Acidic Hydrolysis as a Mechanism for the Cleavage of the Glu$^{298} \rightarrow$ Asp Variant of Human Endothelial Nitric-oxide Synthase

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The 894G→T polymorphism within exon 7 of the human endothelial nitric-oxide synthase (eNOS) gene codes for glutamate or aspartate, respectively, at residue 298 and has been associated with several diseases of cardiovascular origin. A recent report indicates that Asp$^{298}$-eNOS (E298D) is cleaved intracellularly to 100- and 35-kDa fragments, suggesting a mechanism for reduced endothelial function. Here we have documented the precise cleavage site of the E298D variant as a unique aspartyl-prolyl (Asp$^{298}$–Pro$^{299}$) bond not seen in wild-type eNOS (Glu$^{298}$). We show that E298D-eNOS, as isolated from cells and in vitro, is susceptible to acidic hydrolysis, and the 100-kDa fragment can be generated ex vivo by increasing temperature at low pH. Importantly, cleavage of E298D was eliminated using a sample buffer system designed to limit acidic hydrolysis of Asp–Pro bonds. These results argue against intracellular processing of E298D-eNOS and suggest that previously described fragmentation of E298D could be a product of sample preparation. We also found that eNOS turnover, NO production, and the susceptibility to cellular stress were not different in cells expressing WT versus E298D-eNOS. Finally, enzyme activities were identical for the respective recombinant enzymes. Thus, intracellular cleavage mechanisms are unlikely to account for associations between the exon 7 polymorphism and cardiovascular diseases.

Endothelium-derived nitric oxide (NO) plays a prominent role in regulating systemic blood pressure and maintaining vascular homeostasis. NO has also been recognized as an important mediator of structural changes in the vasculature, including flow and injury evoked vascular remodeling and angiogenesis (1–3). Within endothelial cells that line the lumen of all blood vessels, endothelial nitric-oxide synthase (eNOS) cata-

lyzes calcium-calmodulin-dependent NO synthesis through the conversion of l-arginine to l-citrulline and NO (4). Dysfunction of the endothelium, often associated with a reduction in the activity or expression of eNOS or NO bioreactivity, is a hallmark of cardiovascular diseases such as hypertension, diabetes, heart failure and atherosclerosis (5). Given the importance of eNOS to cardiovascular function, the investigation into whether mutations or polymorphisms within the eNOS gene correlate with increased risk of cardiovascular disease has become an active area of research.

The human eNOS gene has 26 exon regions and covers 21 kilobase pairs on the long arm of chromosome 7 (6). To date, there are no positive studies demonstrating that mutations in the eNOS gene are causally linked to a disease process using traditional linkage analysis (7). However, in patients with coronary spasm (8) or renal disease (9), polymorphisms within the eNOS promoter have been postulated to impact levels of mRNA and protein, whereas in patients with coronary artery disease (10) or hypertension (11) polymorphisms within exon regions of eNOS may affect enzyme function. Conversely, many other studies do not show associations of the above polymorphisms with the disease (7).

The exon 7 polymorphism (894G→T) that specifies either a glutamate (E) or aspartate (D) residue at position 298 in the human eNOS protein has been analyzed in several patient populations with coronary artery disease, hypertension, and cerebral vascular disease (12–14). This polymorphism is of particular interest because this conservative amino acid substitution within the oxygenase domain of eNOS may influence NO synthesis. Within each disease category, there is evidence both for and against this polymorphism influencing eNOS and/or endothelial function. For example, two groups have differing results concerning the association of the exon 7 polymorphism and essential hypertension in the Japanese population (11, 15).

Based upon the crystal structure of the oxygenase domain of eNOS, the substitution of a glutamate residue for an aspartate at position 298 within the protein is unlikely to alter protein conformation to an appreciable extent (16). However, a recent report demonstrated that eNOS, as isolated from patients with an 894T allele (coding for aspartate at residue 298), was cleaved intracellularly by an unknown protease, thus providing a possible mechanism to explain an impairment in eNOS function (17). Because most aspects of the proposed intracellular cleavage remain to be determined, we sought to: 1) elucidate the mechanism whereby the Glu$^{298} \rightarrow$ Asp variant of eNOS (E298D) is cleaved and 2) further characterize enzymatic differences between wild-type eNOS and cleaved E298D eNOS in cells and in vitro.

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¶ The abbreviations used are: NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; WT, wild type; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; LDS, lithium dodecyl sulfate.
**EXPERIMENTAL PROCEDURES**

Generation of Wild-type (WT) Human eNOS and E298D eNOS cDNA—Total RNA from single-donor human umbilical vein endothelial cells was isolated with TriZol reagent and subjected to reverse transcriptase-PCR using an oligo(dT)$_17$ primer. The reverse transcriptase-PCR product corresponding to human eNOS was PCR-amplified using the forward primer 5′-cccccgaagttgacagactatccgagacattga- gagggccc-3′ and the reverse primer 5′-ggggattctagctggtggagagctcagagtcggctg-3′. The amplified product was ligated into (HindIII and XbaI) pcDNA3.1 (Invitrogen) mammalian expression vector. The wild-type human eNOS sequence was verified by DNA sequencing. The generation of E298D eNOS cDNA was performed by site-directed mutagenesis (QuikChange, Stratagene) according to the manufacturer’s protocol. Complementary primers were used for mutagenesis (± strand oligonucleotide) and the reaction products were treated with varying conditions to evoke cell stress. To examine the effects of hypoxia, the transfected cells were placed into an incubator and equilibrated with 1% oxygen for 48 h, as described previously (19). To trigger cellular apoptosis, COS cells were treated with staurosporine (1 μM) for 6 h (20). To initiate oxidative stress, transfected cells were incubated with H$_2$O$_2$ (5 mM) for 30 min. At the end of each incubation period, samples were prepared for electrophoresis using the NUPAGE buffer system (described below).

Western Blot of Total Cell Lysates—For all experiments on lysates (except when intentionally altering pH), 48 h post-transfection COS-7 cells were lysed in modified radioimmune precipitation buffer (50 mM Tris-Cl, pH 7.4, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 0.1% deoxycholic acid, 1 mM Pefabloc, 1 mM ml aprotonin, 1 μg/ml leupeptin, and 2 μg/ml leupeptin). Sample preparation for SDS-PAGE was performed using either the traditional Laemmli method (21) or the commercial NUPAGE system (Novex/Invitrogen) that was designed to limit in vitro degradation of protein samples. A Laemmli-style loading buffer (6× concentrated SDS-Tris-based buffer/dye at pH 6.8 (22 °C) with β-mercaptoethanol) was added to the samples, and the samples were boiled at 100 °C for 5 min. Alternatively, we followed the protocols of the NUPAGE system, where a lithium dodecyl sulfate (LDS) sample buffer (0.2 mM dithiothreitol, pH 8.5) was mixed with fresh dithiothreitol and added to samples. The LDS samples were then heated to 70 °C for 10 min. All cell lysates were separated by electrophoresis on 7.5% SDS-PAGE and transferred to nitrocellulose membranes. WT and the E298D eNOS were detected using monoclonal antibody (Transduction Laboratories, N30020) directed at the carboxyl terminus of human eNOS, an epitope conserved in WT and E298D eNOS.

Isolation and Sequencing of 100-kDa Fragment—Ten plates of COS-7 cells (100-m plates) were transfected with WT or E298D eNOS cDNA. Cells were lysed with modified radioimmune precipitation buffer, and eNOS was isolated using 2-5% ADP-Sepharose (Amersham Pharmacia Biotech) as described previously (22). Bound proteins were eluted by adding one volume of Laemmli-style sample buffer to the Sepharose bed and boiling for 5 min in a 100 °C block heater. Eluted proteins were subjected to a 7.5% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. Proteins were visualized by staining the membrane with a Coomassie Brilliant Blue stain (Sigma), and the unique 100-kDa band generated from the E298D mutant was sequenced by Edman degradation (W. M. Keck Biotechnology Resource Center at Yale University School of Medicine).

**Acidic Hydrolysis of Glu$^{298}$ → Asp eNOS**

![Image](http://www.jbc.org/)

**FIG. 1.** Glutamate to aspartate substitution at residue 298 creates a labile Asp-Pro bond in human eNOS. A, COS-7 cells were transiently transfected with vectors coding for the WT or Glu$^{298}$ → Asp variant (E298D) of eNOS. Lysates were Western blotted using a carboxyterminal eNOS monoclonal antibody. An arrow indicates a unique 100-kDa band in the E298D lysate. In B, Edman sequencing of the purified 100-kDa fragment confirms cleavage at the Asp$^{26675}$-Pro$^{26676}$ bond. harvested using the above pH lysis solutions (buffers plus 1% Nonidet P-40, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% SDS, 1 mM Pefabloc, 1 μg/ml aprotonin, 1 μg/ml leupeptin, and 2 μg/ml leupeptin). Lysates were rotated for 10 h at 37 °C. Lysates were adjusted to pH 7.4 using sodium hydroxide and prepared for SDS-PAGE using NUPAGE sample preparation.

**NO Release Assay**—Chemiluminescent measurement of nitrite in media was performed as described previously (25). Basal levels of NO release were acquired by sampling media from cells just prior to harvesting (48 h post-transfection).

**Biochemical Assays on Purified eNOS**—Hemoglobin capture assays and cytochrome c reduction assays were all performed as described previously (22).

**Pulse-Chase Experiments**—Twelve hours post-transfection, confluent COS-7 cells from 100 mm cell culture plates were trypsinized and divided equally into 60-mm cell plates. Cells were grown to 70% confluency in complete medium. Medium was changed to methionine/cysteine-free Dulbecco’s modified Eagle’s medium before harvesting at various time points. Lysates were immunoprecipitated using anti-eNOS antibody (Transduction Laboratories) and protein A-Sepharose. Eluted proteins were separated by SDS-PAGE. Gels were dried for 3 h and then exposed to film at −80 °C for 12 h. Band intensity at 135 kDa was assayed by densitometry (26).

**RESULTS AND DISCUSSION**

The allele frequency of the 894G polymorphism is greater than the 894T variant in populations studied to date (e.g. 67.5% in Australians and 92.2% in Japanese (27)). For this reason we have termed eNOS with glutamate at 298 as wild type and eNOS with aspartate at 298 as E298D below.

Preparation of Cell Lysates at Acidic pH—Phosphate-citrate buffer solutions were mixed to pH 7.6, 5.0, and 3.0. Transfected cells were...
present in cell lysates containing the E298D eNOS, as previously reported (17). As the antibody used was raised against an epitope derived from the extreme carboxyl terminus of human eNOS, it was apparent that the cleavage took place within the amino-terminal domain of the protein. To determine the precise location of the cleaved bond, we isolated the 100-kDa fragment and sequenced the amino terminus by Edman degradation. The sequenced portion of the fragment corresponds precisely to residues Pro299 through Val310 of eNOS, showing that E298D eNOS was cleaved on the carboxyl side of the aspartate at position 298, as depicted in Fig. 1B.

Because the cleavage site occurred at the Asp–Pro bond, we searched for proteases that may cleave at this site and found none. However, it has been recognized for many years that Asp–Pro bonds are particularly susceptible to acid hydrolysis (28). In fact, one criticism of using the method of Laemmli when preparing samples for SDS-PAGE is that proteins are boiled in an acidic solution, a combination of conditions that can cause significant protein degradation (29). Although the Tris-based loading buffers commonly used in SDS-PAGE are near neutral pH (6.8), the solutions become significantly more acidic when heated. (For example, a 50 mM Tris buffer with pH 6.8 at 22 °C will drop to pH 5.3 at 100 °C.) Therefore, before investigating an in vivo mechanism of eNOS fragmentation, we investigated whether the cleavage phenomenon could be an artifact of our sample preparation method. As shown in Fig. 2A, when we used Laemmli-style buffers and increased the boiling time during sample preparation, we saw increased generation of the 100-kDa fragment of interest in E298D samples (arrow; compare 100-kDa fragment in lanes 3–8). All other degradation bands were found in both WT and E298D eNOS. Importantly, when we used a commercially available sample buffer containing LDS formulated to maintain a more basic pH throughout sample preparation, we observed absolutely no fragmentation of E298D (compare lanes1 and 2 with lanes 3–8). This suggests that the unique 100-kDa fragment generated in E298D eNOS may arise from acid hydrolysis of the Asp-Pro bond.

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Having established acid hydrolysis as a likely mechanism of
cleavage of E298D eNOS, we investigated whether acidic conditions alone, without boiling, could account for fragmentation of the enzyme. To separate the heating of samples from the accompanying acidification, we switched to cell lysis solutions buffered by citrate-phosphate mixtures. The citrate-phosphate lysis solutions were much more resistant to acidification upon heating than Tris-based buffers (e.g., a pH 7.0 solution of citrate-phosphate buffer at 22 °C remained at pH 7.0 when temperature was raised to 100 °C, whereas Tris buffers commonly dropped 1–2 pH units upon heating to 100 °C). Experiments in total cell lysates were performed to test whether fragmentation of E298D could occur upon chronic exposure to acidic conditions. Cells were transfected with appropriate cDNAs and lysates processed and further incubated in various pH solutions while rotating, at 37 °C for 10 h. As seen in Fig. 3A, fragmentation of both E298D and WT eNOS occurs markedly at pH 3.0. The fragmentation pattern of E298D in pH 3.0 includes a unique 100-kDa band (to the right of the asterisk) just below a larger, more prominent band that is also seen in the WT eNOS, pH 3.0 lane (lanes 3 and 6). At pH 5.0, the larger, prominent band seen at pH 3.0 is not present. With a darker exposure, a unique band present in the E298D, the pH 3.0 lane, becomes apparent at pH 5.0 (to the left of the asterisk). The unique 100-kDa band in E298D is consistent in size with the fragment of interest seen in Figs. 1 and 2. These experiments provide evidence that only upon chronic exposure to nonphysiologic pH ranges (10 h at less than pH 5.0) will E298D be partially cleaved. Heating the enzyme to levels beyond 37 °C without substantial acidification does not cause significant cleavage (i.e., see Fig. 2A, lanes 1 and 2, where the samples are heated to 70 °C), thus suggesting that both a change in pH and temperature (acidic hydrolysis) were responsible for the cleavage. However, to test whether various manipulations to promote cellular stress could evoke the cleavage of eNOS, transfected COS cells were exposed to a variety of stimuli and samples were processed in LDS buffer. As seen in Fig. 3B exposure of cells to hypoxia (48 h post-transfection in 1% O2 incubators), hydrogen peroxide (5 mM, 30 min), and staurosporine (1 μM, 6 h). Blots are representative of at least three experiments.

Collectively, the above data suggest that the cleavage of the E298D eNOS to the 100-kDa species may be artifically generated in vitro. However, we cannot rule out the possibility that in the uncleaved state, E298D eNOS may have properties and activities different from wild-type eNOS that may account for higher cardiac disease risk in carriers of the 894T allele. Thus, COS cells were transfected with WT and E298D eNOS cDNA and the amount of NO produced over a 16-h period was quan-
tified using NO-specific chemiluminescence. As shown in Fig.
4, A and B, the production of NO2 and the levels of eNOS
protein expression in these experiments were not different
from cells expressing the two forms eNOS. Next, we cloned both
WT and E298D eNOS cDNAs into a bacterial expression vec-
tors and produced and purified the recombinant proteins. As
seen in Fig. 5A, both proteins were more than 85% pure based
on Coomassie staining. Direct analysis of the catalytic activity
of recombinant WT or E298D eNOS using hemoglobin capture
of NO as an assay to assess the rate of NO production or
cytochrome c reduction as an assay to assess the ability of the
eNOS reductase domain to transfer electrons to an artificial
acceptor showed that these proteins were indistinguishable
enzymatically (see Fig. 5B). To confirm that cleavage of E298D
is a product of sample preparation rather than proteolytically
cleaved intracellularly, we prepared purified recombinant en-
yzymes for Western blotting using NUPAGE (LDS buffers)
or Laemmli-style methods to prepare samples. Fig. 5C demon-
strates that recombinant E298D is cleaved in a fashion similar
to that from eukaryotic cell lysates prepared similarly.

Here we show that the extensively documented E298D poly-
morphism in eNOS does not appear to influence the stability,
half-life, or biologic activity of the enzyme isolated from cells or
the enzymatic activity of the recombinant protein. Most impor-
tantly, isolation of samples in standard Laemmli buffer for
SDS-PAGE will generate a 100-kDa fragment cleaved precisely
at the Asp–Pro bond, suggesting that the presence of the 100-
kDa fragment in human tissue from carriers of the E298D
polymorphism is likely to arise because of sample preparation.
However, we cannot rule out the possibility that this fragment
can be generated in an in vivo context by an unknown proteo-
lytic mechanism. In preliminary studies, we generated a 100-
kDa protein corresponding to the fragment derived from the
cleavage of E298D (Figs. 1 and 2) and expressed it as a re-
combint protein in E. coli. Functional analysis of this protein
missing the heme domain showed that it was an active reduc-
tase (based on cytochrome c reduction). If this cleavage can
occur in a cellular context, it is possible for this protein to
reduce oxygen to superoxide, which can contribute to endothe-
lial dysfunction. Alternatively, because eNOS is subjected to
various levels of regulation including protein-protein interac-
tions, fatty acylation, and phosphorylation, these control steps
may be influenced by the polymorphism. In conclusion, a caus-
itive linkage between the common polymorphism E298D in
eNOS and the incidence of cardiovascular disease cannot be
explained based on the cleavage or impaired function of the
enzyme.

**Fig. 4.** Both WT and E298D eNOS produce NO to the same
extent. A, COS-7 cells were transfected with eNOS cDNAs (WT or
E298D), and 48 h post-transfection, the release of nitric oxide, quanti-
fied as NO2, over 48 h was measured via NO-specific chemilumines-
cence. B, representative Western blot of eNOS levels from NO release
experiments documenting equal levels of expression of the two proteins.
Data are presented as the mean ± S.E., with n = 21–23 individual
transfectants.

**Fig. 5.** Enzyme activity and cleav-
age of recombinantly expressed WT
and E298D eNOS proteins. A, repre-
sentative Coomassie Blue-stained SDS
gel of recombinant WT or E298D eNOS,
purified from E. coli. B, comparison of
catalytic activities of WT and E298D
eNOS as determined by hemoglobin cap-
ture and cytochrome c reduction, respec-
tively. In C, recombinant WT or E298D
was prepared for Western analysis using
either LDS or Laemmli sample buffer.
The results shown in B are the mean ±
S.E. of triplicate observations.

|                          | WT          | E298D       |
|--------------------------|-------------|-------------|
| Hemoglobin capture min^{-1}| 19.72 +/- 2.4 | 27.2 +/- 6.0 | p>0.05 NS (n=3) |
| Cytochrome C reduction min^{-1} | 47.20 +/- 28.9 | 54.89 +/- 30.6 | p>0.05 NS (n=3) |
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