Enhanced Electron Flux and Reduced Calmodulin Dissociation May Explain “Calcium-independent” eNOS Activation by Phosphorylation*

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Bovine endothelial nitric oxide synthase (eNOS) is phosphorylated directly by the protein kinase Akt at serine 1179. Mutation of this residue to the negatively charged aspartate (S1179D eNOS) increases nitric oxide (NO) production constitutively, in the absence of agonist challenge. Here, we examine the potential mechanism of how aspartate at 1179 increases eNOS activity using purified proteins. Examination of NO production and cytochrome c reduction resulted in no substantial changes in the $K_m$/EC$_{50}$ for l-arginine, calmodulin, and calcium, whereas there was a 2-fold increase in the rate of NO production for S1179D and a 2–4-fold increase in reductase activity (based on cytochrome c reduction). The observed increase in activity for both assays of NOS function indicates that a faster rate of electron flux through the reductase domain is likely the rate-limiting step in NO formation from eNOS. In addition, S1179D eNOS did show an increased resistance to inactivation by EGTA compared with wild type eNOS. These results suggest that a negative charge imposed at serine 1179, either by phosphorylation or by replacement with aspartate, increases eNOS catalytic activity by increasing electron flux at the reductase domain and by reducing calmodulin dissociation from activated eNOS when calcium levels are low.

Nitric oxide synthases (NOS) are composed of modular domains, with the amino-terminal oxygenase domain containing binding sites for heme, BH$_4$, arginine, and zinc and the carboxyl-terminal reductase domain containing sites for FMN, FAD, and NADPH. These two domains are joined by a calmodulin (CaM)-binding domain, and the reductase domain contains an autoinhibitory loop in the carboxyl-terminal domain, which regulates electron flow in the protein. Calcium-dependent CaM activation is essential for NO production by purified endothelial and neuronal NOS isoforms. In all NOS isoforms, NO is generated by a two step process utilizing the electrons donated by NADPH to reduce the heme iron, permitting activation of molecular oxygen followed by oxidation of a guanidino N group of arginine to form NO, l-citrulline, and H$_2$O (1).

For activation of eNOS, there is a consensus that calcium-activated CaM is necessary for eNOS activity in vitro (2, 3). In a cellular context, there is growing evidence that for some agonists, such as acetylcholine (4) or vascular endothelial growth factor (5), a rise in intracellular calcium is necessary for NO production, whereas with other forms of stimuli, such as fluid shear stress (6), estrogen (7), and insulin (8), a rise in calcium is not required for NO production. Although the molecular basis for these observations is not known, it is likely that CaM is bound under both circumstances because CaM is an essential regulator of intra- and interdomain electron flux (9), a required process for NO generation.

Recently, it was shown that wortmannin and LY294002, two distinct inhibitors of a phosphatidylinositol-3-kinase, attenuated vascular endothelial growth factor- (5, 10), insulin-(11) and shear stress (12, 13)-stimulated NO production, suggesting that pathways downstream of phosphatidylinositol-3-kinase may regulate eNOS. Furthermore, the serine/threonine kinase Akt was identified as the downstream kinase that specifically phosphorylates eNOS (at serines 1179 and 1177 for bovine and human eNOS, respectively (10, 12–14)). Phosphorylation on this residue is associated with an increase in NO production and activation of the enzyme at lower calcium/CaM concentrations than nonphosphorylated eNOS (10, 14). Mutation of serine 1179 to aspartate (S1179D eNOS), to mimic the negative charge afforded by phosphate, increased NO production in the absence of agonist challenge, whereas mutation to alanine (i.e. S1179A) blocked Akt-dependent eNOS phosphorylation and NO release (12, 14). Thus, the purpose of this study was to investigate the potential mechanisms of how aspartate at 1179 increases NOS activity and basal NO production using purified proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trizma base, EDTA, EGTA, l-arginine, calmodulin, NADPH, HEPES, Dowex AG50WX8, and cytochrome c were obtained from Sigma. Tetrahydrobiopterin was acquired from J. B. Schircks (Jona, Switzerland). The Bradford protein assay kit was purchased from Bio-Rad. 2′,5′-ADP Sepharose 4B was from Amersham Pharmacia Biotech. L-[2,3,4,5-3H]Arginine (55 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). The proteinase K was purchased from Roche Molecular Biochemicals.

**eNOS Constructs and Protein Purification**—Wild type bovine eNOS in the plasmid pcW was expressed as described previously with groELS in E. coli BL21 cells (15). The S1179D mutant eNOS for expression in E. coli was generated as follows. eNOS S1179D in pCW 3 (14) was digested with XhoI/XbaI, subcloned into the identical sites of eNOS in pcW, and co-expressed with groELS. Isolation of recombinant eNOS was preformed as reported previously (16, 17), with the following modifications. eNOS was eluted from 2′,5′-ADP Sepharose with either 10
mm NADPH or 10 mm 2-AMP. The amount of eNOS was quantitated using the peak absorbance at 409–412 nm, with an extinction coefficient for heme content of 0.1 μM−1 cm−1. The purity of eNOS was determined by 7.5% SDS-PAGE followed by Coomassie staining. Low temperature SDS-PAGE was performed identically, except that samples were not boiled and the electrophoresis was carried out at 4 °C in a slurry of ice/water (18). In experiments in which NOS cofactors (L-arginine, calmodulin, and NADPH) were titrated, they were omitted from the purification and storage of the enzymes and were incubated as described below.

Assay for NOS Activity—NO production was measured by the hemoglobin capture assay as described (19). Briefly, the reaction mixture contained eNOS (0.5–2.5 μg), oxyhemoglobin (8 μM), L-arginine (100 μM), BH₄ (5 μM), CaCl₂ (120 μM), calmodulin (120–200 nm), NADPH (100 μM) in HEPES buffer (50 mM) pH 7.4. In the determination of calcium EC₅₀ value for eNOS, the above reaction mixture was modified as follows: MOPS buffer (10 mM, pH 7.6), KCl (100 mM), and CaM (250 mM) were substituted. Under these conditions, free calcium was calculated using the WinMAXC program, version 1.8 (Stanford University), with a Kᵦ of 2.2 × 10⁻⁸ M. The precise free calcium concentration was achieved by mixing an appropriate proportion of 10 mM K₄EGTA and 10 mM CaEGTA stock solutions (Molecular Probes). NOS activity was monitored for linearity over 2 min at 401 nm, and NO production was calculated based on the change of absorbance using the extinction coefficient of 60 mM−1 cm−1. All reactions were carried out at 23 °C, and each data point represents 3–5 observations. The extinction coefficient of 0.0033 μM⁻¹ cm⁻¹ at 276 nm was used for determination of calmodulin concentration. The production of NO using this method was completely blocked by the addition of nitro-L-arginine (1 mM). When the inactivation of eNOS was determined by the addition of EGTA (200–800 μM) to the reaction mixture, chelator was added 1 min after initiation of the reaction by NADPH. Identical conditions were used when NADPH-cytochrome c reductase activity was examined. These reactions contained CaM (120 mM) and CaCl₂ (200 μM) in a 0.5-mL volume with eNOS (0.5 μg).

The conversion of L-arginine to L-citrulline was assayed as described previously (20). Briefly, eNOS (0.25–2 μg) was incubated for 3–10 min at 22 °C in the following reaction mixture: 3 pmol of L-[³H]arginine (55 Ci/mmol), 10–300 μM arginine, 1 mM NADPH, 120–200 nm calmodulin, 2 mM CaCl₂, and 30 μM BH₄ in a final reaction volume of 50–100 μL. The reaction was terminated by the addition of 0.5 mL of 20 mM HEPES, pH 5.5, containing 2 mM EGTA and EDTA. The reaction mixture was placed over Dowex AG50W×8, and the flow-through was counted on a Packard 1500 liquid scintillation analyzer.

Analytical Methods—NADPH-cytochrome c reductase activity and 2,6-dichlorophenolindophenol (DCIP) reduction were measured as a change in absorbance at 550 nm as described previously (17, 21), using an extinction coefficient of 0.521 μM⁻¹ cm⁻¹ for both cytochrome c and DCIP. Briefly, a reaction mixture (1 mL) contained either cytochrome c (90 μM); DCIP (36 μM); HEPES buffer (50 mM) at pH 7.6, NaCl (250 mM), NADPH (100 μM), calmodulin (120 mM), and CaCl₂ (200 μM); or other substances as indicated. The reaction was incubated for 60 s (at 23 °C) after the addition of eNOS. When inactivation of reductase activity was determined by the addition of EGTA (200–800 μM), chelator was added 1 min after initiation of the reaction and monitored for an additional 1 min. The reaction contained HEPES buffer (50 mM) at pH 7.6, CaM (120 mM), and CaCl₂ (200 μM) and was initiated with NADPH (100 μM). No NADCl was added in experiments that examined EGTA inactivation of eNOS to mimic the conditions used in the hemoglobin capture experiments. The addition of NO inhibitors did not influence the rate of cytochrome c reduction (not shown). Determination of the calcium EC₅₀ for eNOS was performed as described above for the hemoglobin capture assay.

Data Analysis and Statistics—All data are expressed as mean ± S.E. At least triplicate determinations were performed with a minimum of three different batches of enzymes for each data set. Wild type and mutant enzymes were purified simultaneously to control for activity variations between preparations. Statistical significance was determined using Student’s t test, and p < 0.05 was considered statistically significant.

RESULTS

Expression and Purification of eNOS—Both wild type and S1179D eNOS were expressed and purified from E. coli. In a culture of 1.6 liters, approximately 2.5–4.0 mg of eNOS was typically recovered using 2’5’-ADP Sepharose 4B chromatography. As seen in Fig. 1A, both enzymes were >90% pure based on Coomassie staining. These results are typical, as seen from seven independent preparations of both wild type and S1179D eNOS prepared side-by-side. Both enzymes were primarily in their dimeric form, as shown by low temperature SDS-PAGE (Fig. 1B).

NOS S1179D Has Greater NO Synthase and Reductase Activities Than Does Wild Type eNOS—Next we compared the activities of wild type and S1179D eNOS by measuring the rate of NO production. S1179D eNOS exhibited a higher turnover number (under optimal conditions) compared with wild type enzyme (84 ± 6 versus 27 ± 1 min⁻¹, n = 6 separate and paired preparations of enzymes). The Kₐ values for L-arginine were similar for wild type and S1179D eNOS (Fig. 2A, 1.8 versus 2.5 μM, respectively; see Table I).

Because the rate of electron flux from the reductase domain to the oxygenase domain is critical for NOS catalysis, we examined whether this increase in S1179D eNOS activity could be attributed to enhanced reductase activity. When both DCIP and cytochrome c reduction were examined, a significant increase in activity for S1179D compared with wild type eNOS was observed (Fig. 2B), suggesting that a possible mechanism for the increased activity may lie within the reductase domain. Furthermore, this increase was accentuated by the presence of CaM, which increased the overall activity for both enzymes. Basal cytochrome c reduction, in the absence of CaM, was 4-fold higher for S1179D compared with wild type eNOS. The magnitude of CaM-stimulated cytochrome c reduction was higher for S1179D eNOS (749 ± 35 versus 1272 ± 55 min⁻¹ for wild type and S1179D eNOS, respectively, n = 3–5); however, the level of stimulation by CaM was 8-fold for wild type eNOS compared with only 3-fold for S1179D eNOS.

Next, we examined the possibility that S1179D eNOS may produce more superoxide than wild type eNOS, which could reduce cytochrome c. As expected, no superoxide dismutase inhibitable cytochrome c reduction was observed (as an index of superoxide anion generation) in the absence of CaM, as reported earlier for wild type eNOS (86 ± 6 versus 95 ± 8 min⁻¹) and for S1179D eNOS (278 ± 9 versus 288 ± 7 min⁻¹, n = 3–5). However, in the presence of CaM, superoxide dismutase reduced the rate of cytochrome c reduction for both wild type (610 ± 51 versus 866 ± 8 min⁻¹) and S1179D (1179 ± 43 versus 1518 ± 19 min⁻¹) eNOS. The relative decrease in cytochrome c activity in the presence of superoxide dismutase was similar with both enzymes (30% for wild type and 22% for S1179D eNOS), suggesting that the enhanced reductase activity of...
S1179D compared with wild type eNOS (assayed by cytochrome c reduction) was not due to uncoupling of the enzyme.

**NADPH-dependent NO Formation and Reductase Activities Are Not Different for Wild Type and S1179D eNOS**—The NADPH dependence of NO production and cytochrome c reduction were examined because the NADPH binding site lies close in proximity to the Ser-1179 in eNOS. S1179D eNOS had a higher maximum turnover number (k_{cat}) than did wild type enzyme based on NO production, assayed in the presence of CaM (Fig. 3A). The increased k_{cat} was associated with a 4-fold increase in the K_m for NADPH for S1179D eNOS compared with wild type eNOS (36 versus 8 μM, respectively). The k_{cat} for NADPH-dependent cytochrome c reduction in the absence of CaM was greater for eNOS S1179D than wild type eNOS (290 versus 70 min^{-1}, respectively; Fig. 3B). In the presence of CaM, the k_{cat} for cytochrome c reduction was considerably higher for S1179D compared with wild type eNOS (840 versus 460 min^{-1}, respectively). The K_{m} values for NADPH were unchanged in the absence or presence of CaM (0.40 and 0.75 μM for wild type eNOS and 2.0 and 1.9 μM for S1179D eNOS in the absence and presence of CaM, respectively).

**EC_{50} Values for CaM Are Unchanged between Wild Type and S1179D eNOS**—To assess whether the increased activity of S1179D eNOS was attributable to changes in the affinity of the enzyme for CaM, we examined the kinetics of NOS activity and cytochrome c reduction assayed in the presence of all NOS cofactors in excess as a function of CaM concentration. The k_{cat} for CaM activation of NOS activity was 22 min^{-1} for wild type and 43 min^{-1} for S1179D eNOS. Transformation of the data, normalizing for the differences in k_{cat}, revealed a slight shift in the curve to the left for S1179D eNOS but little difference in the EC_{50} values for CaM, consistent with reported data on phosopho-eNOS (10). The EC_{50} values were 8 nM for wild type and 7 nM S1179D eNOS (Fig. 4A). We then measured NADPH-mediated cytochrome c reduction. The k_{cat} for CaM activation of cytochrome c reduction was about 2-fold higher for S1179D eNOS compared with wild type enzyme (1140 and 620 min^{-1} for S1179D and wild type eNOS, respectively). Transformation of the data normalized for the differences in k_{cat} revealed small differences in the EC_{50} values for CaM between wild type and S1179D eNOS (21 versus 13 nM; Fig. 4B).

Comparison of Calcium Activation and Inactivation of eNOS—In previous experiments, it was demonstrated that the “apparent calcium sensitivity” of eNOS was enhanced in cells expressing either a majority of phosopho-eNOS or S1179D eNOS, suggesting that phosphorylation changed the affinity of calcium/CaM activation (12, 14). As seen in Fig. 4C, after normalization for the differences in maximal activity, the calcium dependence was slightly increased for S1179D eNOS (p < 0.05, two-way analysis of variance). The EC_{50} values for calcium with wild type and S1179D eNOS were slightly different also (310 and 250 nM calcium, respectively), as determined by NO production (in the presence of 250 nM CaM). As seen in the inset to Fig. 4C, increasing concentrations of free calcium did indeed enhance S1179D eNOS turnover to a greater extent than that seen with wild type enzyme. Furthermore, the EC_{50} value for calcium assaying cytochrome c reduction were similar to those obtained measuring NO production (Fig. 4D; 290 and 220 nM for wild type and S1179D eNOS, respectively). Again, the V_{max} for calcium activation of S1179D eNOS turnover was greater than wild type (Fig. 4D, inset).

To examine whether the inactivation of S1179D eNOS was different than that of wild type enzyme, we measured the decay in eNOS activity after chelation of calcium with EGTA. In these experiments, all NOS cofactors were added in the presence of calcium (200 μM), and NO production was monitored for 1 min, followed by the addition of different concentrations of EGTA and monitoring for an additional 1 min. As seen in Fig. 5A, the addition of EGTA to wild type and S1179D eNOS reduced NO production in a concentration-dependent manner. However, NO production from S1179D eNOS was less sensitive to the addition of EGTA; i.e. wild type eNOS activity declined more rapidly at lower concentrations of EGTA than did S1179D eNOS activity. The greatest difference in activity between the enzymes was seen at 400 μM EGTA. Furthermore, at 600 μM EGTA, no activity was detected for wild type eNOS, whereas residual activity was still detected for S1179D eNOS. Similar results were obtained using cytochrome c reduction (Fig. 5B), with significant differences in activity seen with 400 and 600 μM chelator added to the reaction. However, at the highest concentration of EGTA, residual reductase activity was found for both wild type and S1179D eNOS.

**DISCUSSION**

In a cellular context, the production of NO from eNOS is regulated by calcium-calmodulin (2), subcellular localization (22, 23), protein-protein interactions (24, 25), and, most recently, phosphorylation on serine 1179 (in bovine eNOS or serine 1177 in human eNOS) by the protein kinase Akt (10, 12–14) and AMP kinase (26). Mutation of serine 1179 to asparagine 1177 in human eNOS (10) mediates cytochrome c reductase activity (27, 28), and AMP kinase (26). Mutation of serine 1179 to asparagine 1177 in human eNOS (10) mediates cytochrome c reductase activity (27, 28), and AMP kinase (26). Mutation of serine 1179 to asparagine 1177 in human eNOS (10) mediates cytochrome c reductase activity (27, 28), and AMP kinase (26). Mutation of serine 1179 to asparagine 1177 in human eNOS (10) mediates cytochrome c reductase activity (27, 28), and AMP kinase (26).
results in a form of eNOS that constitutively produces NO when the mutant cDNA is transfected into cells (12, 14). Moreover, increasing the ratio of phospho- to phospho-eNOS by infection of endothelial cells with an adenovirus that expresses constitutively active Akt results in greater NOS activity at lower free calcium levels, whereas a dominant negative kinase reduces calcium-dependent activation of eNOS (12, 14). In order to elucidate the mechanism of how phosphorylation may regulate NO synthesis, we have compared the activities of wild type and S1179D eNOS. Here, we show that, using recombinantly expressed and purified proteins, mutation of serine 1179 to aspartate increases the \( k_{\text{cat}} \) of eNOS without substantially changing the apparent affinities for L-arginine, CaM, and calcium. However, the increase in the \( k_{\text{cat}} \) of S1179D eNOS may be explained, in part, by markedly increased basal and CaM-stimulated reductase activities and reduced inactivation by EGTA. These data suggest that aspartate 1179 can influence electron flux through the reductase domain and CaM dissociation during catalysis.

The low specific activity of eNOS relative to inducible and neuronal NOSs is a subject of intense investigation. Recent studies have suggested that the rate of cytochrome \( c \) reduction and electron flux through eNOS is much slower than that measured in inducible NOS and neuronal NOS, suggesting that regulation of electron flux from NADPH through the flavins in the reductase portion of the protein to the heme domain in the oxygenase domain may control the rate of NO synthesis (27–29). It has been shown for all NOS isoforms that CaM can regulate electron flux from the flavin centers to heme, as well as electron flow within the reductase domain (1). The presence of a CaM autoinhibitory loop in the FMN domain of eNOS and neuronal NOS, missing in inducible NOS (30), suggests that removal of this loop is necessary prior to CaM binding to the canonical CaM binding motif and activation of NOS as seen in other CaM requiring proteins (31). This issue has been recently addressed by Nishida and Ortiz de Montellano (27), who have shown that deletion of the autoinhibitory loop in eNOS increases basal cytochrome \( c \) reductase activities while reducing CaM-stimulated reductase activity, and Daff et al. (32), who demonstrated that deletion of the 42-amino acid loop abolished the calcium/CaM dependence of neuronal NOS. The former authors also demonstrated that “loopless” eNOS has a 3-fold higher specific activity and exhibits a greater sensitivity toward free calcium assayed at fixed CaM levels relative to wild type enzyme. This suggests that the loop, per se, negatively regulates NO synthesis even in the presence of CaM and affects the calcium dependence of eNOS.

In the present study, mutation of serine 1179 to aspartate in eNOS increased the rate of NO synthesis (2-fold) and basal cytochrome \( c \) reductase activity (2–4-fold), consistent with loopless eNOS. Moreover, CaM-stimulated reductase activity was always greater in S1179D eNOS, but the fold enhancement of CaM-stimulated cytochrome \( c \) reduction was less for S1179D eNOS compared with the wild type protein. Simply stated,
mutation of serine to aspartate improves basal electron flux through the reductase domain, and augments CaM-stimulated activation of reductase activity to a level greater than that seen with maximally activated wild type eNOS, analogous to loopless eNOS. In contrast to deletion of the autoinhibitory loop, the increase in specific activity of S1179D eNOS occurred without a substantial change in the apparent affinity of eNOS for calcium, CaM, and l-arginine. In experiments using loopless eNOS, the $K_{app}$ for calcium was 20 nM, compared with 150 nM for wild type enzyme, whereas the differences in calcium activation in the present experiments were not as great for wild type versus S1179D eNOS. These data imply that the increase in basal NO production in cells transfected with S1179D eNOS or after phosphorylation at this site is unlikely, due to complete displacement of the autoinhibitory domain (mimicked by loopless eNOS), but more likely is due to a higher rate of turnover with little change in the apparent calcium sensitivity of the enzyme. However, we cannot rule out the possibility that additional sites of phosphorylation or other posttranslational control mechanisms may regulate autoinhibitory loop displacement, in vivo.

The only observed difference in cofactor utilization was for NADPH. S1179D eNOS had a 4-fold higher $K_m$ for NADPH than did wild type eNOS when NO production was measured and a 2–5-fold higher $K_m$ when cytochrome $c$ reduction was quantified. Although the apparent affinity of NADPH was lower in S1179D eNOS, the rates of NO production and reductase activity were greater than those of wild type eNOS. The reasons for this are not known, but they may relate to the equilibrium of NADPH/NADP$^+$ with the enzyme.

Another interesting finding of this paper is that eNOS inactivation by EGTA was dramatically different in S1179D eNOS compared with wild type enzyme. Because the rate of NO production was linear during our measurements, the ability of EGTA to slow NOS activity was likely due to rapid chelation of free calcium in the buffer followed by changing the equilibrium of calcium-dependent activation of CaM and the subsequent binding of activated CaM to the enzyme. The relative EGTA resistance of S1179D eNOS can be interpreted in at least two ways, i.e. either that aspartate at 1179 prevents the ability of EGTA to access calcium tightly bound to CaM or that the negative charge at 1179 slows the dissociation of CaM from NOS when free calcium levels are diminishing. Regardless of the precise mechanism, aspartate at 1179 results in greater NOS activity at any free calcium level, consistent with greater NO release and NOS activity in cells transfected with S1179D or in endothelial cells expressing constitutively active Akt.

In summary, our findings suggest that phosphorylation of eNOS at serine 1179 (mimicked by aspartate) increases NOS catalytic activity by enhancing electron flux through the reductase domain and by reducing CaM dissociation. This novel mechanism may explain, in part, why S1179D eNOS and phospho-eNOS produce NO at resting levels of calcium. Our data also implies that the "calcium-independent NO production" following shear stress or insulin activation of Akt and eNOS phosphorylation may be more correctly stated as NO production that occurs without a further rise in intracellular calcium because phosphorylation permits greater eNOS activation to occur at any level of calcium without substantially changing the affinity of calcium-activated CaM for eNOS. At this time, we cannot rule out the possibility that removal of the autoinhibitory loop occurs, in conjunction with eNOS phosphorylation, in vivo. However, phosphorylation at Ser-1179 is critical for NO release because mutations that block Akt-induced phosphorylation also block agonist- and shear-induced NO release. As a corollary to this hypothesis, it is possible that Akt-dependent phosphorylation of serine 1179 stimulated by vascular endothelial growth factor, a calcium/CaM-dependent agonist for NO synthesis (5), permits transient, burst-like NO release that is contemporaneously attenuated by a calcium/CaM-dependent inactivation process. This inactivation process would remove CaM tightly bound to eNOS and would most likely be different.

**Fig. 4.** Calmodulin- and calcium-dependent activation of NOS and reductase activities are slightly enhanced for S1179D eNOS. Calmodulin-dependent hemoglobin capture (A) and cytochrome $c$ reduction (B) were performed on both wild type (filled symbols) and S1179D eNOS (open symbols). The rate of NO production detected by hemoglobin capture method is in the presence of all NOS cofactors, whereas cytochrome $c$ reduction was performed in the absence of arginine and BH$_4$. In C and D, identical experiments were performed in the presence of increasing concentrations of free calcium. The insets in C and D depict the calcium-dependent turnover of S1179D and wild type eNOS in both NO production and cytochrome $c$ assays. The maximal turnover rates were as follows for wild type and S1179D eNOS, respectively: A, 22 and 43 min$^{-1}$; B, 620 and 1400 min$^{-1}$; C, 58 and 100 min$^{-1}$; and D, 1930 and 3810 min$^{-1}$. Values are mean ± S.E., $n$ = 3–6 determinations from at least three enzyme preparations.
from the inactivation mechanism triggered by shear stress, which is a persistent stimulus for NO release in the absence of a change in cytoplasmic calcium. This concept is supported by results demonstrating that CaM binding to eNOS increases its phosphorylation by Akt (10) and that dominant negative Akt attenuates both calcium-independent (i.e. shear stress, (12) and calcium-dependent agonist triggered NO production (i.e. vascular endothelial growth factor (10, 14), histamine, and acetylcholine; data not shown). Future studies delineating the structural basis for these observations will shed light on the role of phosphorylation in controlling electron flux through eNOS and hence, NO production from endothelial cells.

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