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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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There is evidence that endothelial nitric-oxide synthase (eNOS) is regulated 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 spatial and temporal signals, namely subcellular targeting to the Golgi complex and lipid rafts/caveolae by protein-protein interactions and 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 function (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 NOS 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

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[1] The abbreviations used are: eNOS, endothelial nitric-oxide synthase; NO, nitric oxide; HEK, human embryonic kidney; BAEC, bovine aortic endothelial cell; PMA, phorbol 12-myristate 13-acetate; VEGF, vascular endothelial growth factor; mAb, monoclonal antibody; HA, hemagglutinin; WT, wild-type; L-NAME, L-nitroarginine methyl ester.

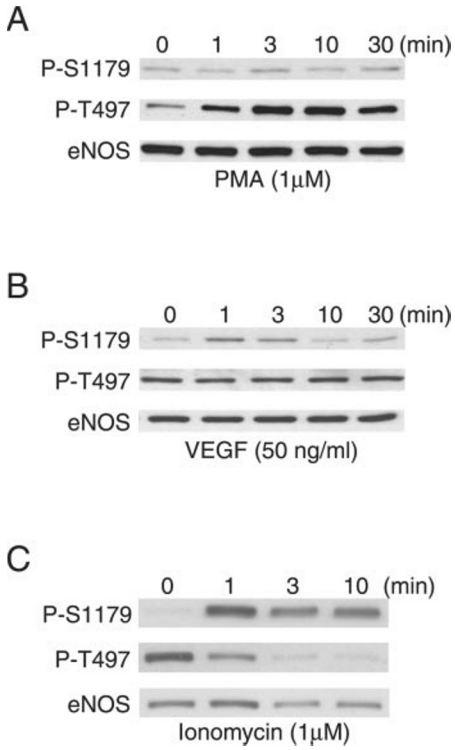

FIG. 1. **Time course of the agonist-induced changes in the phosphorylation of serine 1179 and threonine 497 in BAECs and transfected COS-7 cells.** BAECs were stimulated with PMA (1 μM, *A*), VEGF (50 ng/ml, *B*), or ionomycin (1 μM, *C*) for the times indicated. eNOS was isolated by 2′–5′-ADP-Sepharose chromatography and Western blotted for phospho-Ser^{1179}, phospho-Thr^{497}, and total eNOS. Shown here is a representative blot from three separate experiments.

S1179D) generates an extremely active enzyme (eNOS T497A/S1179D) but neutralizes the ability of S1179D eNOS to produce basal and stimulated NO and promotes eNOS-dependent superoxide anion release. Thus, the phosphorylation of threonine 497 is a critical residue that may be an intrinsic mechanism to balance whether eNOS generates NO *versus* superoxide anion in cells.

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 mg/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) at various time points.

eNOS Constructs—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, Ala^{495}, and Asp^{495} 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 Ca^{2+} and Mg^{2+}) and subsequently lysed in buffer that contained 50 mM Tris, pH 7.5, 0.1 mM EGTA, 0.1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholate, 20 mM NaF, 1 mM $Na_4P_2O_7$, 0.3 mg/ml Pefabloc SC, 20 mM Na_2MoO_4, 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 $\times$ *g* for 10 min. In some experiments, after normalizing for equal protein concentration, lysates supernatant were either collected or precleared with protein G-Sepharose beads. Pre-cleared samples were then immunoprecipitated with an anti-HA antibody (Roche Applied Science) to immunoprecipitate HA-tagged hsp90 followed by protein G-Sepharose incubation. In other experiments, eNOS and associated proteins were concentrated by affinity binding to 2′–5′-ADP-Sepharose beads after samples had been precleared 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 Ser^{1179} (Cell Signaling) or Thr^{497} (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 nitrite (NO_2^-), a stable breakdown product of NO in aqueous solution, by NO-specific chemiluminescence (8). Samples collected from basal overnight accumulation of NO_2^- 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 preagonist nitrite for 30 min at 37 °C. In some experiments, cells were incubated with 1 mM L-NAME during the 30-min preagonist nitrite accumulation. Subsequently, the cells were incubated with agonists (1 μM ionomycin, 10 μM ATP or 5 μM A23187, Sigma) for another 30 min to allow post-agonist nitrite accumulation. The medium was then harvested, and nitrite accumulation was measured using NO-specific chemiluminescence. The cells were lysed with either 1 M 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 the different eNOS mutants was monitored by conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline. Transfected COS-7 cells were lysed in the lysis buffer described above without Na_2MoO_4 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 activity assay buffer containing 50 mM Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 100 nM calmodulin (Sigma), 1 mM NADPH (Sigma), 15 μM BH_4 (*6R*-tetrahydro-L-biopterin, Sigma), 8.6 μM L-arginine (Sigma), 1.4 μM L-[^{14}C]arginine (348 mCi/mmol), and 2.5 mM $CaCl_2$ (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 transected 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 Axiovert 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 confluency prior to analysis for NO release or superoxide anion production measured by ferricytochrome *c* reduction (15). Cells were incubated with 1 mM L-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 mM L-NAME in Hanks' Balanced Salt Solution for another 30 min. The same experiment was carried out in the presence of 1000 units/ml superoxide

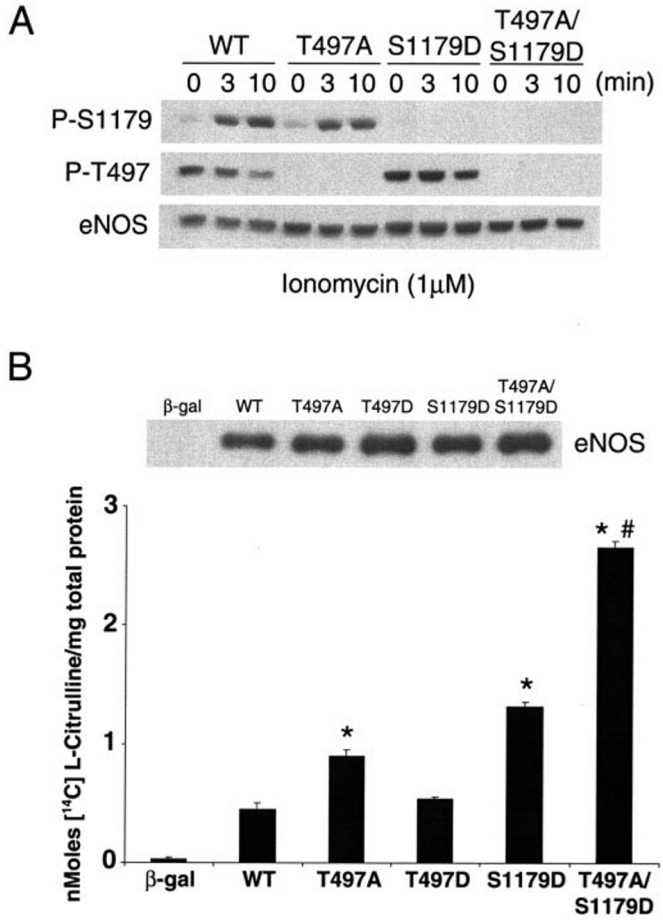

FIG. 2. **Phosphorylation and enzymatic activity of eNOS mutants transfected into COS-7 cells.** *A*, WT eNOS and the mutants were transfected into COS-7 cells and transfected cells stimulated with ionomycin (1 μM) for various times. Cell lysates were analyzed for changes in phosphorylation at Ser^{1179} and Thr^{497} and for total eNOS levels. *B*, similarly transfected cells were used to determine eNOS enzyme activity by conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline under V_{max} 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. Sample lysates were Western blotted with total eNOS mAb antibody to document equal levels of eNOS protein in the transfectants. *, $p < 0.05$ compared with WT; #, $p < 0.05$ compared with S1179D mutant.

dismutase. Rates of superoxide anion release were calculated from superoxide dismutase-inhibitable ferricytochrome *c* absorbance at 550 nm using the molar extinction coefficient $\epsilon = 21{,}000\ M^{-1}\ cm^{-1}$.

RESULTS AND DISCUSSION

To characterize the relationship between eNOS phosphorylation on Thr^{497} and Ser^{1179}, 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. 1*A*, PMA increased the extent of phosphorylation on Thr^{497}, a known protein kinase C phosphorylation site, but did not influence phosphorylation on Ser^{1179}. VEGF, an agonist that signals via a receptor tyrosine kinase, stimulated the time-dependent phosphorylation on Ser^{1179} that was maximal 1–3 min after agonist challenge but did not influence Thr^{497} phosphorylation/dephosphorylation (Fig. 1*B*). Treatment of BAEC with ionomycin to promote the release of calcium and capacitive calcium entry into cells stimulated the time-dependent phosphorylation on Ser^{1179} and dephosphorylation of Thr^{497}. Thus, distinct agonists exert differential regulation of Thr^{497} *versus* Ser^{1179}.

It has been suggested that the dephosphorylation of Thr^{497} is a prerequisite for the phosphorylation of Ser^{1179}. 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^{1179} and Thr^{497}. The rationale was that since T497A exhibits enhanced calcium sensitivity and calmodulin binding (7) 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. 2*A*, the basal phosphorylation on Ser^{1179} is very low at time 0 (in cells transfected with WT and T497A eNOS), whereas the basal phosphorylation on Thr^{497} 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^{1179} and a decrease in phosphorylation on Thr^{497}, consistent with the data in BAEC (Fig. 1*C*). A similar increase in phosphorylation on Ser^{1179} was seen in cells transfected with T497A eNOS showing that the level of Thr^{497} phosphorylation has no significant influence on the extent of Ser^{1179} phosphorylation in this reconstituted system. The constitutively active eNOS S1179D was highly phosphorylated on Thr^{497} 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^{497}.

We next examined NOS enzymatic activity of the different eNOS constructs. Transfection of COS cells with WT eNOS increased NOS activity (under V_{max} conditions) in detergent-solubilized cell lysates (Fig. 2*B*). Both T497A and S1179D eNOS exhibited greater enzyme activity (2–2.5-fold) than WT eNOS, whereas the activity of T497D eNOS was comparable with that of WT enzyme. Transfection with the double mutant, T497A/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_2^-) from COS cells transfected with the different eNOS constructs. Transfection with WT eNOS increased basal (Fig. 3*A*) and ATP-stimulated (Fig. 3*B*) NO_2^- release from cells. Transfection with constitutively active S1179D eNOS enhanced both basal and stimulated NO_2^- release from cells compared with WT eNOS. Surprisingly, transfection with T497A, T497D, and T497A/S1179D all resulted in reduced basal accumulation of NO_2^- (Fig. 3*A*). In contrast, upon challenge with ATP, equivalent amounts of NO_2^- 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_2^- 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_2^- (Fig. 3*C*) was reduced in cells transfected with T497A, T497D and T497A/S1179D eNOS and following stimulation (Fig. 3*D*), the deficit in NO production was partially compensated or rescued. These data suggest that the lack of phosphorylation on Thr^{497} in eNOS impairs the ability of eNOS to produce NO in the absence of an agonist challenge ("calcium-independent"

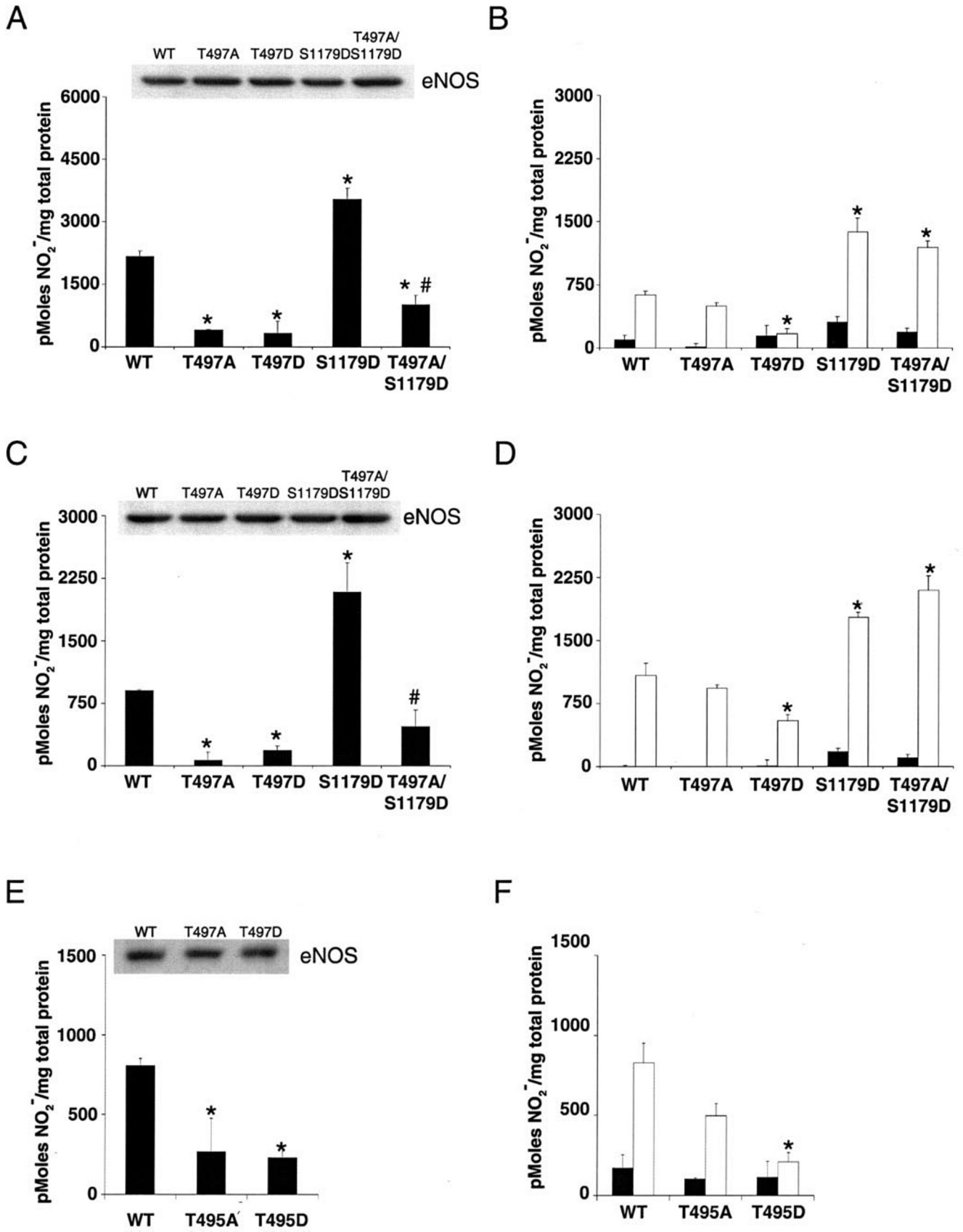

FIG. 3. **Basal and agonist induced NO release from cells transfected with eNOS mutants.** NO release (measured as NO_2^-) from COS-7 cells transfected with bovine eNOS (*A–D*) or human eNOS (*E* and *F*) cDNAs. In *A*, *C*, and *E*, the accumulation of NO_2^- in the medium was quantified after 24 h. In *B*, cells were washed and preincubated for 30 min in serum-free medium (prestimulation values) (*closed bar*) followed by stimulation with ATP (10 μM) (*open bar*) for an additional 30 min. In *D*, the exact experiment was performed using ionomycin (1 μM) 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.

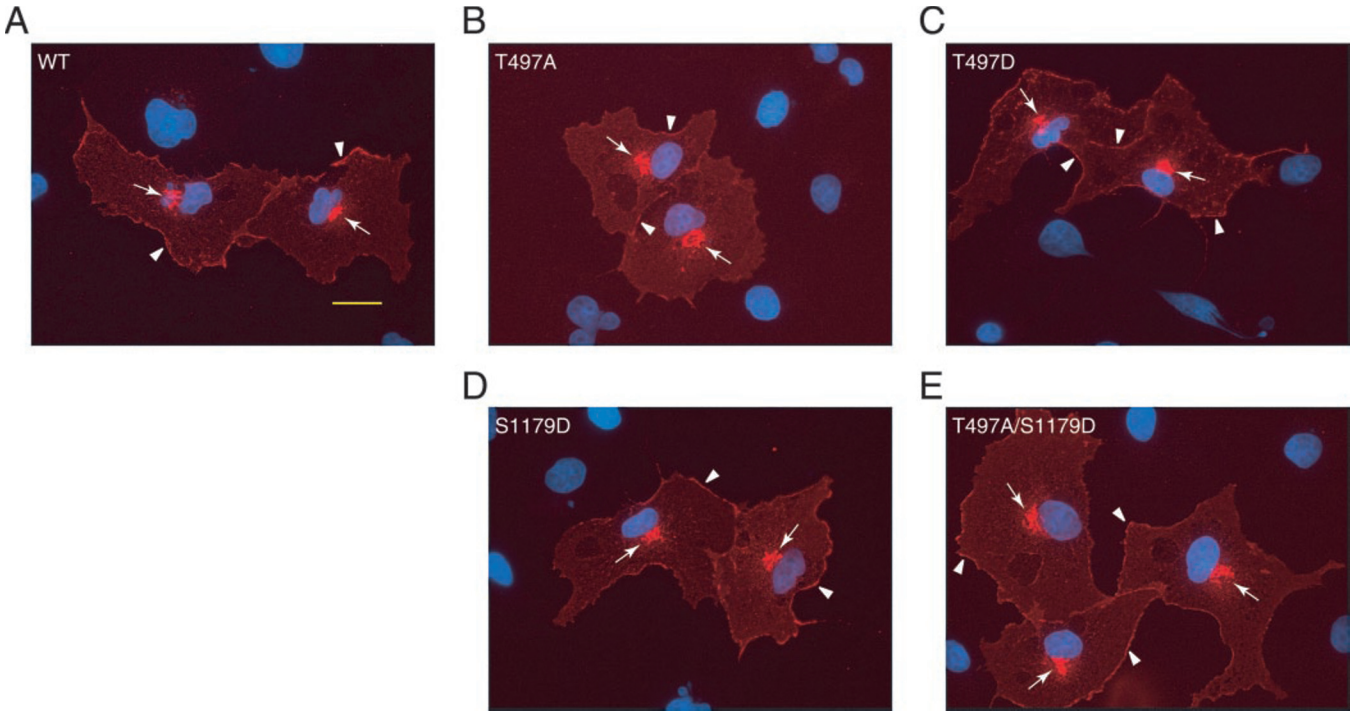

FIG. 4. **eNOS mutants localize to the Golgi and plasma membrane in a manner similar to WT eNOS.** COS-7 cells were transfected with the respective eNOS cDNAs (WT (*A*), T497A (*B*), T497D (*C*), S1179D (*D*), and T497A/S1179D (*E*)), and eNOS distribution was examined by indirect immunofluorescence using an eNOS mAb (Biomol). eNOS was found in both the Golgi region (*arrows*) and plasma membrane (*arrowheads*) with all of the constructs tested. The nuclei are counterstained with DAPI to delineate the nucleus of the transfectants, and the scale bar = 20 μm.

activation), but is functionally "normalized" by stimulation with two distinct calcium-mobilizing agonists. We also compared basal and stimulated NO_2^- release from COS cells transfected with human eNOS constructs (WT, T495A, T495D) to assess if the above effects were particular to bovine eNOS. As seen in Fig. 3, *E* and *F*, both T495A and T495D human eNOS exhibited reduced basal accumulation of NO_2^-, an effect partially overcome by the stimulation of cells with ionomycin. Thus, the enhanced NOS activity observed with T497A and T497A/S1179D eNOS and comparable activities between WT and T497D eNOS do not precisely correlate with basal or stimulated NO generation of NO_2^-, as is typically seen with WT and S1179D eNOS.

One possible explanation for the inability of Thr^{497} mutants to produce NO synthesis may be because of impaired localization of eNOS. Indeed, eNOS localization is necessary for the fidelity of stimulus-dependent NO release (16, 17). To examine the localization of eNOS, WT, T497A, T497D, S1179D, and T497A/S1179D eNOS constructs were transfected into COS cells and eNOS localization was examined by immunofluorescence microscopy using an eNOS mAb. As seen in Fig. 4, all of the eNOS constructs localized to the perinuclear region of cells and the plasmalemma. Thus, all of the constructs tested localized in a similar fashion to WT eNOS.

We next rationalized that the apparent discrepancy in NOS activity assays *versus* NO release may be explained by a defect in the coupling of L-arginine metabolism to NO synthesis. Clearly, the rate of electron flux from the reductase domain of eNOS is tightly coupled to NO synthesis in the oxygenase domain. Enhancement of electron flux by calmodulin binding or by a phosphomimetic substitution of Ser^{1179} with aspartate (S1179D eNOS) results in an equivalent increase in electron flux and NO synthesis. Moreover, both WT and S1179D eNOS exhibit low level superoxide dismutase-inhibitable cytochrome *c* reduction under conditions of L-arginine deprivation (8, 13) 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. 5*A*, 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

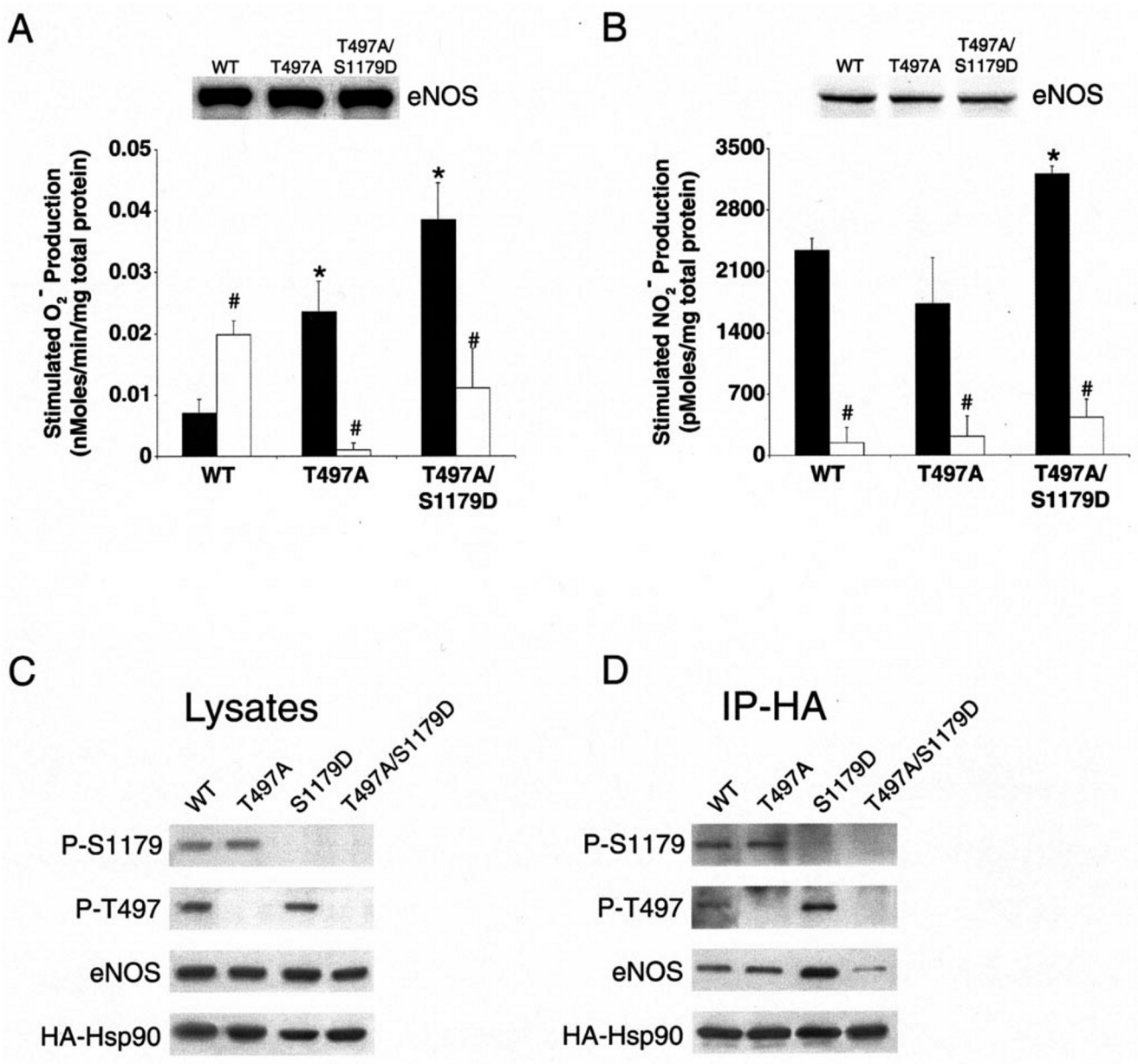

FIG. 5. **T497A and T497A/1179D eNOS produces more superoxide than WT eNOS and has decreased hsp90 interaction.** *A*, HEK 293 cells were stably transfected with WT, T497A, or S1179D/T497A eNOS constructs, and ionophore-stimulated superoxide anion release was measured in the absence (*closed bar*) or 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 NO_2^- 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 μM ionomycin for the indicated times. Cells were then lysed, and total cell lysates were probed with phosphospecific Ser^{1179} and Thr^{497} 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.

with WT in cells expressing T497A eNOS or T497A/S1179D eNOS (Fig. 5*B*). Thus, the mutation of Thr^{497} 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

and interaction with hsp90 were examined. As seen in Fig. 5*C*, 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. 5*D*). 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^{497} in bovine eNOS or Thr^{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 *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^{497} is phosphorylated in WT and S1179D eNOS. Upon stimulation, WT and S1179D eNOS are dephosphorylated on Thr^{497} and the level of NO release increases as expected.

With T497A and T497A/S1179D eNOS, the apparent hysteresis of NOS activity *versus* NO release is complex and difficult to fully understand mechanistically. Clearly, the activity of T497A eNOS (assayed under V_{max} 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 *in vitro* NOS 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^{1179} phosphorylation (at least for T497A). The decrease in basal NO release may be explained by the alanine substitution at Thr^{497} reducing the affinity of a key NOS 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 transfectants. 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^{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^{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^{497} dephosphorylation. These data with T49A eNOS are consistent with a recent report (14) showing that adenoviral transduction of endothelial cells with hsp90 increases both basal and stimulated Ser^{1179} and Thr^{497} phosphorylation concomitantly with enhanced basal and stimulated NO release, suggesting that phosphorylation of Thr^{497} promotes the coupling of eNOS and NO synthesis.

In summary, our data imply that the phosphorylation of Thr^{497} in eNOS may be important for the coupling of L-arginine metabolism to NO synthesis. It is possible that upon dephosphorylation of Thr^{497}, eNOS may serve a generator of both superoxide and NO. Moreover, the ability of eNOS to maintain its ability to produce NO in excess over superoxide anion production may reside in the recruitment of eNOS regulatory proteins, protein phosphorylation, and the correct alignment of the oxygenase/reductase domains of the enzyme. Our data suggest that the importance of Thr^{497} in eNOS is unlikely to be explained exclusively by regulating calmodulin binding to NOS. Future experiments examining how the phosphorylation of Thr^{497} influences NADPH-dependent electron flux through the reductase domain to the oxygenase domain and the heme environment of eNOS are clearly warranted.

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