Real-Time PCR Assay Optimization for Allelic Discrimination of a Glu298Asp Polymorphism in the Constitutive Endothelial Nitric Oxide Synthase Gene

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Introduction

Nitric oxide (NO) is synthesized from L-arginine by a family of NO synthase (NOS) isoforms. Constitutively expressed NOS isoforms, endothelial NOS (eNOS) and neuronal NOS (nNOS), are likely the major contributors to whole-body NO production. Many studies have strongly associated polymorphisms of the *eNOS* gene with increased risk of hypertension, cardiovascular disease, coronary spastic angina, myocardial infarction, and stroke, but the results are not always conclusive (Wang and Wang 2000). In particular, Yoshimura et al. (1998) identified a Glu298Asp variant in exon 7 of the *eNOS* gene that was more frequent in patients with coronary spasm, but in studies of an elderly population in Australia, Liyou et al. (1998) could find no association of this variant with coronary artery disease. Recently, various eNOS polymorphisms have been reported to be associated with type 2 diabetes and the insulin resistance syndrome (Monti et al. 2003). Moreover, NO impairment has been hypothesized as an early step in the development of metabolic syndrome (Nisoli et al. 2003). Metabolic syndrome is a collection of cardiovascular risk factors, including insulin resistance, abdominal obesity, hypertension, and hypertriglyceridemia, that increase the chance of developing heart disease, stroke, and diabetes. The question of whether subpopulations of humans suffering from metabolic syndrome have defects in *eNOS* gene expression awaits an answer from genetic analysis. Our aim was to investigate the relation between the Glu298Asp polymorphism in *eNOS* exon 7 and metabolic syndrome. For this purpose, we set up a fast and reliable real-time PCR allelic discrimination method.

Methods

Most assays for mutation detection combine amplification of the DNA sequence that spans the target polymorphic nucleotide with allele-specific oligonucleotide (ASO) probes (Wallace et al. 1979). Real-time PCR assays can be devised to combine DNA amplification with probing of specific sequences, allowing amplification and detection in a homogeneous, all-in-one-tube system with reliable genotype assignment. Real-time PCR also minimizes the time needed for experiments, since 48 patient samples can be analyzed simultaneously in duplicate. To validate such a real-time PCR genotyping system, a comparison should be made to established methods. For this comparison, we analyzed samples from 20 patients and 20 control subjects by means of both a classical PCR-RFLP method and real-time PCR allelic discrimination.

Genomic DNA (gDNA) was purified from peripheral blood leukocytes as previously reported (Miller et al. 1988). For PCR-RFLP, a specific *eNOS* genomic fragment covering the Glu298Asp polymorphism was amplified in the iCycler iQ® system followed by endonuclease digestion. Primer pairs were as follows: sense 5'-TCCCTGAGGAGGGCATGAGGCT-3'; and antisense 5'-TGAGGGTCACACAGGTTCCT-3'. The amplification mix (25 µl) consisted of 12.5 µl 2x iQ™ supermix, 500 ng gDNA, and 300 nM of each primer. After initial denaturation and hot-start DNA polymerase activation (3 min at 95°C), amplification was performed for 30 cycles (1 min at 94°C, 1 min at 61°C, and 1 min at 72°C). The resulting 457 bp products were incubated at 37°C for at least 20 hr with 8 U of the restriction enzyme *Ban*II. In case of polymorphism at position 1917 of the *eNOS* gene, typically a G to T substitution, the *Ban*II restriction site is lost.

For real-time PCR experiments, primers and ASO hydrolysis probes were designed by Beacon Designer 2.1 software (PREMIER Biosoft International) and purchased from Qiagen GmbH (Hilden, Germany); they are shown in Table 1. Forward and reverse primers were used in a PCR fluorescent detection system with the intercalating dye SYBR Green I to check for product specificity. Each sample (25 µl) contained 12.5 µl iQ™ SYBR® Green supermix, 0.1 µM of each primer, and scalar quantities of gDNA (200, 100, 50, and 25 ng). PCR cycles were programmed on an iCycler iQ real-time PCR detection system and consisted of hot-start incubation (3 min at 95°C) and amplification for 40 cycles (10 sec at 95°C and 10 sec at 62°C). Generation of a melt curve starting at 55°C with increments of 0.5°C every 10 sec followed the amplification program.

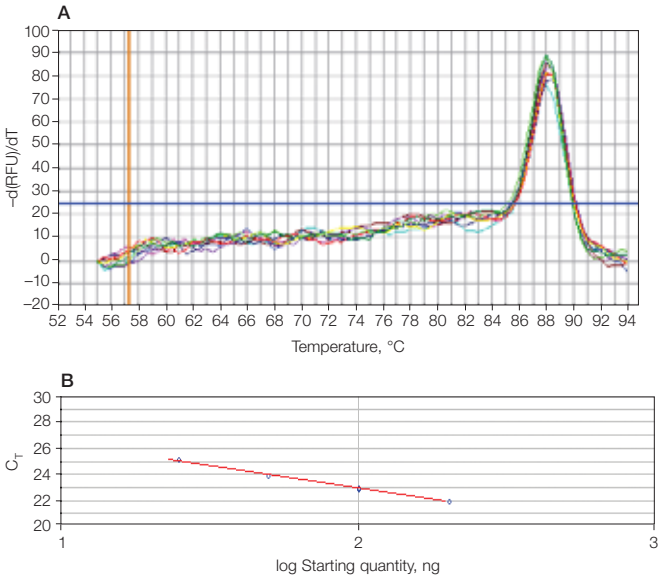

Fig. 1. Specificity of PCR amplification. A, melt curve; B, standard curve. Results using a reaction mix with SYBR Green I and the primers shown in Table 1. Standard curve had slope = –3.492, intercept = 29.39, r^2 = 0.997.

In order to find the specific discriminating conditions for the ASO probes, the iCycler iQ gradient feature was used to optimize annealing temperature (Ugozzoli 2003). Duplicate PCR reactions were carried out in 25 µl containing 12.5 µl iQ supermix, 0.1 µM of each primer, 0.075 mM of each ASO probe, and 50 ng human gDNA. PCR conditions were the same as for the SYBR Green I test, except for annealing/extension temperature. This was performed at 58.0, 59.0, 60.5, 61.0, 61.5, 62.0, 62.5, and 63.0°C (temperature gradient). All subsequent analyses for test validation were performed with the same conditions except for the annealing/extension temperature, fixed at 62.0°C.

Table 1. Sequence of primers and ASO probes for Glu298Asp *eNOS* polymorphism analysis. The polymorphic site is underlined. Abbreviations: WT, wild type; BHQ, Black Hole Quencher.

Name	Sequence
eNOS-Rev primer	5'-GGGGGCAGAAGGAAGAGT-3'
eNOS-Fwd primer	5'-CCAGGAAACGGTCGCTTC-3'
eNOS-WT probe	5'-[Texas Red]-CTGGGGGCTCATCT-GGG-[BHQ2]-3'
eNOS-Mutant probe	5'-[6-FAM]-CTGGGGGATCATCTGGG-[BHQ1]-3'

Results

PCR Specificity

To check the specificity of PCR amplification using the primers shown in Table 1, we amplified some samples in the presence of SYBR Green I. The iCycler iQ system was programmed to perform a two-step (denaturation and annealing/extension) PCR protocol, and melt-curve analysis followed amplification. Figure 1A clearly shows a single sharp peak, indicating amplification of a single product and the absence of nonspecific fragments and primer-dimers at an annealing temperature of 62°C. In addition, high PCR efficiency and a good dynamic range were observed (Figure 1B). Primer-dimers and nonspecific PCR fragments were also not observed within the temperature range used subsequently (59–64°C; data not shown).

Assay for Allelic Discrimination

The differentially labeled wild-type (WT) and mutant ASO probes were mixed in the same reaction with the PCR primers and subjected to an annealing temperature gradient from 59 to 64°C. Some results from the optimization experiment are shown in Figure 2. For both the WT and mutant probes, reactions worked well within the range 62–64°C. Results obtained using annealing temperatures lower than 62°C showed lack of specificity, especially for the WT ASO probe. For example, the amplification plot generated at 60°C (Figure 2A) shows that the WT probe labeled with Texas Red hybridized to the WT PCR product (red trace), but also cross-hybridized to the homozygous mutant PCR product (blue trace).

Validity

After assay optimization, 20 metabolic syndrome patients and 20 healthy control subjects were genotyped using the real-time PCR method described. The results (Figure 3) were compared to those obtained by PCR-RFLP analysis (Figure 4). The genotyping results were the same for all 40 samples analyzed (100% accordance), confirming the validity of the method. Table 2 summarizes the results for both methods.

Table 2. Number and percentage of metabolic syndrome patients and control subjects showing the different *eNOS* variants.

Genotype	Metabolic Syndrome Patients	Control Subjects
WT (GG)	8 (40%)	10 (50%)
Heterozygote (GT)	9 (45%)	8 (40%)
Mutant (TT)	3 (15%)	2 (10%)
Total	20 (100%)	20 (100%)

Conclusions

We have shown a robust assay for allelic discrimination of the Glu298Asp polymorphism in the *eNOS* gene. This assay shows the accuracy, specificity, reliability, and processivity of a real-time PCR genotyping system. Typically, 48 samples can be processed in 1.5 hr in duplicate, so hundreds of patients can be genotyped in a few days. The data reported in Table 2 show a very slight prevalence of *eNOS* mutants and heterozygotes in metabolic syndrome patients compared with control subjects. In any case, the number of subjects tested in this preliminary study is too few to draw any conclusions. Our group has so far recruited more than 400 metabolic syndrome patients and 200 healthy control subjects. These will be part of a wider study that we hope will shed light on the possible involvement of *eNOS* defects in the genesis of metabolic syndrome.

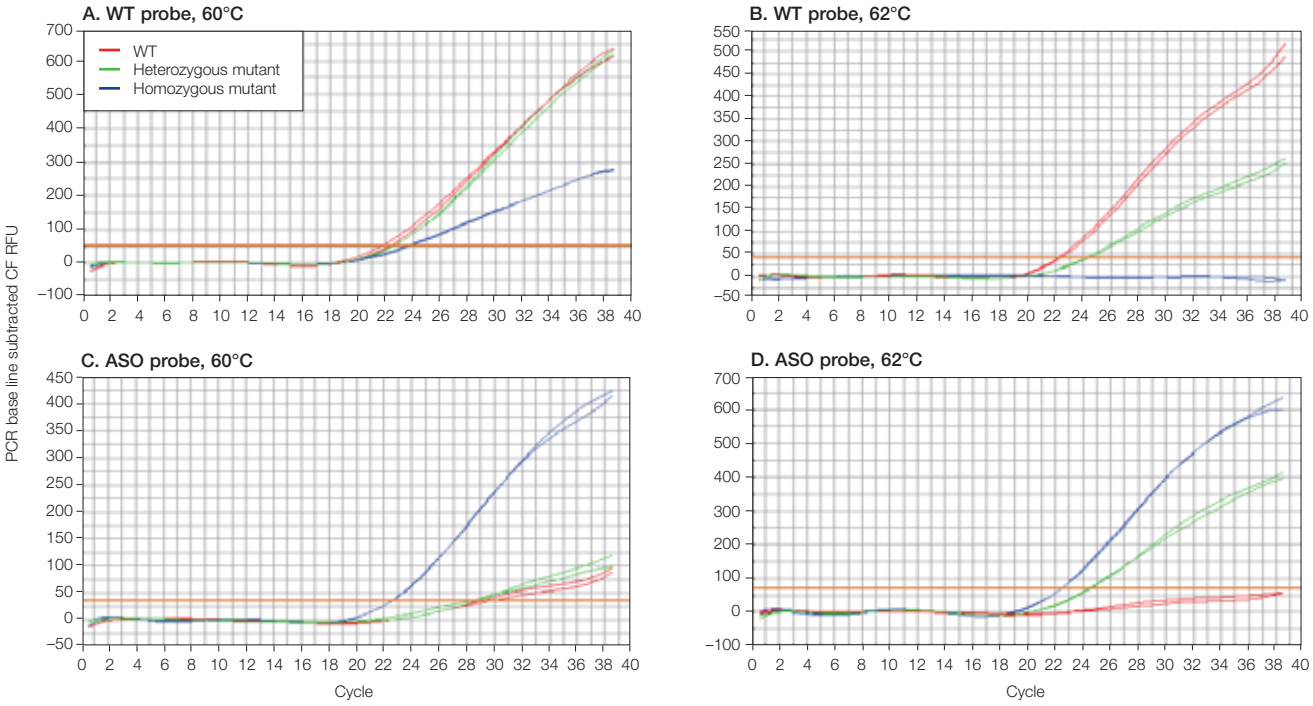

Fig. 2. Amplification plots for WT (A and B) and mutant (C and D) ASO probes. PCR was carried out with an annealing/extension step at 60°C (A and C) or at 62°C (B and D). The traces for each genotype were clearly distinguishable at 62°C.

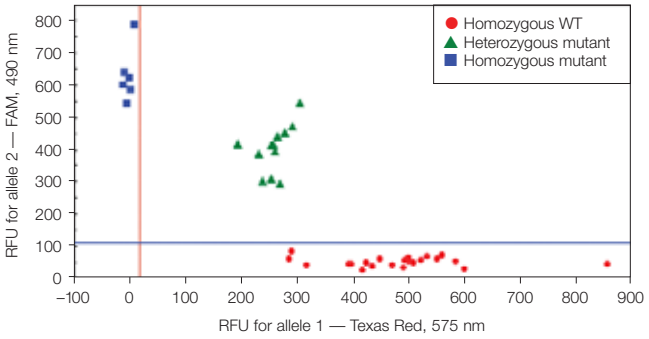

Fig. 3. Example of an allelic discrimination graph for the Glu298Asp polymorphism in the *eNOS* gene. Twenty samples were analyzed in duplicate in a single analysis.

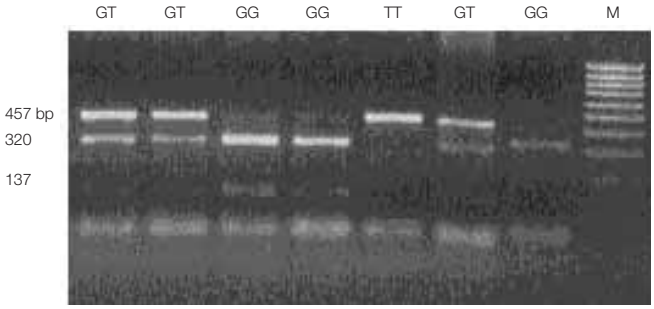

Fig. 4. Example of a classical PCR-RFLP analysis for Glu298Asp polymorphism in the *eNOS* gene. Samples from seven individuals were run on a 2% agarose gel, stained with ethidium bromide, and photographed under UV transillumination. GG = WT homozygote; GT = heterozygote; TT = mutant homozygote; M, DNA size markers.

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