraiosis phenotype (P=0.52).

NO_x Levels

Plasma NO_x levels were lower in cerebral SVD, but this was not statistically significant (SVD, 13.95 μmol/L [SD, 5.4 μmol/L]; controls, 15.80 μmol/L [SD, 5.40 μmol/L]; P=0.23). There were no significant differences in plasma NO_x levels when the 2 SVD subgroups were compared with each other and with controls (P=0.36, ANOVA).

Functional Significance of Polymorphisms

An allele dosage effect with NO_x levels increasing across the 3 different T-786C genotypes was observed (P=0.03; P=0.06, multivariate analysis). There was a similar linear trend at the intron 4 locus (P=0.17) but not at the G894T locus (P=0.65; Figure 2, top). Because the haplotype distribution indicated that the combined presence of -786C and intron 4a was protective, the effects of both loci on NO_x concentration were determined. Levels in patients with the -786CC genotype were higher in the presence of the intron 4a allele (Figure 2b, bottom). Additionally, NO_x levels increased across T-786C genotype only in the presence of the intron 4a allele (P=0.07 for interaction).

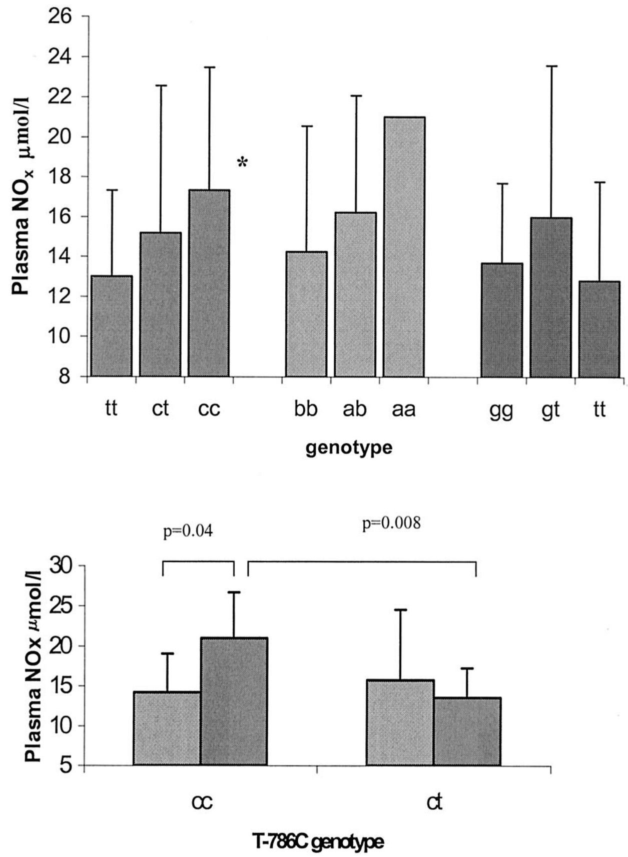

Figure 2. Top, Plasma NO_x levels (SD) according to eNOS genotype at T-786C (red), Intron 4ab (blue), and G894T (green) loci. *P=0.03 for trend. Bottom, Plasma NO_x levels at T-786C locus according to presence or absence of 4a allele. Analysis is restricted to -786 CC and CT genotypes because no individuals were found carrying -766TT genotype and intron 4a allele (red, intron 4a present; blue, intron 4a absent).

Discussion

The main finding in this study is that the intron 4a allele of the eNOS gene was protective against cerebral SVD, an effect confined to isolated symptomatic lacunar infarction. The haplotype analysis confirmed the role of the 4a variant, because the c-a-t and c-a-g haplotypes were particularly underrepresented in disease. Our results are consistent with the role of endothelial dysfunction in cerebral SVD, specifically a role of endothelium-derived NO.

In our study, we found that NO_x levels were lower in SVD, but the difference was not statistically significant. This finding probably reflects the smaller number of patients for whom NO_x measurements were available and variability in NO_x measurements in controls, despite reducing the influence of diet on the plasma pool by collecting only fasting plasma samples and allowing subjects to drink only nitrate-free water. Although we were unable to demonstrate an association between NO_x levels and SVD, we did find evidence of linear associations between NO_x measurements and eNOS genotypes, particularly at the T-786C locus. At this position there was evidence of an allele dosage effect, with NO_x levels increasing with number of C alleles. Our findings would be consistent with the location of this polymorphism within the promoter region and in vitro functional work indicating that the 786C allele is associated with increased gene transcription.[14] A similar trend was found between NO_x levels and the intron 4ab genotype, with higher levels associated with the presence of the 4a allele, a finding consistent with earlier reports.[17,18]

There are a number of possible explanations for the association between the 4a allele and disease. The intron 4 locus could act simply as a marker for another functional polymorphism in linkage disequilibrium. Such a variant would have to be different from the polymorphisms we tested (-786C and 894T). Another possibility is that the intron 4a allele has intrinsic functional significance because there was a weak association with plasma NO_x concentrations in our study. Although this variant lies within an intron, an insertion/deletion polymorphism could affect mRNA stability and enzyme levels. A third possibility is that the intron 4 locus modulates the effects of a variant in linkage disequilibrium. Because the combination of -786C and intron 4a was protective in our study, this haplotype could have a particular functional role. Consistent with this hypothesis, the intron 4a allele led to a significant increase in NO_x levels associated with the -786CC genotype and an increase in levels across the different T-786C genotypes. One potential explanation is that the intron 4 27-bp repeat element has a *cis* regulatory role enhancing transcription activity at the -786 locus.[14]

Two previous studies examined the G894T variant as a risk factor in SVD. In the Étude du profil Génétique de l'Infarctus Cérébral (GÉNIC)[19] study, the 896GG genotype was found to be a risk factor for lacunar stroke but not other stroke subtypes, a finding that we and others[20] have not been able to reproduce. Furthermore, we found not even a trend for this locus to be associated with NO_x levels, which would argue that it is nonfunctional.

Conversely, a number of earlier studies have suggested that the 4a allele is a risk factor for vascular diseases. In relation to stroke, Hou and colleagues[21] recently reported that the intron 4a allele was a risk factor for all stroke subtypes, including lacunar stroke, which would be at variance with our observation that the intron 4a allele protected against SVD. It is possible that our divergent findings reflect differences in genetic backgrounds, because the frequency of eNOS polymorphisms has been shown to vary markedly among different ethnic groups,[30] or differences in environmental exposure, which has been shown to modify the influence of eNOS variants on disease risk.[31]

Interestingly, in our study, the protective effect of eNOS genotype was confined to only the group with isolated lacunar infarction, not those with leukoaraiosis, which would support the existence of heterogeneity within SVD and different etiopathogeneses of the 2 phenotypes.[3,23,24] Availability of endothelial NO could be particularly important in relation to development of microatheroma at the origin of perforating arterioles and subsequent occlusion. Further studies using well-characterized phenotypes may help to clarify the molecular genetic mechanisms involved in cerebral SVD and provide insights into disease pathogenesis.

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