

Endothelial Nitric Oxide Synthase Gene Polymorphism Is Associated with Systemic Lupus Erythematosus

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ABSTRACT. Objective. In systemic lupus erythematosus (SLE), endothelial nitric oxide synthase (eNOS) gene locus has been found to be suggestive of linkage with disease, nitric oxide (NO) is produced in significant amounts, and endothelial cell dysfunction is observed. eNOS gene polymorphism may affect both the synthesis of eNOS protein and its enzymatic activity. We examined the influence of eNOS gene polymorphisms on susceptibility to SLE.

Methods. Genomic DNA from 88 Northwestern Colombian women with SLE, as well as 199 controls matched for sex, age, and ethnicity, was genotyped for the $-786T \rightarrow C$ polymorphism in the promoter region, the intron 4 variable number of tandem repeats, and the Glu298Asp polymorphism in exon 7 of the eNOS gene by polymerase chain reaction and restriction fragment length polymorphism techniques. Haplotype and allele frequency comparisons, a Hardy-Weinberg equilibrium test, and linkage disequilibrium (LD) analysis were performed.

Results. The intron 4b allele was associated with SLE (OR 2.2, 95% CI 1.29–3.60, $p_c = 0.005$) as was the 4bb genotype (OR 2.9, 95% CI 1.61–5.33, $p_c = 0.0009$), while the 4a allele was protective (OR 0.4, 95% CI 0.26–0.76, $p_c = 0.005$), as was the 4ab genotype (OR 0.29, 95% CI 0.15–0.56, $p_c < 0.0001$). In controls, all loci were in linkage disequilibrium ($p < 0.02$). In patients, intron 4 was in Hardy-Weinberg disequilibrium, due to an excess of homozygotes ($p = 0.01$).

Conclusion. eNOS polymorphism influences SLE predisposition. Since intron 4bb genotype is responsible for higher levels of eNOS synthesis and intron 4 ab genotype is associated with lower synthesis, our results might provide insight into the elevated levels of NO observed in SLE patients. (J Rheumatol 2004;31:2163–8)

Key Indexing Terms:

SYSTEMIC LUPUS ERYTHEMATOSUS

POLYMORPHISM

LUPUS NEPHRITIS

ENDOTHELIAL NITRIC OXIDE SYNTHASE

AUTOANTIBODIES

GENETICS

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that can affect diverse organ systems including the vascular system. Vascular manifestations typically associated with SLE span a broad range, including vasculitis, vasculopathy, vasospasm, and thromboembolism. Endothelial cell dysfunction is characteristic of

patients with SLE. Decreased fibrinolytic capacity and increased nitric oxide (NO), von Willebrand factor, fibronectin, and thrombomodulin levels have all been documented as indicators of endothelial cell dysfunction in patients with SLE^{1–6}. This endothelial cell dysfunction might be induced by immune complex deposition into the endothelial cells^{1,4}, by antiendothelial⁵ and anti-DNA antibodies⁷, and by increased levels of homocysteinemia⁸. Nevertheless, some investigators have argued that plasma markers of endothelial cell damage in SLE seem to be an epiphenomenon and may simply be related to excess of inflammation⁹. However, elevated levels of activated circulating endothelial cells found in SLE patients indicate that vascular injury occurs in SLE and that endothelial cells are potential participants in the inflammatory processes that contribute to tissue damage in SLE¹⁰.

NO is an important biologically active molecule that participates in inflammatory and autoimmune responses, as well as in host defense against microbes and tumor cells¹¹. It is produced by the conversion of L-arginine to L-citrulline by NO synthase (NOS), an enzyme present in 3 isoforms: a constitutive NOS expressed in neuronal tissue; an inducible enzyme found in macrophages and other cells that

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plays a role in NO-induced cytotoxicity; and a constitutively active endothelial form (eNOS)¹². The inducible form of NOS (iNOS) is also present in endothelial cells¹². NO is readily transformed into nitrite and nitrate, both of which are excreted into the urine.

The major physiological stimulus for the continuous production of NO *in vivo* is shear stress. The cellular action of NO is due to the activation of guanyl cyclase and the formation of cyclic guanosine 3':5'-cyclic monophosphate (cyclic GMP). Whereas NO is quite unstable, the formation of S-nitrosothiols in the presence of oxygen and thiols provides a NO stable reservoir¹³.

One of the 3 identified isoforms of NOS, eNOS is critical to vascular homeostasis and therefore participates in the pathogenesis of endothelial dysfunction¹⁴. Normal endothelial function is characterized by a dynamic balance between NO and other oxidants, including O₂⁻ and H₂O₂¹⁵. Most of the NO released from endothelial cells is elaborated abuminally, where it acts on the smooth muscle cell to cause vasodilation. However, some NO may also enter the lumen and thereby diffuse into platelets. Prostacyclin and NO can act synergistically to reverse platelet aggregation¹⁶.

Activated endothelial cells are sources of excessive NO production in SLE¹. Overproduction of NO could contribute to tissue injury given its capacity to increase vascular permeability, generate toxic free radicals such as peroxynitrite, and induce cytotoxicity^{1,17}. Most studies focusing on NO in SLE have considered upregulated iNOS as the enzymatic mechanism explaining NO production. In particular, iNOS has been implicated in the pathogenesis of nephritis¹⁸, endothelial cell dysfunction¹, and cutaneous involvement in SLE¹⁹. No association with iNOS gene polymorphism, located on chromosome 17 at 17q11.2-q12, was observed in Spanish patients with SLE²⁰. In contrast, iNOS promoter polymorphism was found to be a risk factor for SLE in African-American patients²¹.

The eNOS gene, located on chromosome 7q36.1, is composed of 26 exons spanning 21 kb, and is polymorphic²². Several single nucleotide polymorphisms (SNP) and variable number of tandem repeats (VNTR) have been described, some of which may regulate eNOS expression. It has been shown that intron 4 VNTR influences both the eNOS protein synthesis and the enzyme activity²³. Notably, eNOS locus has been found to be suggestive of linkage with SLE²⁴. We examined the -786T→C SNP in the promoter region, the intron 4 VNTR, and the Glu298Asp SNP in exon 7 of the eNOS gene in patients with SLE from Northwestern Colombia.

MATERIALS AND METHODS

Study population. This was an association study in which we analyzed 88 women with SLE, whose clinical and immunological characteristics are shown in Table 1. All patients fulfilled 4 or more of the classification criteria for SLE²⁵. Patients were seen in the Rheumatology Unit at the Clínica Universitaria Bolivariana, in Medellín, Colombia. Controls were 199 per-

Table 1. General characteristics of 88 women with SLE.

Characteristic	
Age, yrs	34.4 ± 11.6
Duration of SLE, yrs	5.9 ± 6.5
Clinical manifestations, %	
Musculoskeletal involvement	89
Cutaneous involvement	85
Raynaud's phenomenon	48
Cardiopulmonary	30
Nephritis	40
Hypertension	50
Neurologic involvement	45
Hematologic	75
Antiphospholipid syndrome	22
SLICC > 1	19
Autoantibodies, %	
Antinuclear	100
Anti-DNA	72
Anti-Ro	33
Anti-La	14
Anti-Sm	35
Anti-RNP	55

SLICC: Systemic Lupus International Collaborating clinics.

sons without inflammatory or autoimmune disease or history of chronic infectious disease, including tuberculosis or human immunodeficiency virus infection. They were matched to patients by age, sex, ethnicity, and socioeconomic status and were unrelated to the patients. Their mean age was 41 ± 15 years. This research was conducted in compliance with Resolution No. 008430 (1993) from the Ministry of Health of the Republic of Colombia, was classified as research with minimal risk, and had local Ethics Committee approval.

Definition of clinical and laboratory features. The clinical and laboratory variables associated with SLE, including each feature of the revised American College of Rheumatology criteria, were evaluated²⁵. Each clinical and serological finding was recorded as present or absent for each patient at any time in the course of the disease. These clinical manifestations were defined as follows: (1) arthritis: non-erosive arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion; (2) malar rash; (3) photosensitivity; (4) alopecia; (5) discoid lupus; (6) Raynaud's phenomenon; (7) renal involvement, as evidenced by a positive renal biopsy result, or active urinary sediment, or proteinuria > 500 mg/24 h; (8) neurologic involvement, as evidenced by seizures without any other definable cause, or psychosis lacking any other definable cause, or other conditions such as peripheral neuropathy, stroke, transverse myelitis, chorea, or other central nervous system lesions directly attributable to SLE in the absence of other causes; (9) pleuritis: pleural rub and/or effusion and/or typical pleuritic pain; (10) pericarditis: documented by electrocardiogram, rub, or evidence of pericardial effusion; (11) autoimmune hemolytic anemia, with a hematocrit < 35% and a reticulocyte count > 4%; (12) leukopenia, white cells < 4000/mm³; (13) thrombocytopenia, platelets < 100,000/mm³; (14) arterial or venous thrombosis diagnosed on clinical grounds and confirmed by complementary tests. The presence or absence of arterial hypertension (blood pressure levels > 140/90) was also recorded, as was the presence of antiphospholipid syndrome²⁶.

Severity of disease. The severity of the disease and the organic damage was evaluated using the Systemic Lupus International Collaborating Clinics (SLICC) damage index²⁷.

Serologic studies. Antinuclear antibodies (ANA) were determined by immunofluorescence using Hep-2 cells as substrate. Anti-dsDNA antibody

ies and precipitating antibodies to extractable nuclear antigens (ENA), including Sm, U1-RNP, Ro/SSA, and La/SSB as well as anticardiolipin antibodies were detected by immunoenzymatic methods (ELISA Quantalite, Inova, San Diego, CA, USA) according to manufacturer's instructions. C3 and C4 were measured by immunodiffusion.

eNOS genotyping. Genomic DNA was extracted from 10 ml of EDTA-anti-coagulated blood sample using the standard salting-out technique. The G894T polymorphism in exon 7 of the eNOS gene was genotyped by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis with the primer pairs 5'-AGG AAA CGG TCG CTT CGA CGT GCT G 3' and 5'-CCC CTC CAT CCC ACC CAG TCA ATC C 3' and allele-specific restriction enzyme digestion²⁸. 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 s. 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.

Genotypes for the intron 4 polymorphism were determined by PCR using the primers 5'-AGG CCC TAT GGT AGT GCC TTT-3' and 5'-TCT CTT AGT GCT GTG GTC AC-3'. PCR was performed for 35 cycles in a volume of 40 µl²⁹. The PCR reaction mixtures were heated to 94°C for 4 min for initial denaturation and underwent 35 cycles at 94°C for 30 s for denaturation, at 63°C for 30 s for annealing, and at 72°C for 1 min for extension. Finally, extension was conducted at 72°C for 5 min. The direct PCR products were analyzed by electrophoresis. Fragments of 393, 420, and 447 bp corresponded to the eNOS alleles 4a, 4b, and 4c, respectively.

The variants in the 5'-flanking region were assessed by PCR amplification using the primers 5'-TGG AGA GTG CTG GTG TAC CCC A-3' and 5'-GCC TCC ACC CCC ACC CTG TC-3' with the same temperature cycles described for polymorphism in intron 4²⁹. The amplified products were digested with *Msp I* for 1.5 h at 37°C, producing fragments of 140 and 40 bp for the wild-type allele, or 90, 50, and 40 bp in the case of a polymorphic variant. These fragments were analyzed by electrophoresis. All DNA were coded. The genotyping was blinded to case or control status. The code was broken when the genotyping was completed. For quality control, 10% of the samples were subject to repeat PCR and genotyping, and no discrepancies were detected.

Statistical analysis. Data were managed and stored using the SPSS program (V9.05 for Windows, SPSS, Chicago, IL, USA)³⁰. Differences between allele and genotype frequencies were determined by chi-square and Fisher's exact test as appropriate. Hardy-Weinberg equilibrium testing, linkage disequilibrium testing, and estimates of haplotype frequencies were performed using Arlequin software³¹ as described³². Because neither family studies nor sequencing was systematically performed, designated haplotypes in this study are likely haplotypes based on mathematical likelihoods. Crude odds ratios (OR), as estimates of the relative risk, were calculated with 95% confidence intervals (CI). A p value < 0.05 was considered statistically significant.

RESULTS

In SLE patients as well as in the control group, -786 T, intron 4 b, and Glu298Asp G were the most frequent alleles (Table 2). The estimated haplotype frequencies among the -786 SNP, intron 4, and Glu298Asp inter-loci combination disclosed the TbG haplotype as the most frequently observed in both patients and controls (Table 3). Significant differences in intron 4 genotype and allele frequencies were detected (Table 2). Allele intron 4b was found more frequently in patients than in controls (88% vs 77%; OR 2.16, 95% CI 1.29–3.60, $p_c = 0.005$), while the intron 4a allele was less frequent in patients (11% vs 22%; OR 0.45, 95%

Table 2. eNOS allele and genotype frequencies in 88 patients with SLE and 199 controls. Data are expressed in percentages.

Variable	SLE	Controls
Glu298Asp		
G	78	74
T	23	26
GG	61	56
GT	33	37
TT	6	8
Intron 4		
4a	11	22*
4b	88	77**
4c	1	1
4aa	3	3
4ab	15	37†
4ac	1	2
4bb	81	59††
-786		
T	69	69
C	31	31
TT	44	42
TC	51	54
CC	5	4

* OR 0.45, 95% CI 0.26–0.76, $p = 0.0025$. ** OR 2.16, 95% CI 1.29–3.60, $p = 0.0028$. † OR 0.29, 95% CI 0.15–0.56, $p < 0.0001$. †† OR 2.93, 95% CI 1.61–5.33, $p = 0.0003$.

Table 3. Estimated haplotype frequencies. Estimated haplotypes are shown if carried by at least 5% of participants in either patient or control group. Significant differences were not found. Data are percentages.

-786T→C-Intron-4-Glu298Asp	Patients	Controls
TbG	50	49
CbT	15	17
CbG	7	3
TaG	5	11
CaG	4	10
TbT	4	9

CI 0.26–0.76, $p_c = 0.005$). Genotype 4bb was associated with disease (81% vs 59%; OR 2.93, 95% CI 1.61–5.33, $p_c = 0.0009$) (Table 2).

Both patients and controls showed a different pattern for linkage disequilibrium. In the patient group there was no significant association among intron 4 and Glu298Asp and -786 SNP, while in the control group, we observed significant linkage disequilibrium between all loci ($p < 0.02$). Strong deviation from Hardy-Weinberg expected proportion in patients was observed for intron 4. The percentage of observed homozygotes for this locus was greater than expected (84% vs 61%; $p = 0.01$).

We did not find association between eNOS polymorphisms and clinical manifestations, or with the presence of autoantibodies and severity of disease.

DISCUSSION

Our results provide genetic evidence indicating that the eNOS locus influences the susceptibility to SLE in our population. First, a misbalance in intron 4 was clearly seen between patients and controls. The 4b allele and 4bb genotype were associated with SLE. Second, there was greater genetic selection in the intron 4 locus in patients because there was an excess in the number of homozygotes. Third, no linkage disequilibrium was found between intron 4 and Glu298Asp and -786T→C in patients, suggesting a high mutation rate at the intron 4 locus in this group.

Our findings support those observed in a previous genome-wide scan indicating that eNOS locus was suggestive of linkage with SLE²⁴. In an association study, Kim, *et al* assessed Glu298Asp and intron 4 VNTR polymorphisms in Korean patients with Beçhet's disease and other patients with rheumatic diseases with vasculitis, 15 of whom had SLE³³. They did not observe significant differences in eNOS polymorphisms among their SLE patients with vasculitis and 80 healthy controls. In our group not a single patient had vasculitis (Table 1). Investigators from Korea found a weak association between intron 4 ab genotype and lupus nephritis³⁴. In Caucasian and African North American patients with SLE no association with eNOS polymorphisms was observed³⁵. Our study population was from Northwestern Colombia, a group primarily derived from the Spaniards in whom admixture has been shown to be in low proportions with Amerindian and Black populations^{36,37}. Our results therefore provide new data useful for future comparisons on allelic and genotype frequencies and may also contribute to the elucidation of the history of human populations, since the eNOS gene polymorphism varies among populations^{29,35,38}.

We did not observe a significant influence of eNOS polymorphism on the immune response or the course of the disease, although there was an inherent loss of statistical power in these subgroup analyses. SLE is observed in genetically susceptible individuals in whom clinical expression is modified by permissive and protective environments occurring over time. SLE is a complex disease, and its inheritance does not follow a single-gene dominant or single-gene recessive Mendelian law, indicating that it is polygenic. SLE phenotype varies among populations. Since the effects of genotype on phenotype in a particular population may vary depending upon environment and length of its exposure, followup of participants in this study will assess association of eNOS polymorphism with specific disease manifestations.

The intron 4 VNTR has been found to have a consistent influence on eNOS mRNA expression, protein concentration, and enzyme activity^{23,39}. In cultured human umbilical vein endothelial cells, intron 4bb genotype was responsible for higher levels of eNOS synthesis, while intron 4 ab genotype was associated with reduced synthesis²³. Accordingly,

our results might have a functional explanation for the elevated levels of NO, as well as the endothelial dysfunction observed in SLE patients. How intron 4 may influence NO synthesis is still unknown, since this variant is intronic and it is unlikely to be functional in its own right. However, mutation within introns could affect rates of eNOS transcription and/or processing of the primary transcript, and ultimately affect eNOS enzyme levels³⁹. The effect of intron 4 on eNOS protein synthesis could also be indirectly linked to additional variation in its gene structure that produces direct effects²³.

Constitutive eNOS expression is dependent on basal transcription machinery in the core promoter, involving positive and negative protein-protein and protein-DNA interactions⁴⁰. Chromatin-based mechanisms and epigenetic events also regulate expression of eNOS at the transcriptional level in a cell-restricted fashion⁴⁰. Although constitutively active, important physiological and pathophysiologic stimuli alter eNOS gene transcription rates. There are numerous factors known to affect the basal expression level and activity of the enzyme, such as hypoxia⁴¹, shear stress⁴², hormones such as estrogen⁴³, oxidized low-density lipoproteins⁴⁴, and mechanical forces⁴⁵. In contrast, tumor necrosis factor alpha, a cytokine implicated in the pathophysiology of SLE⁴⁶, has been reported to downregulate eNOS mRNA steady-state, stability, and transcription in endothelial cells^{40,47}.

Endothelial cell dysfunction in SLE has been evaluated in disease activity. Most studies agree concerning lack of association between endothelial dysfunction and activity of disease, regardless of methods used^{3,48}. Belmont, *et al* observed that endothelial cell expression of constitutive NOS (i.e., eNOS) was independent of SLE activity¹. These findings were consistent with those of Awada, *et al*, indicating that vascular endothelial injuries occurring during disease flares persist throughout the inactive phases of the disease³. On the other hand, Clancy, *et al* found that levels of activated circulating endothelial cells are associated with SLE activity¹⁰.

While there is compelling evidence indicating that endothelial cell dysfunction occurs in SLE, scarce information exists concerning eNOS synthesis/activity in SLE. Furusu, *et al* observed that glomerular eNOS expression was significantly higher in SLE kidneys with proliferative glomerulonephritis disclosing minimal to mild lesions in comparison with normal kidneys⁴⁹. In the same study, a reciprocal expression pattern of eNOS and iNOS was observed, leading the authors to speculate that the induction of iNOS may compensate the production of eNOS⁴⁹. As reviewed by Abramson, *et al*⁵⁰, excessive NO production has been reported in patients with SLE; however, the role of eNOS in this NO overproduction and its ensuing cytotoxic effects in specific tissues have not been fully examined. The effects of NO vary depending upon the local concentrations,

cellular source, and target^{11,17,50-52}. Although NO constitutively produced by endothelium is believed to play a protective role in the microvasculature^{50,51}, an important contribution of eNOS to inflammatory processes has also been reported⁵².

In conclusion, polymorphism, chromosomal position, and function/tissue expression make the eNOS gene a candidate to confer susceptibility for SLE in our population.

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