Intracellular processing of endothelial nitric oxide synthase isoforms associated with differences in severity of cardiopulmonary diseases: Cleavage of proteins with aspartate vs. glutamate at position 298

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An endothelial nitric oxide synthase (eNOS) polymorphism in exon 7 (894 G/T) resulting in glutamate or aspartate, respectively, at position 298 on the protein is correlated with severity of cardiopulmonary diseases. Because glutamate and aspartate are considered to be conservative replacements, the polymorphism was thought to be a marker for a functional locus elsewhere in the gene. We now show in transfected cells, primary human endothelial cells, and human hearts, that eNOS with aspartate, but not glutamate, at position 298 is cleaved, resulting in the generation of 100-kDa and 35-kDa products. Recombinant or native eNOS was examined by immunoblotting either in lysates (COS7) or after partial purification over 2′,5′-ADP-Sepharose and calmodulin-Sepharose. Immunoblotting after SDS/PAGE with a carboxyl-terminal antibody showed a single major protein band in the predicted position for eNOS at 135 kDa. An additional band at approximately 100 kDa was present only in the recombinant 298Asp eNOS and in the eNOS synthesized by primary cells and heart tissue with a G/T genotype. Using an eNOS amino-terminal-specific antibody, an immunoreactive band at approximately 35 kDa, corresponding to the residual N-terminal cleavage fragment, was observed in those cells with a T genotype. Thus, eNOS with aspartate but not glutamate at position 298 is cleaved, resulting in the generation of N-terminal 35-kDa and C-terminal 100-kDa fragments. Thus, the eNOS gene with polymorphisms at nucleotide 894 generates protein products with differing susceptibility to cleavage, suggesting that, in contrast to prior predictions, this polymorphism has a functional effect on the eNOS protein.

Nitric oxide, a ubiquitous messenger molecule with important regulatory functions, is synthesized by a family of enzymes called nitric oxide synthases (NOS). Three NOS isoforms have been identified: two constitutive, the neuronal (nNOS, type I) and endothelial (eNOS, type III) enzymes, and one inducible (iNOS; type II). All have an amino-terminal heme- and arginine-binding domain, a central calmodulin-binding region, and a carboxyl-terminal reductase domain, with an NADPH-binding site (1–4).

The eNOS gene is located on chromosome 7q35–36 and comprises 26 exons spanning 21 kb (1). In view of the physiological and pathophysiological importance of NO, the potential role of eNOS in the pathogenesis of various human diseases has been examined using its polymorphic variants as potential disease markers.

Various genetic polymorphisms of the eNOS gene have been reported as "susceptibility genes" in a number of cardiovascular and pulmonary diseases. A $T^{-786}\rightarrow C$ mutation in the 5′-flanking region of eNOS gene was associated with coronary spasm in a Japanese population (5); whereas a 27-bp repeat in intron 4 at the 5′-end of the eNOS gene was correlated with a smoking-dependent risk of coronary disease in an Australian population (6). The frequency of the 774 T and 894 T alleles, which exhibit genetic disequilibrium, was associated with severity of lung disease in α-1-antitrypsin deficiency (7). 894 G→T was also correlated with increased coronary spasm (8), myocardial infarction (9, 10), and essential hypertension (11). In particular, the data from the Cambridge Heart Antioxidant Study (CHAOS) show significantly more Asp^{298} homozygosity among patients with coronary heart disease (12). Although the 774 C/T substitution is silent, the 894 G/T substitution results in a glutamate or aspartate, respectively, at position 298 in the eNOS protein. Because glutamate and aspartate are conservative substitutions, it has been postulated that the polymorphism serves as a marker for a functional effect elsewhere in the eNOS gene or in its vicinity. In the present study, we demonstrate that the eNOS gene product with an aspartate, but not a glutamate, at position 298 is subject to cleavage in normal tissue and in cells overexpressing eNOS. Thus, in contrast to prior conclusions, the coding region polymorphism has functional consequences.

Materials and Methods

Plasmid Constructs. Human aorta eNOS cDNA insert 893 A/894 G (a kind gift from T. Quertermous, Harvard Medical School, Massachusetts General Hospital, Boston) was ligated into the PBK-CMV phagemid vector (Stratagene) at the *Eco*RI restriction site. Two mutant constructs, 894T and 893C were prepared by using QuickChange Site-Directed Mutagenesis Kit (Stratagene). The oligonucleotides used in the mutagenesis for 894T and 893C were 5′-GCAGGCCCCAGATGA**T**CCCCCA-GAACTCTTCC-3′ and 5′-GCAGGCCCCAGATG**C**GC-CCCCAGAACTCTTCC-3′, respectively. Sequences of all constructs were confirmed twice by DNA sequencing (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit; Perkin–Elmer).

Cell Culture and Transient Transfection. Primary cultures (passage 4 to 10) of all human primary endothelial cells were grown by using media and conditions supplied by the manufacturer (Clonetics, San Diego). COS7 cells (American Type and Culture Collection) grown in high glucose DMEM (Life Technologies, Rockville, MD; cat. no. 11965-092) supplemented with 10% FBS and 40 units/ml penicillin-streptomycin, were transfected with 4

Abbreviations: NOS, nitric oxide synthase; eNOS, endothelial NOS.

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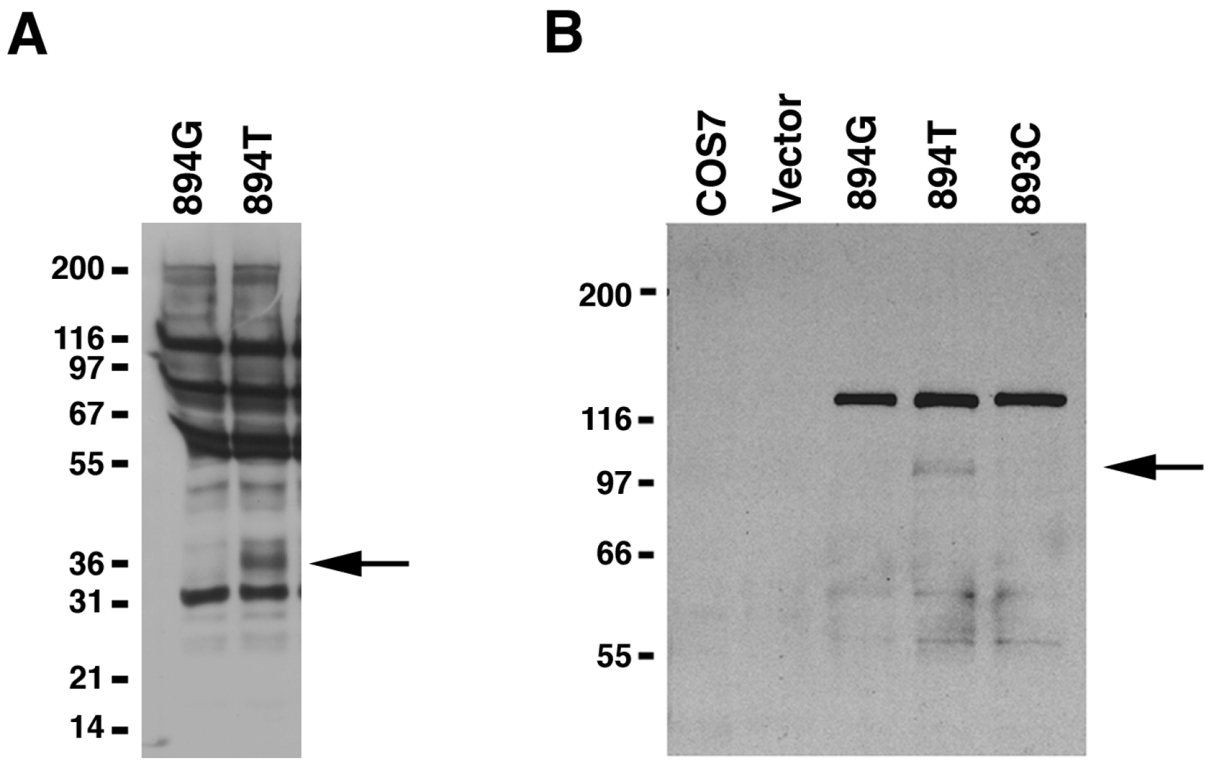

Fig. 1. Identification of ≈100-kDa and ≈35-kDa eNOS fragments in COS7 cells transfected with eNOS cDNA containing 894T. Samples (20 μg of protein per lane) of lysates of COS7 cells transfected with vector, 894G, 894T, or 893C were subjected to SDS/PAGE and immunoblot analysis as outlined in *Materials and Methods*. Blots reacted with anti-human eNOS N-terminal-specific antibodies (*A*) or C-terminal-specific antibody (*B*) revealed 35-kDa and 100-kDa immunoreactive bands only in the lysates from 894T-transfected cells (see arrows). Data are representative of three independent experiments.

μg of total plasmid DNA in 100-mm cell culture plates using Lipofectamine Plus and Lipofectamine according to the manufacturer's protocol (Life Technologies).

eNOS Purification. Purification of eNOS was performed according to the method of Pollock *et al.* (13) with minor modifications. Briefly, whole cell lysates of human heart tissue (quick-thawed from −80°C storage), transfected COS7 cells, or primary human endothelial cells (both harvested on ice), were prepared by homogenization in ≈20 vol of ice-cold Buffer A [50 mm Tris·HCl (pH 7.5)/1 mM EDTA/2 mM β-mercaptoethanol with protease inhibitors (pepstatin A, aprotinin, leupeptin, PMSF, each at 10 μg/ml)] by 15 passes of a Potter–Elvehjem Teflon/glass motorized homogenizer at medium speed. After centrifugation (150,000 × *g*, 1 h, 4°C), the pellet was homogenized as above but in an equal volume of Buffer A with 1 M KCl and 10% (vol/vol) glycerol (Buffer B) and recentrifuged (150,000 × *g*, 30 min, 4°C). The resulting pellet was homogenized in an equal volume of Buffer A with 10% (vol/vol) glycerol and 1% (wt/vol) deoxycholic acid, sodium salt (Buffer C), mixed at 4°C for 20 min and then centrifuged (150,000 × *g*, 30 min, 4°C). The supernatant was incubated (overnight, 4°C) with 20 mg (dry weight) of 2′,5′-ADP-Sepharose 4B (Amersham Pharmacia) equilibrated in Buffer C. ADP-Sepharose beads were collected by centrifugation and washed three times with Buffer C. For human primary endothelial cell preparations, ADP-Sepharose beads were then washed twice in Buffer A, heated at 100°C for 5 min in 40 μl of 1× Laemmli sample buffer, and eluted proteins were subjected to SDS/PAGE and immunoblot analysis. For COS7 cell transfectants and human heart tissue preparations, ADP-Sepharose beads were eluted by mixing at 4°C for 1 h with 20 mM NADPH in 1 ml of Buffer C. Buffer exchange was accomplished with 2 vol of Buffer D [50 mM Tris·HCl (pH 7.5)/150 mM NaCl/10 mM β-mercaptoethanol/1 mM magnesium acetate/1 mM imidazole/2 mM $CaCl_2$/0.05% Triton X-100] by centrifugation (1500 × *g*, 75 min, 4°C) in a Microcon-30 microconcentrator (Amicon). The sample retentate was adjusted to 1.4 ml total volume in a 1.5-ml microfuge tube and mixed (overnight, 4°C) with 50 μl of calmodulin affinity resin slurry (Stratagene) that had been preequilibrated in Buffer D. The beads were washed three times in Buffer D and pelleted, the supernatant was removed and then heated to 100°C for 5 min in 1× Laemmli sample buffer for subsequent SDS/PAGE or immunoblotting.

Immunoblots. Samples of whole cell lysates or partially purified proteins were subjected to SDS/PAGE (8% Tris/glycine gels; NOVEX, San Diego) and transferred to nitrocellulose for immunoblotting. Membranes for analysis with eNOS C-terminal-specific antibody were incubated (3 h, room temperature) in blocking buffer [50 mM Tris (pH 7.5)/150 mM NaCl/3% (wt/vol) BSA/1% (vol/vol) Tween-20], followed by incubation (overnight, 4°C) with mouse anti-human eNOS antibody (Transduction Laboratories, Lexington, KY) diluted 1:4,000 in blocking buffer. Membranes for analysis with eNOS N-terminal-specific antibody were incubated (2 h, room temperature) in blocking buffer [50 mM Tris (pH 7.5)/150 mM NaCl/5% (wt/vol) nonfat dry milk/0.3% (vol/vol) Tween-20], followed by incubation (1 h, room temperature) with rabbit anti-human eNOS antibody (Santa Cruz Biotechnology) diluted 1:400 with blocking buffer. Membranes were then washed with five blocking buffer washes, each for 5 min, incubated (1 h, room temperature) with secondary antibody diluted 1:4,000 in blocking buffer using either anti-murine (Promega; cat. no. W402B) or anti-rabbit (Promega; cat. no. W401B) IgG horseradish peroxidase, exten-

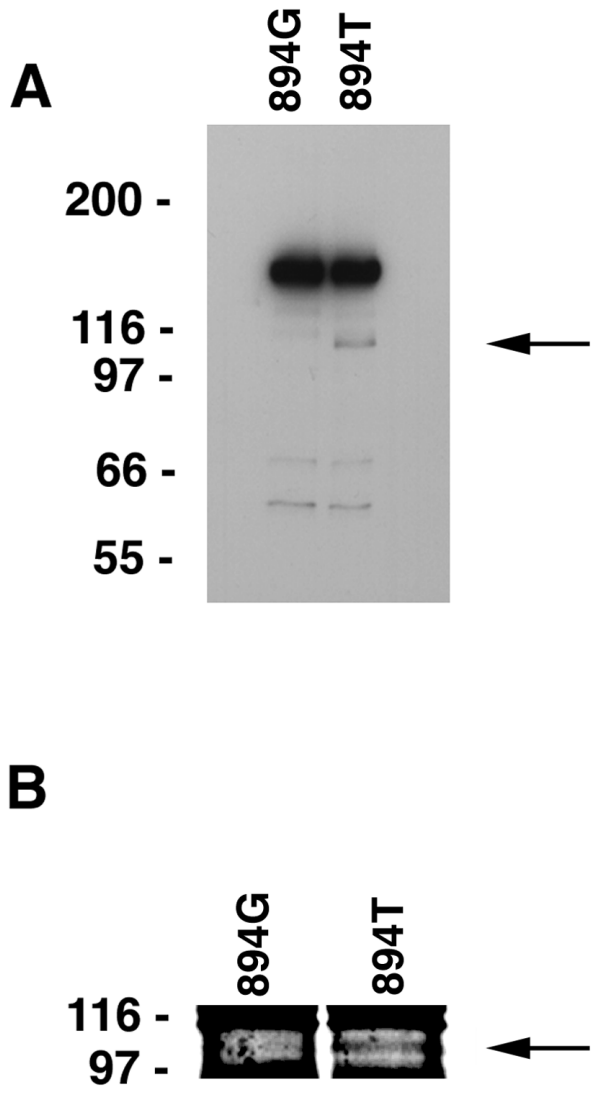

Fig. 2. Immunoblot and protein analysis of affinity-purified eNOS and eNOS fragments from COS7 cells transfected with eNOS cDNA containing 894G or 894T. Partially purified proteins were prepared as detailed in *Materials and Methods* using ADP-Sepharose followed by calmodulin-Sepharose. Immunoblot analysis (*A*) was performed with equal amounts (≈0.5 μg) of partially purified 135-kDa eNOS protein from COS7 cells transfected with eNOS cDNA containing either 894G or 894T. Immunoreactive ≈100-kDa protein was observed only in 894T transfectants. Protein analysis (*B*) was performed by silver staining of 8% SDS-polyacrylamide gels with equal amounts of partially purified 135-kDa eNOS from 130 100-mm culture plates of COS7 cells transfected with eNOS cDNAs containing either 894G or 894T. A unique band at ≈100-kDa was observed in partially purified protein from 894 T cells (see arrow).

sively washed, and finally immunoreactive proteins were detected by chemiluminescence (ECL, Amersham Pharmacia; cat. no. RPN2106).

eNOS Enzymatic Assays. eNOS-catalyzed conversion of L-[^{3}H]-arginine to L-[^{3}H]citrulline was performed with whole cell lysates of COS7 cells transiently transfected with 894G, 894T, or 893 C cDNA in presence of saturating cofactors according to the method of Bredt and Snyder (14).

Genotyping. Genotyping of 894G/T polymorphic sites from genomic DNA was performed by PCR-RFLP as described by Novoradovsky *et al.* (7).

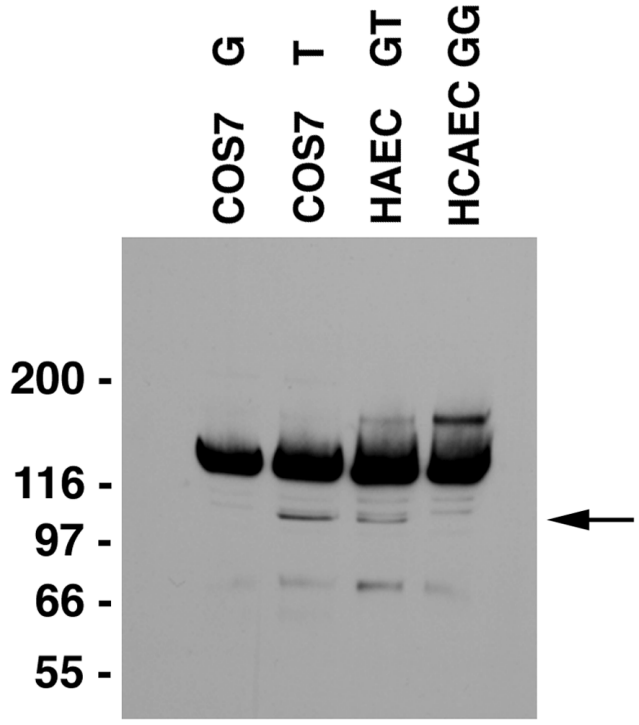

Fig. 3. Identification of the ≈100-kDa eNOS fragment in human endothelial cells of the 894G/T, but not 894G/G, genotype. Lysates of COS7 cells transfected with 894G or 894T cDNA, human primary endothelial cells [either human aortic endothelial cells (HAEC) or human coronary artery endothelial cells (HCAEC)] were collected on ice. eNOS, partially purified from each by 2′,5′-ADP-Sepharose chromatography, as detailed in *Materials and Methods,* was subjected to SDS/PAGE and immunoblotting with C-terminal-specific anti-eNOS antibodies. The eNOS band at 135 kDa served as a standard and was present at ≈0.6 μg per lane (as determined by silver staining with BSA as the standard; data not shown). An ≈100-kDa immunoreactive protein was seen only in extracts of cells of the 894T or 894G/T genotype (see arrow). Data were replicated in a second independent experiment.

Results and Discussion

Comparision of COS7 Transfectant Whole Cell Lysates for eNOS Immunoreactivity. To determine whether eNOS with glutamate or aspartate at position 298 was subject to differential regulation, COS7 cells were transiently transfected with three pBK-CMV eukaryotic expression vector constructs, eNOS 894G, 894T, and 893C, resulting in eNOS 298 glutamate, aspartate, and alanine, respectively. eNOS enzymatic activities, as measured by conversion of [^{3}H]arginine to [^{3}H]citrulline, in whole cell homogenates, showed no significant differences among all three constructs (data not shown). The cell proteins were then separated by SDS/PAGE, followed by transfer to nitrocellulose and immunoblotting with amino-terminal and carboxyl-terminal antibodies. As expected, all transfectants contained the 135-kDa eNOS band. The eNOS with aspartate at position 298 gave rise to additional bands at 35 kDa and 100 kDa that reacted with amino- and carboxyl-terminal antibodies, respectively (Fig. 1; also see Fig. 2*A*). The "degraded" 100-kDa eNOS is only a small fraction of the total. This finding, however, does not permit the conclusion that only a small fraction of eNOS is degraded, since the 100-kDa fragment may itself be subject to proteolysis. To determine whether the 100-kDa band was generated *in vivo* and *in vitro*, trichloroacetic acid was added directly to COS7 cells to precipitate proteins immediately. Subsequent Western blotting analysis showed that the 100-kDa band was only present in cells containing the eNOS 298 Asp protein (data not shown).

Because the 100-kDa fragment is predicted to have intact

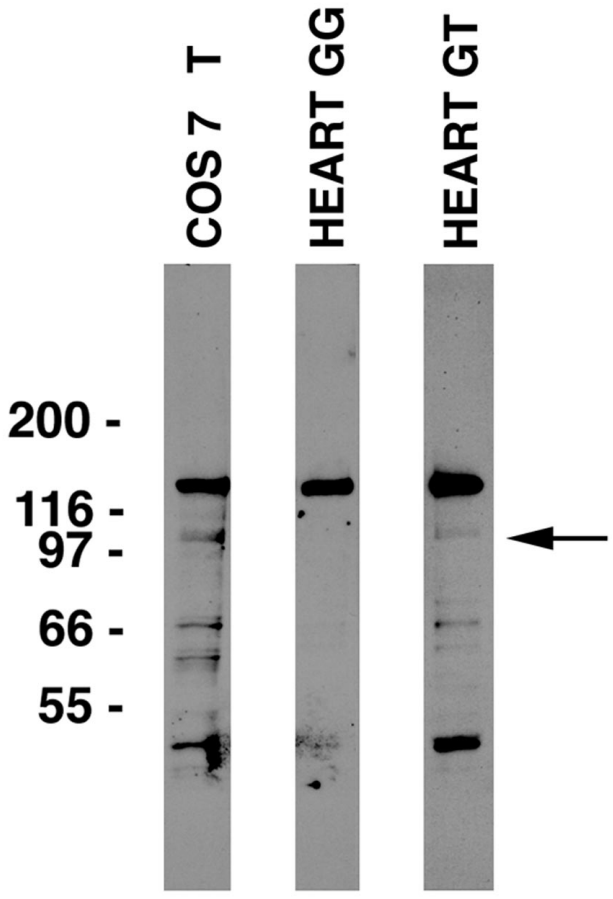

Fig. 4. Identification of the ≈100-kDa eNOS fragment in human heart tissue of the 894G/T genotype. Samples (20 μg of protein) of lysate of COS7 cells transfected with 894T cDNA, or eNOS partially purified from 400 mg of human heart tissue by chromatography with 2′,5′-ADP-Sepharose followed by calmodulin-Sepharose, was subjected to SDS/PAGE and immunoblot analysis as outlined in *Materials and Methods*. Immunoblotting with C-terminal-specific anti-human eNOS antibodies revealed a unique band at ≈100-kDa observed only in 894T COS7 lysates and human heart 894G/T, but not 894G/G, genotype. Data from one experiment is shown. In an independent experiment (data not shown), intact eNOS 298 Asp and its 100-kDa fragment were immunoprecipitated from 100 mg of human heart tissue. Thus, the presence of the 100-kDa band was confirmed by two independent procedures. Anonymous human tissues were obtained from the National Disease Research Interchange (NDRI), Philadelphia, PA, and the University of Miami, Brain and Tissue Bank for Developmental Disorders, Miami, FL.

calmodulin-binding (amino acids 493–509) and reductase domains, further analysis of the eNOS 298 Asp was performed using a two-step batch-affinity purification with 2′,5′-ADP-Sepharose, which interacts with the NADPH site, followed by calmodulin-Sepharose. By this procedure, the 100-kDa fragment was partially purified from eNOS 298 Asp, but not eNOS 298 Glu, and could be visualized by silver staining (Fig. 2*B*). These data are consistent with the hypothesis that the 100-kDa fragment contains the reductase and calmodulin-binding domains, as predicted from its immunoreactivity, and is preferentially generated from eNOS 298 Asp.

Immunoreactivity of eNOS in Primary Cells and Heart Tissue. To determine whether the preferential degradation of eNOS 298 Asp was a result of its overexpression in COS7 cells, cells in primary culture and human hearts of known G/T genotype at position 894 were similarly analyzed by SDS/PAGE and immunoblotting with the carboxyl-terminal antibody. A 100-kDa band was observed in cell lysates from three primary human endothelial cell lines, one with the 894 T/T genotype (microvascular endothelial), and two with the 894 G/T genotype (aortic endothelial and pulmonary artery endothelial), but not in one with the 894 G/G genotype (coronary artery endothelial) (data not shown). To establish further the identity of the 100-kDa band in the endothelial lines, the eNOS band was purified based on the presence of its NADPH binding site. A 100-kDa band was observed with eNOS purified by 2′,5′-ADP-Sepharose chromatography from cell lysates of human aortic endothelial cells with an 894 G/T genotype, but not from human coronary artery cells with an 894 G/G genotype (Fig. 3). An additional 75-kDa band was also observed, but it was not unique to the eNOS 894 G/T genotype. Human heart eNOS was analyzed by immunoblotting following sequential 2′,5′-ADP-Sepharose and calmodulin-Sepharose chromatography. A 100-kDa immunoreactive band was observed in samples from human hearts with the G/T, but not the G/G, genotype (Fig. 4). These data are consistent with the hypothesis that, in normal tissues and cells, eNOS containing aspartate, but not glutamate, at position 298 is processed to a 100-kDa fragment.

This report demonstrates that eNOS isoforms are processed differently depending on the presence of aspartate or glutamate at position 298. Based on the appearance of 35-kDa amino-terminal and 100-kDa carboxyl-terminal fragments, it appears that eNOS 298 Asp is cleaved in the vicinity of amino acid 298. It was somewhat surprising that differential cleavage should result from a glutamate to aspartate replacement. Of note, however, Chou-Fasman secondary structure predictions show significant potential structural changes, even with this seemingly small conservative replacement. Thus, the 894 G/T polymorphism is not silent, and may possibly be perceived by an endogenous protease as a target for cleavage.

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