Phosphorylation of Threonine 497 in Endothelial Nitric-oxide Synthase Coordinates the Coupling of l-Arginine Metabolism to Efficient Nitric Oxide Production*

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Endothelial nitric-oxide synthase (eNOS) regulates by reciprocal dephosphorylation of Thr 497 and phosphorylation of Ser 1179. To examine the interrelationship between these sites, cells were transfected with wild-type (WT), T497A, T497D, S1179D, and T497A/S1179D eNOS and activity, NO release, and eNOS localization were assessed. Although eNOS T497A, S1179D and T497A/S1179D eNOS had greater enzymatic activity than did WT eNOS in lysates, basal production of NO from cells was markedly reduced in cells transfected with T497A and T497A/S1179D eNOS but augmented in cells transfected with S1179D eNOS. Stimulating cells with ATP or ionophore normalized the loss of function seen with T497A and T497A/S1179D eNOS to levels observed with WT and S1179D eNOS, respectively. Despite these functional differences, the localization of eNOS mutants were similar to WT. Because both T497A and T497A/S1179D eNOS exhibited higher enzyme activity but reduced production of NO, we examined whether these mutations were “uncoupling” NO synthesis. T497A and T497A/S1179D eNOS generated 2–3 times more superoxide anion than WT eNOS, and both basal and stimulated interactions of T497A/S1179D eNOS with hsp90 were reduced in co-immunoprecipitation experiments. Thus, the phosphorylation/dephosphorylation of Thr 497 may be an intrinsic switch mechanism that determines whether eNOS generates NO versus superoxide in cells.

Endothelial nitric-oxide synthase (eNOS)1 produces the free radical gas, nitric oxide, which has been implicated in the regulation of cardiovascular homeostasis. Due to its importance to overall cardiovascular health, nature has evolved multiple control mechanisms that tightly regulate the enzymatic function of eNOS. eNOS is a prototype for proteins regulated by multiple control mechanisms that tightly regulate the enzymatic function of eNOS. eNOS is a prototype for proteins regulated by phosphorylation events (1). These complex post-translational mechanisms control the state of eNOS activation/inactivation and the fidelity of electron flux through the reductase domain of the protein to the oxygenase domain where the chemistry of NO synthesis occurs. Dysregulation of the synthesis of eNOS cofactors, protein-protein interactions, and phosphorylation could potentially “uncouple eNOS,” i.e. electron transfer reactions result in the production of superoxide, instead of NO.

eNOS can be phosphorylated on serines 116, 617, 635, and 1179 and phosphorylated on threonine 497 (bovine amino acids) (2–9). The phosphorylation of serines 617, 635, and 1179 all result in the activation of eNOS function, whereas the phosphorylation of serine 116 and threonine 497 may reduce eNOS activity (3, 4, 7, 10). There is a good correlation between threonine 497 (bovine/threonine 495 (human) dephosphorylation and NO production (measured as cGMP) but not Ser 1179 phosphorylation when bradykinin is used as an agonist for NO production, suggesting that Thr 497 dephosphorylation is sufficient for eNOS activation (3, 4, 7). Using cells transfected with a de-phosphomutant of eNOS (T495A in human eNOS), there is an enhanced interaction of the essential allosteric regulator calmodulin, which correlates well with increased eNOS calcium sensitivity in vitro. In addition, it was suggested that the reciprocal dephosphorylation of threonine 497 and phosphorylation of serine 1179 were essential for eNOS activation and that dephosphorylation may be a prerequisite for serine 1179 phosphorylation (3, 4, 7, 11).

Because the T495A eNOS mutant (de-phospho) demonstrated enhanced calcium sensitivity compared with T495D eNOS (potential phosphomimetic) and the rate of electron flux through the reductase domain of eNOS critically determines eNOS activity and the potential for uncoupling eNOS (12, 13), we sought to examine the relationship between threonine 497 dephosphorylation (mimicked by T497A mutation) and the balance of NO generation versus superoxide anion production in intact cells. Here we show that T497A eNOS increases NO activity in cell lysates but reduces basal NO production in intact cells. Combining eNOS T497A with a mutation of Ser 1179 that renders eNOS constitutively active to produce NO (eNOS...
**Fig. 1.** Time course of the agonist-induced changes in the phosphorylation of serine 1179 and threonine 497 in BAECs and transfected COS-7 cells. BAECs were stimulated with PMA (1 μM, A), VEGF (50 ng/ml, B), or ionomycin (1 μM, C) for the times indicated. eNOS was isolated by 2M NaOH or lysis buffer as described above from 1 mg/ml NaNO3 and precipitated with an anti-HA antibody (Roche Applied Science) to immunoprecipitate HA-tagged hsp90 cDNA construct (14) or mutants) with or without an HA-tagged hsp90 cDNA construct (14) using LipofectAMINE 2000 (Invitrogen) as per manufacturer’s protocol. Transiently transfected cells were used 48 h post-transfection. Stably transfected HEK 293 cells were used in superoxide anion measurements and were selected in 1 mg/ml G418 for 2 passages.

**Materials and Methods**

**Cell Culture Conditions**—Bovine aortic endothelial cells (BAECs), passages 4–8, COS-7, and HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% (v/v) fetal calf serum. All of the experiments using these cells were done when cells reached confluency in 100-mm dishes for BAECs or in 6-well plates for COS-7 and HEK 293 cells. Prior to agonist stimulation, BAECs or transfected COS-7 cells were rendered quiescent in serum-free Dulbecco’s modified Eagle’s medium for 16 or 6 h, respectively, before the addition of 1 μM ionomycin (Sigma), 1 μM PMA (Calbiochem), 10 μM ATP (Sigma), or 50 ng/ml VEGF (Genentech). The mutant bovine eNOS constructs were generated by standard cloning methods. The S1179D, T497A, and T497D mutant eNOS constructs were generated by site-directed mutagenesis (Stratagene). The T497A/S1179D double mutant eNOS construct was generated by subcloning the XhoI/XbaI fragment of S1179D mutant eNOS into the T497A construct. All of the constructs were verified by sequencing. Human eNOS constructs (wild-type, Ala495, and Asp495 also called T495A and T495D in this paper) were generated as described previously (7).

**Transient Transfection and Generation of Stable Cell Line**—COS-7 or HEK 293 cells were transfected with the bovine eNOS cDNA (wild-type or mutants) with or without an HA-tagged hsp90 cDNA construct (14) using LipofectAMINE 2000 (Invitrogen) as per manufacturer’s protocol. Transiently transfected cells were used 48 h post-transfection. Stably transfected HEK 293 cells were used in superoxide anion measurements and were selected in 1 mg/ml G418 for 2–3 weeks.

**Immunoprecipitation and Immunoblotting**—Immediately after agonist stimulation, cells were washed with ice-cold phosphate-buffered saline (without Ca2+ and Mg2+) and subsequently lysed in buffer that contained 50 mM Tris, pH 7.5, 0.1 M EGTA, 0.1 M EDTA, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholate, 20 mM NaF, 1 mM Na3P04, 0.3 mg/ml Pefabloc SC, 20 mM Na3MoO4, 5 mM ATP, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 2 μg/ml pepstatin A. Cell lysates were Dounce-homogenized (50 strokes) and rotated at 4°C for 15 min before the insoluble materials were removed by centrifugation at 12,000 × g for 10 min. In some experiments, after normalizing for equal protein concentration, lysates supernatant were either collected or preclarified with protein G-Septarose beads. Pre-clear sed samples were then immunoprecipitated with an anti-HA antibody (Roche Applied Science) to immunoprecipitate HA-tagged hsp90 followed by protein G-Septarose incubation. In other experiments, eNOS and associated proteins were concentrated by affinity binding to 2–5’-ADP-Septarose beads after samples had been preclarified with Sepharose. Proteins in both the cell lysates and immunoprecipitate were heated in SDS sample buffer before separation by SDS-PAGE. Following overnight transfer of the proteins onto nitrocellulose membranes, Western blots were performed using anti-HA or phospho-specific antibodies against Ser1179 (Cell Signaling) or Thr497 (3). The blots were then stripped and re-probed for total eNOS (9D10, Zymed Laboratories Inc.).

**NO Release from Transfected Cells**—48 h after transfection of eNOS mutants into COS-7 or HEK 293 cells, the media were processed for the measurement of nitrate (NO2−), a stable breakdown product of NO in aqueous solution, by NO-specific chemiluminescence (8). Samples collected from basal overnight accumulation of NO2− in serum media were treated with 100% cold EtOH to precipitate serum proteins. After removing serum proteins by centrifugation, sample supernatants were subjected to speed vacuum to remove EtOH. The same cells were then incubated with serum-free Dulbecco’s modified Eagle’s medium for 6 h, and fresh medium was added to accumulate proaeronitrite for 30 min at 37°C. In some experiments, cells were incubated with 1 μM T-NAME during the 30-min proaeronitrite accumulation. The medium was then harvested, and nitrite accumulation was measured using NO-specific chemiluminescence. The cells were lysed with either 1% NaOH or lysis buffer as described above to determine the total protein concentration. Cell lysates were subjected to Western blotting analysis to demonstrate equal eNOS expression in all of the samples.

**NOS Activity Assay**—The activity of different eNOS mutants was monitored by conversion of L-[14C]arginine to L-[14C]citrulline. Transfected COS-7 cells were lysed in the lysis buffer described above without Na3MoO4 or ATP. After removing the insoluble material by centrifugation, the supernatant was assayed to normalize protein concentration. 100 μg of total protein was used per assay reaction and was diluted in a total volume of 100 μl of assay buffer containing 50 mM Tris, pH 7.5, 0.1 M EGTA, 0.1% SDS, 1% Triton X-100, 1 mM NADPH (Sigma), 15 μM BH4 (6-mercaptopurine riboside, Sigma), 9.8 μM l-arginine (Sigma), 1.4 μM L-[14C]arginine (348 mCi/mmol), and 2.5 mM CaCl2 (Sigma). All of the reaction samples were kept on ice until incubation at 37°C for 10 min. The reaction was stopped by adding 1 ml of ice-cold stop buffer containing 20 mM HEPES, pH 5.5, 2 mM EDTA, and 2 mM EGTA to each sample. The samples were then passed through Dowex AG50WX8 cation exchange resin, and the flow-through was counted on a liquid scintillation analyzer.

**eNOS Localization**— transiently transfected COS-7 cells were fixed in 3% paraformaldehyde for 10 min followed by cell permeabilization using 0.1% Triton X-100. Prior to incubation with anti-eNOS mAb (clone H32, Biomol), cells were blocked with 5% goat serum for 20 min. Immunofluorescent staining of eNOS was visualized using an AlexaFluor 568 goat anti-mouse secondary antibody (Molecular Probes). Cells were then counterstained with 4’,6-diamidino-2-phenylindole to visualize the nuclei. Z-series images were captured using the Zeiss Axiowert 200 m microscope and deconvolved using the Openlab 3.1.3 image analysis software (Improvision).

**Measurement of Superoxide Anion**—Stably transfected HEK 293 cells were grown to confluence prior to analysis for NO release or superoxide anion production measured by ferricytochrome c reduction. (15). Cells were incubated with 1 μM T-NAME for 30 min before washing with Hanks’ Balanced Salt Solution and incubated with 50 μM ferricytochrome c and 5 μM A23187 with or without 1 μM T-NAME in Hanks’ Balanced Salt Solution for another 30 min. The same experiment was carried out in the presence of 1000 units/ml superoxide dismutase.
It has been suggested that the dephosphorylation of Thr<sup>497</sup> is a prerequisite for the phosphorylation of Ser<sup>1179</sup>. To examine the interrelationship between these sites in a defined reconstituted system, COS cells were transfected with WT eNOS and the following mutants, T497A, S1179D, and T497A/S1179D eNOS and then stimulated with ionomycin to examine the time course of phosphorylation/dephosphorylation on Ser<sup>1179</sup> and Thr<sup>497</sup>. The rationale was that since T497A exhibits enhanced calcium sensitivity and calmodulin binding (17) and S1179D is a constitutively active form of eNOS that also lowers the threshold for calcium-calmodulin activation (13), combining the two mutants should create synergy between the sites. As seen in Fig. 2A, the basal phosphorylation on Ser<sup>1179</sup> is very low at time 0 (in cells transfected with WT and T497A eNOS), whereas the basal phosphorylation on Thr<sup>497</sup> is high (in cells transfected with WT and S1179D eNOS). Ionomycin stimulation of cells transfected with WT eNOS resulted in a time-dependent increase in phosphorylation on Ser<sup>1179</sup> and a decrease in phosphorylation on Thr<sup>497</sup>, consistent with the data in BAEC (Fig. 1C). A similar increase in phosphorylation on Ser<sup>1179</sup> was seen in cells transfected with T497A eNOS showing that the level of Thr<sup>497</sup> phosphorylation has no significant influence on the extent of Ser<sup>1179</sup> phosphorylation in this reconstituted system. The constitutively active eNOS S1179D was highly phosphorylated on Thr<sup>497</sup> and was partially dephosphorylated upon challenge with ionomycin. In cells transfected with T497A/S1179D eNOS, no phosphosignal was present documenting the specificity of the antibodies. Thus, both WT and S1179D eNOS are highly phosphorylated on Thr<sup>497</sup>.

We next examined NOS enzymatic activity of the different eNOS constructs. Transfection of COS cells with WT eNOS increased NOS activity (under V<sub>max</sub> conditions) compared with eNOS released from cells. Transfection with constitutively active S1179D eNOS resulted in a large increase in enzymatic activity (5-fold) compared with WT eNOS and the other mutants. Thus, combining T497A and S1179D mutations generates a highly active form of the enzyme.

Because the rate of eNOS activity correlates well with the production of NO for WT and S1179D eNOS, we examined basal (24-h accumulation) and ATP-stimulated (30-min collection period) NO release measured as nitrite (NO<sub>2</sub>) from COS cells transfected with the different eNOS constructs. Transfection with WT eNOS increased basal (Fig. 3A) and ATP-stimulated (Fig. 3B) NO<sub>2</sub> release from cells. Transfection with constitutively active S1179D eNOS enhanced both basal and stimulated NO<sub>2</sub> release from cells compared with WT eNOS. Surprisingly, transfection with T497A, T497D, and T497A/S1179D all resulted in reduced basal accumulation of NO<sub>2</sub> (Fig. 3A). In contrast, upon challenge with ATP, equivalent amounts of NO<sub>2</sub> were produced from T497A eNOS compared with WT enzyme, whereas NO release from T497D eNOS remained depressed. Challenging cells expressing either T497A/S1179D eNOS or S1179D eNOS with ATP resulted in similar amounts of NO<sub>2</sub> release. We repeated the above experiments using ionomycin as an agonist for NO release and obtained similar results (Fig. 3, C and D). Basal accumulation of NO<sub>2</sub> (Fig. 3C) was reduced in cells transfected with T497A, T497D and T497A/S1179D eNOS and following stimulation (Fig. 3D), the deficit in NO production was partially compensated or rescued. These data suggest that the lack of phosphorylation on Thr<sup>497</sup> in eNOS impairs the ability of eNOS to produce NO in the absence of an agonist challenge (“calcium-independent”

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**RESULTS AND DISCUSSION**

To characterize the relationship between eNOS phosphorylation on Thr<sup>497</sup> and Ser<sup>1179</sup> BAECs were challenged with three agonists, PMA, VEGF, or ionomycin and the time course of changes in phosphorylation was examined using phosphospecific antibodies. As seen in Fig. 1A, PMA increased the extent of phosphorylation on Thr<sup>497</sup>, a known protein kinase C phosphorylation site, but did not influence phosphorylation on Ser<sup>1179</sup>. VEGF, an agonist that signals via a receptor tyrosine kinase, stimulated the time-dependent phosphorylation on Ser<sup>1179</sup> that was maximal 1–3 min after agonist challenge but did not influence Thr<sup>497</sup> phosphorylation (Fig. 1B). Treatment of BAEC with ionomycin to promote the release of calcium and capacitive calcium entry into cells stimulated the time-dependent phosphorylation on Ser<sup>1179</sup> and dephosphorylation of Thr<sup>497</sup>. Thus, distinct agonists exert differential regulation of Thr<sup>497</sup> versus Ser<sup>1179</sup>.

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**Fig. 2. Phosphorylation and enzymatic activity of eNOS mutants transfected into COS-7 cells.** A, WT eNOS and the mutants were transfected into COS-7 cells and transfected cells stimulated with ionomycin (1 μM) for various times. Cell lysates were analyzed for changes in phosphorylation at Ser<sup>1179</sup> and Thr<sup>497</sup> and for total eNOS levels. B, similarly transfected cells were used to determine eNOS enzyme activity by conversion of L-[<sup>14</sup>C]arginine to L-[<sup>14</sup>C]citrulline under V<sub>max</sub> conditions as described under “Materials and Methods.” The data are the mean ± S.E. of triplicate samples and represent data obtained in three separate experiments. Serum lysates were Western blotted with total eNOS mAb antibody to document equal levels of eNOS protein in the transfecants. *, p < 0.05 compared with WT; #, p < 0.05 compared with S1179D mutant.
**Fig. 3.** Basal and agonist induced NO release from cells transfected with eNOS mutants. NO release (measured as NO\textsubscript{2}/NO\textsubscript{3}) from COS-7 cells transfected with bovine eNOS (A–D) or human eNOS (E and F) cDNAs. In A, C, and E, the accumulation of NO\textsubscript{2}/NO\textsubscript{3} in the medium was quantified after 24 h. In B, cells were washed and preincubated for 30 min in serum-free medium (prestimulation values) (closed bar) followed by stimulation with ATP (10 mM) (open bar) for an additional 30 min. In D, the exact experiment was performed using ionomycin (1 mM) as an agonist. In E and F, similar experiments were performed with human eNOS cDNAs and ionomycin was used as an agonist. The insets depict equal eNOS expression by all of the mutants by Western blotting with eNOS mAb antibody. All of the data represent the mean ± S.E. of triplicate samples (three different transfectants) repeated in three separate experiments. *, p < 0.05 compared with WT; #, p < 0.05, compared with S1179D mutant.
eNOS Is Regulated by Phosphorylation of Threonine 497

Mutation of the serine residue Ser^{1412} to aspartate in neuronal NOS, a site equivalent to the phosphorylation site Ser^{1179} in eNOS, accelerates electron flux in the neuronal NOS reductase domain and results in less NO release, suggesting an optimal coupling of electron flux to NO generation (18). These results strongly suggest that the rate of electron flux into the heme domains of different NOS isoforms regulates the coupling of l-arginine metabolism to NO synthesis. Perhaps if Thr^{497} phosphorylation regulates the fidelity of electron transfer coupled to NO synthesis, mutations preventing the phosphorylation of Thr^{497} may permit eNOS to generate superoxide anions.

To test this hypothesis, stable cell lines were generated in HEK 293 cells expressing equivalent amounts of WT, T497A, and T497A/S1179D eNOS and the production of superoxide anion was assessed by superoxide dismutase-sensitive ferricytochrome c reduction and NO_{2} was quantified. As seen in Fig. 5A, the three cell lines expressed similar levels of eNOS protein by Western blotting. The basal levels of superoxide produced by the cells were lower than the limits of detection for this assay. Stimulation of cells with ionophore resulted in barely detectable superoxide anion production from cells expressing WT eNOS (solid bar). However, the addition of l-NAME enhanced the detection of superoxide (open bar), presumably by eliminating the quenching action of eNOS-derived NO on superoxide anion generated from non-NOS sources. In contrast, cells expressing T497A eNOS and T497A/S1179D eNOS produced much more superoxide, an effect reduced by treatment of the cells with l-NAME. Similar to cells transiently transfected with these constructs, basal NO_{2} release was less for cells stably transfected with T497A or T497A/S1179D eNOS (14.4 ± 0.5, 8.3 ± 0.4*, and 10.9 ± 0.6† nmol NO_{2}/mg protein for cells expressing WT, T497A, and T497A/S1179D eNOS, respectively; p < 0.05, compared with WT (*) and compared with T497A †). Moreover, ionophore-stimulated NO_{2} was comparable...
with WT in cells expressing T497A eNOS or T497A/S1179D eNOS (Fig. 5B). Thus, the mutation of Thr497 to a residue that cannot be phosphorylated, perhaps mimicking the de-phosphostate, augments the ability of eNOS to serve as a superoxide anion generator at the expense of NO synthesis. The ability of T497A and T497A/S1179D eNOS to produce superoxide anion instead of NO synthesis may explain, in part, the discordance between the elevated NOS activity in broken cell lysates and reduced NO production from cells transfected with these constructs. If T497A eNOS and T497A/S1179D eNOS were not uncoupled, the production of NO under basal and stimulated conditions should have exceeded the levels obtained from cells expressing WT or S1179D eNOS, respectively (see Fig. 3).

Recent evidence suggests that the association of eNOS with the molecular chaperone, hsp90, may influence many functions of eNOS including coupling L-arginine metabolism to NO biosynthesis (19–23). To examine the interaction of eNOS with hsp90, WT eNOS and different eNOS mutants were co-transfected with HA-tagged hsp90 and the levels of phosphorylation

Fig. 5. T497A and T497A/S1179D eNOS produces more superoxide than WT eNOS and has decreased hsp90 interaction. A, HEK 293 cells were stably transfected with WT, T497A, or S1179D/T497A eNOS constructs, and ionophore-stimulated superoxide anion release was measured in the absence (closed bar) or presence of 1 mM L-NAME (open bar). The inset depicts equal eNOS expression in the cell lines by Western blotting with eNOS mAb antibody. All of the data represent the mean ± S.E. of triplicate samples, and each reading was analyzed in duplicates. This experiment was repeated four additional times with qualitatively similar results. *p < 0.05 compared with WT, #, p < 0.005 compared with samples in the absence of L-NAME. B, stable transfectants were stimulated, and cells were washed and preincubated for 30 min in serum-free medium and stimulated with ionophore for an additional 30 min and NO2 was measured in the absence or presence of l-NAME as in A. *, p < 0.05 compared with WT; #, p < 0.05 compared with samples in the absence of l-NAME. C, eNOS and HA-tagged hsp90 were co-transfected into COS-7 cells and stimulated with 1 μmol ionomycin for the indicated times. Cells were then lysed, and total cell lysates were probed with phosphospecific Ser1179 and Thr497 antibodies followed by total eNOS and total HA antibodies to depict equal levels of protein expression. D, cell lysates from C were immunoprecipitated with anti-HA antibody and Western blotted for total eNOS to determine relative co-association of the two proteins. The levels of HA-tagged hsp90 were identical (lower panel). The Western blots are representative of three separate experiments.
and interaction with hsp90 were examined. As seen in Fig. 5C, the levels of eNOS and HA-hsp90 expression in lysates were identical with the expected time-dependent changes in phosphorylation similar to data in Fig. 2. Immunoprecipitation of HA-hsp90 in non-stimulated cells (time 0) resulted in equal amounts of eNOS recovered from cells expressing WT and T497A eNOS. However, the interaction of eNOS with hsp90 was increased in cells transfected with S1179D eNOS and virtually eliminated with cells expressing T497A/S1179D eNOS (Fig. 5D). After stimulation with ionophore, the interaction of WT and T497A eNOS with hsp90 were similar, whereas the enhanced interaction of S1179D with hsp90 was maintained. In contrast, both basal and stimulated interaction of T497A/S1179D eNOS with hsp90 was less than that seen with the other constructs. Thus, an altered interaction with hsp90 may be an additional mechanism to account for the elevated capacity of T497A/S1179D eNOS to produce superoxide anion.

Collectively, the data suggest that the phosphorylation/dephosphorylation of Thr\textsuperscript{497} in bovine eNOS or Thr\textsuperscript{495} in human eNOS does not change eNOS subcellular localization but may regulate NO production and determine the generation of NO versus superoxide anion upon stimulation of cells. Most strikingly, combining the T497A mutation with the well characterized “gain-of-function” mutant S1179D eNOS results in a very active enzyme \textit{in vitro}. The basal release of NO from cells expressing T497A/S1179D eNOS is effectively lower than WT eNOS and markedly less than that observed with S1179D eNOS. However, upon agonist challenge, both T497A eNOS and T497A/S1179D eNOS behave similarly to WT and S1179D eNOS, respectively, despite their higher activities in broken cell assays. This finding suggests that at rest, in the absence of a marked increase in cytoplasmic calcium, the S1179D mutation removes the autoinhibitory loop located in the carboxyl tail of eNOS (13, 24), thus increasing electron flux, improving the calcium sensitivity of the enzyme and its interaction with hsp90, and resulting in an enhanced basal NO release. This occurs despite the fact that Thr\textsuperscript{497} is phosphorylated in WT and S1179D eNOS. Upon stimulation, WT and S1179D eNOS are dephosphorylated on Thr\textsuperscript{497} and the level of NO release increases as expected.

With T497A and T497A/S1179D eNOS, the apparent hysteresis of NO activity versus NO release is complex and difficult to fully understand mechanistically. Clearly, the activity of T497A eNOS (assayed under \textit{V}_{\text{max}} \text{conditions with optimal amounts of cofactors}) is greater than WT eNOS and comparable with that of S1179D eNOS. Moreover, the activity of the combined mutant T497A/S1179D eNOS is far greater than T497A and S1179D eNOS. Despite the high \textit{in vitro} NO activities of the mutant enzymes, in contrast to WT and S1179D eNOS, T497A and T497A/S1179D eNOS produce less basal NO despite equal levels of Ser\textsuperscript{1179} phosphorylation (at least for T497A). The decrease in basal NO release may be explained by the alanine substitution at Thr\textsuperscript{497} reducing the affinity of a key NO cofactor/regulator from performing its function in a cellular context or changing the alignment of the reductase and oxygenase domains, effects overcome by agonist stimulation. This possibility is predicated on the data that eNOS is normally localized in the cells (Fig. 4) and the reasonable assumption that the cellular levels of l-arginine and tetrahydrobiopterin are similar in the different transfecteds. Evidence supporting an altered protein-protein interaction are the data demonstrating enhanced co-association of hsp90 with S1179D eNOS and reduced co-association of hsp90 with T497A/S1179D eNOS under basal conditions. However, the interaction of T497A with hsp90 and phosphorylation on Ser\textsuperscript{1179} are similar to WT eNOS. Thus, changes in the interaction of hsp90 with eNOS cannot fully explain this phenomenon. Another possibility is that Thr\textsuperscript{497} phosphorylation regulates the fidelity of electron flux coupled to NO synthesis under basal conditions (i.e. resting levels of calcium in the cell), and upon agonist challenge, enhanced calmodulin binding, regulatory protein-protein interactions, and phosphorylation of other sites maintain NO release despite the capacity to produce superoxide triggered by Thr\textsuperscript{497} dephosphorylation. These data with T49A eNOS are consistent with a recent report (14) showing that adenosinal transduction of endothelial cells with hsp90 increases both basal and stimulated Ser\textsuperscript{1179} and Thr\textsuperscript{497} phosphorylation concomitantly with enhanced basal and stimulated NO release, suggesting that phosphorylation of Thr\textsuperscript{497} promotes the coupling of eNOS and NO synthesis.

In summary, our data imply that the phosphorylation of Thr\textsuperscript{497} in eNOS may be important for the coupling of l-arginine metabolism to NO synthesis. It is possible that upon dephosphorylation of Thr\textsuperscript{497}, eNOS may serve a generator of superoxide anion.

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