Site-Specific Dephosphorylation of Endothelial Nitric Oxide Synthase by Protein Phosphatase 2A: Evidence for Crosstalk between Phosphorylation Sites†

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ABSTRACT: The endothelial isoform of nitric oxide synthase (eNOS) is a calcium/calmodulin-dependent enzyme that catalyzes the synthesis of nitric oxide, a key mediator of vascular homeostasis. eNOS undergoes a variety of posttranslational modifications, including phosphorylation on at least three residues: serines 116 and 1179 and threonine 497. Although the agonist-modulated protein kinase pathways that lead to eNOS phosphorylation have been studied in detail, the signaling pathways governing eNOS dephosphorylation remain less well characterized. The present study identifies protein phosphatase 2A (PP2A) as a key determinant of eNOS dephosphorylation and enzyme activity. We transfected bovine aortic endothelial cells (BAEC) with epitope-tagged cDNAs encoding wild-type eNOS or a series of phosphorylation-deficient eNOS mutants, immunoprecipitated [$^{32}P_i$] biosynthetically labeled recombinant proteins using antibodies directed against the epitope tag and treated the [$^{32}P_i$]-phosphorylated eNOS with protein phosphatases. We found that PP2A dephosphorylates eNOS residues threonine 497 and serine 1179 but not serine 116 and that an eNOS mutant lacking these three established phosphorylation sites is robustly labeled when expressed in BAEC and is dephosphorylated by PP2A. An inhibitor of PP2A increases eNOS enzymatic activity and augments overall levels of eNOS phosphorylation, specifically increasing phosphorylation of serines 116 and 1179. When transfected into BAEC or COS-7 cells, a "phospho-mimetic" eNOS mutant in which threonine 497 is changed to aspartate shows attenuated phosphorylation at serine 1179 as well as reduced enzyme activity in COS-7 cells. Our results indicate that regulation of eNOS dephosphorylation may be a key point for control of nitric oxide-dependent signaling pathways in vascular endothelial cells.

Protein phosphorylation regulates a wide variety of signaling and metabolic pathways. There is a delicate regulatory balance between the actions of protein kinases and phosphatases that, respectively, add and remove phosphate groups from their target proteins. In general, for a given phosphoprotein, much more is known about the identity and regulation of the relevant protein kinases than of the involved protein phosphatases. Specifically for endothelial nitric oxide synthase (eNOS),[1] a key signaling protein that is the focus of the present study, the protein kinases that mediate eNOS phosphorylation have been extensively characterized, whereas the protein phosphatases involved in eNOS dephosphorylation are less well understood.

eNOS catalyzes the conversion of L-arginine to L-citrulline and nitric oxide, a gas that profoundly influences vascular homeostasis (*1*). eNOS is composed of three domains: an amino-terminal heme-binding domain, a carboxyl-terminal reductase domain, and an intervening calmodulin-binding domain (reviewed in refs *2* and *3*). The maximal rate of nitric oxide synthesis is limited by the rate of electron transfer from the reductase domain to the heme moiety of the catalytic domain (*4*). Phosphorylation of eNOS modulates the enzymatic activity and at least three eNOS residues are phosphorylated: serines 116 (*5*) and 1179 (*5*−*7*) and threonine 497 (*8*). Multiple agonists, including vascular endothelial growth factor (VEGF) (*6*, *7*), sphingosine-1-phosphate (*9*), insulin (*10*), estrogen (*11*), and hydrogen peroxide (*12*), promote activation of kinase Akt, which phosphorylates eNOS at serine 1179 and induces eNOS enzyme activity. eNOS isolated from endothelial cells exposed to shear stress has been found to be phosphorylated on serines 1179 (*6*, *13*) and 116 (*5*). Phorbol 12-myristate 13-acetate (PMA) is the only compound reported to increase eNOS phosphorylation at threonine 497 (*14*).

Much less is known about pathways regulating eNOS dephosphorylation. Michell et al. have shown that treatment of endothelial cells with the phorbol ester PMA is associated with a decrease in the phosphorylation of serine 1179 (*14*). We have recently observed that VEGF treatment of bovine

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[1] Abbreviations: eNOS, endothelial nitric oxide synthase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B.; VEGF, vascular endothelial growth factor; PMA, phorbol 12-myristate 13-acetate; BAEC, bovine aortic endothelial cells; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; [$^{32}P_i$], [^{32}P] orthophosphoric acid.

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aortic endothelial cells (BAEC) leads to dephosphorylation of serine 116 and that this dephosphorylation is blocked by pretreatment with cyclosporin A (*15*). Dephosphorylation of threonine 497 is induced by the classical eNOS agonist bradykinin (*8*, *16*) and also by hydrogen peroxide (*12*). The phosphatases involved in most of these pathways remain to be definitively identified (*8*, *14*, *16*). In these studies, we explore eNOS dephosphorylation pathways and demonstrate that (i) PP2A dephosphorylates eNOS at threonine 497 and serine 1179 but not serine 116, (ii) in BAEC, eNOS is phosphorylated on an additional residue(s) other than 116, 497, and 1179, which is (are) dephosphorylated by PP2A, (iii) basal PP2A activity in BAEC inhibits eNOS enzymatic activity, and (iv) phosphorylation of threonine 497 attenuates both phosphorylation of eNOS on serine 1179 and cellular eNOS enzyme activity.

EXPERIMENTAL PROCEDURES

Materials. Fetal bovine serum (FBS) was from HyClone (Logan, CT). All other cell culture reagents and media were from Life Technologies, Inc. PCR reagents were from Stratagene. Vascular endothelial growth factor (VEGF) was from Calbiochem (San Diego, CA). Protein phosphatase inhibitors were from Biomol (Plymouth Meeting, PA). Protein phosphatases 1 (PP1) and 2A (PP2A) and the malachite green serine/threonine phosphatase assay kit, as well as antibodies against eNOS phosphoserine 116 and phosphothreonine 495 (corresponding to threonine 497 in the bovine eNOS sequence), were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phosphoserine 1177 eNOS (corresponding to serine 1179 in the bovine eNOS sequence) and anti-phosphoserine 473 Akt antibodies were from Cell Signaling Technologies (Beverly, MA). Anti-eNOS monoclonal antibody was from Transduction Laboratories (Lexington, KY). Polyclonal anti-eNOS serum was raised in rabbits against a synthetic peptide deduced from the sequence of the eNOS cDNA as described previously (*17*). The anti-hemagglutinin (HA) epitope polyclonal antibody was from Santa Cruz Biotechnology, and the anti-HA monoclonal antibody (12CA5) was from Roche Molecular Biochemicals. Antibody stripping reagent was from Chemicon International (Temecula, CA). Protein G- and protein A-Sepharose beads were from Zymed Laboratories (South San Francisco, CA). [^{32}P] orthophosphoric acid ([$^{32}P_i$]) was purchased from ICN (Costa Mesa, CA). The Bio-Rad Protein Assay Kit was used to make protein determinations. All other materials were from Sigma.

Plasmids. HA-tagged full-length wild-type and 116A and 1179A mutant bovine eNOS plasmid cDNAs have been described previously (*15*, *18*, *19*). Site-specific mutations were generated by PCR-based site-directed mutagenesis; all PCR-generated constructs were validated by nucleotide sequence analysis. The 497A and 497D mutants were generated using the wild-type construct as the template, with primer pairs consisting of the EcoR1 forward primer (5′-CGCGAATTCGAAGGAGCCACCATGGGCAACTT-GAAGAG-3′) and either the 497A reverse primer (5′-GGCAGAGATCTTCACCGCGTTGGCCACTTCCTT-AAAGG**C**CTTCTTCCTGG-3′) or the 497D reverse primer (5′-GGCAGAGATCTTCACCGCGTTGGCCACTTCC-TTAAAG**TC**CTTCTTCCTGG-3′), respectively. The bold underlined characters denote the mutation, and the underlined regular font characters denote the relevant restriction enzyme site.

Two step overlap extension PCR was used to construct the 116D mutant. In the first step, the wild-type construct served as the template for two PCR reactions. The two primer pairs were the following: (i) the EcoR1 forward primer and the 116D reverse primer (5′-AGGTCCCGGG**TC**GGGC-CGGGT-3′); and (ii) the forward 116D primer (5′-ACCCG-GCCC**GA**CCCGGGACCT-3′) and the BglII reverse primer (5′-CCGAGATCTTCACCGCGTTG-3′). In the second step, the EcoR1 forward primer and the BglII reverse primer were used to amplify the whole fragment.

The 116A/497A/1179A mutant was generated in two steps. First, the 116A/497A PCR product was amplified via a PCR reaction using the 116A construct as the template and the EcoR1 forward and 497A reverse primers. The PCR product was then subcloned into the 1179A construct. Similarly the 116A PCR product (*15*) was subcloned into the 1179A construct to generate the 116A/1179A mutant.

Cell Culture and Transfection. Bovine aortic endothelial cells (BAEC) were obtained from Cell Systems (Kirkland, WA). BAEC and COS-7 cells were cultured as described previously (*18*, *20*). Transfections were performed with FuGENE6 (Roche Molecular Biochemicals) using the manufacturer's protocol, and cells were studied 48 h following transfection.

Biosynthetic Labeling. BAEC were washed twice with Dulbecco's modified Eagle's medium (DMEM) without phosphate and then incubated with this medium containing 10% dialyzed FBS and 40−80 μCi/mL [$^{32}P_i$] (900Ci/mmol) for 2−4 h. BAEC were then treated with phosphatase inhibitors as indicated, washed twice with phosphate-free DMEM, and harvested. eNOS was immunoprecipitated with polyclonal anti-eNOS serum or monoclonal anti-eNOS antibody and processed as described below.

Preparation of Cellular Lysate and Immunoprecipitation, and Immunoblot Analysis. Following treatment with protein phosphatase inhibitors or agonists as indicated, cells were washed with phosphate-buffered saline, harvested in lysis buffer (50mM Tris−HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 2 μg/mL leupeptin, 2 μg/mL antipain, 2 μg/mL soybean trypsin inhibitor, 2 μg/mL lima bean trypsin inhibitor, 100 nM okadaic acid and 100 nM calyculin A). In experiments with cyclosporin A cellular treatments, 100 nM cyclosporin A was added to the lysis buffer. Cell homogenates were incubated for 20 min, 4°C, and then centrifuged for 10 min. For analysis of lysates, aliquots of the supernatant were reduced by adding SDS sample buffer and boiling. For immunoprecipitation, aliquots of the supernatant were incubated for 1 h at 4°C with anti-eNOS or anti-HA monoclonal antibody at a final concentration of 4 μg/mL or with polyclonal anti-eNOS serum at a final dilution of 1:100. Protein G- or A-Sepharose beads were added for an additional 1 h at 4°C. The immunoprecipitates were then washed three times with wash buffer (20 mM Tris−HCl, pH 7.4, 300 mM NaCl, 20 mM $MgCl_2$, and 1 mM DTT) and eluted by adding SDS sample buffer and boiling. Proteins were resolved by SDS−PAGE, electroblotted onto PVDF or nitrocellulose membranes, and analyzed by autoradiography and Western blotting. For immunoblots, the mem-

branes were probed with the appropriate primary antibody. Membrane-bound primary antibodies were detected using secondary antibodies conjugated with horseradish peroxidase and Super Signal substrate (Pierce) as described previously (*21*). Densitometric analyses of Western blots were performed using a ChemiImager 4000 (Alpha-Innotech).

In Vitro Dephosphorylation. eNOS was immunoprecipitated from BAEC as described above. Following washing, the eNOS immune complexes were incubated with PP1 or PP2A in 50 mM Tris−HCl, pH 7.4, 1 mM DTT at 30°C, as indicated. The reaction was stopped by adding sample buffer and boiling, and the samples were then processed as described above.

Quantitation of Intracellular NO Generation. eNOS enzyme activity was measured as described previously, with minor modifications (*9*). COS-7 cells were incubated in HEPES buffer (25 mM HEPES, pH 7.3, 109 mM NaCl, 5.4 mM KCl, 0.9 mM $CaCl_2$, 1 mM $MgSO_4$ and 25 mM glucose) for 1 h at 37°C. BAEC were incubated in HEPES buffer for 30 min, 37°C, and then for a further 30 min in the presence of a phosphatase inhibitor or vehicle as indicated. Cells were then labeled with L-[^{3}H] arginine (10 μCi/mL) and stimulated with calcium ionophore A23187 as indicated for 10 min, 37°C, and immediately washed two times with ice-cold PBS containing 5 mM EDTA and 5 mM L-arginine. COS-7 cells were scraped into 2 mL of stop buffer (20 mM sodium acetate, pH 5.5, 1 mM L-citrulline, 2 mM EDTA, and 2 mM EGTA) while BAEC were scraped into this same buffer supplemented with 60 mM octyl-glucoside and 1% Triton X-100. The scraped cells were then sonicated. COS-7 cell lysates were analyzed immediately, and the BAEC lysates were analyzed after a 20 min incubation at 4°C. Aliquots of these lysates were withdrawn to determine total cellular ^{3}H incorporation and were also subjected to immunoblot analyses to confirm that eNOS protein abundance was equivalent under the different experimental treatments. For all the eNOS activity assays shown below, the total eNOS protein expression did not vary substantively within a given experiment. L-[^{3}H] citrulline was isolated from the remaining lysate by anion exchange chromatography with AG 50W-X8 resin (Biorad) and quantitated by liquid scintillation counting.

Statistical Analysis. Mean values for individual experiments were expressed as mean ± S.E. Statistical differences were assessed by analysis of variance followed by t-test. A p value less than 0.05 was considered statistically significant.

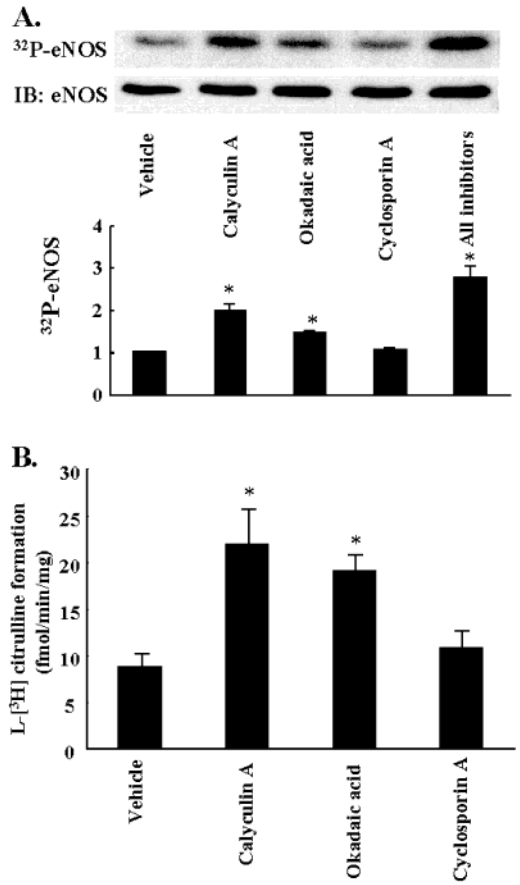

FIGURE 1: Effects of phosphatase inhibitors on [$^{32}P_i$] biosynthetic labeling and activity of eNOS. Panel A shows an autoradiograph and immunoblot of eNOS immunoprecipitated from biosynthetically labeled and protein phosphatase inhibitor-treated BAEC. BAEC were labeled with [^{32}P] orthophosphate and then treated with 100 nM calyculin A, 100 nM okadaic acid, 100 nM cyclosporin A, or a combination of all of these inhibitors for 30 min, 37°C. eNOS was immunoprecipitated, analyzed by SDS/PAGE, and autoradiography and then probed with an antibody directed against eNOS. The experiment shown is representative of three independent experiments. Also shown in Panel A is the densitometric analysis of pooled data, plotted as the fold increase in the ^{32}P-eNOS signal, relative to the signal obtained for eNOS labeling as analyzed in vehicle-treated cells. Panel B shows results of eNOS enzyme activity assays in BAEC. Cells were treated with 10 nM calyculin A, 10 nM okadaic acid, or 100 nM cyclosporin A for 30 min and then with 1 μM A23187 for 10 min, 37°C. Cellular NO synthase activity was measured as described in Experimental Procedures. Each data point represents the mean ± S.E. of five different cellular treatments performed on two separate days. * indicates $p < 0.01$ relative to vehicle treated cells.

RESULTS

Effects of Phosphatase Inhibitors on Basal eNOS Phosphorylation and Enzymatic Activity. We first used protein phosphatase inhibitors in [^{32}P] orthophosphate ([$^{32}P_i$]) biosynthetic labeling experiments to explore the roles of specific phosphatases in maintaining basal eNOS phosphorylation levels in intact endothelial cells. We treated BAEC with the phosphatase inhibitor calyculin A, which inhibits protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), or with okadaic acid, a less potent but more selective inhibitor of PP2A (*22*). As shown in Figure 1A, each of these inhibitors (100 nM) potentiate eNOS phosphorylation in [$^{32}P_i$] biosynthetically labeled BAEC. In contrast, 100 nM cyclosporin A, an inhibitor of the protein phosphatase 2B (calcineurin), has no substantive effect on overall eNOS phosphorylation in [$^{32}P_i$] biosynthetically labeled BAEC. Under similar conditions, we have previously shown that cyclosporin A inhibits VEGF-induced dephosphorylation of eNOS serine 116 while having no effect on the basal phosphorylation of this residue (*15*). These results suggest that PP2A and perhaps PP1, but not PP2B, play a key role in establishing the basal level of eNOS phosphorylation in intact endothelial cells.

We next sought to determine whether the increase in eNOS phosphorylation observed with phosphatase inhibitor treatment is correlated with a change in eNOS activity.

BAEC were preincubated with calyculin A, okadaic acid, cyclosporin A or vehicle for 30 min and then treated with calcium ionophore A23187 (1 μM) for 10 min in the presence

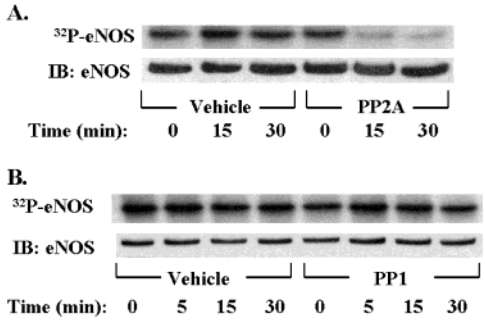

FIGURE 2: In vitro dephosphorylation of [$^{32}P_i$] biosynthetically labeled eNOS. Shown are time courses of protein phosphatase-mediated dephosphorylation of eNOS immunoprecipitated from biosynthetically labeled BAEC. BAEC were incubated with [^{32}P] orthophosphate and then treated with 100 nM calyculin A and 100 nM okadaic acid for 30 min at 37 °C. eNOS was immunoprecipitated and treated with 500 mU PP2A (A) or 500 mU PP1 (B) or vehicle for the indicated times at 30°C. The immunoprecipitated proteins were analyzed by SDS/PAGE and autoradiography and then probed with antibodies directed against eNOS. The experiments shown are representative of two independent experiments.

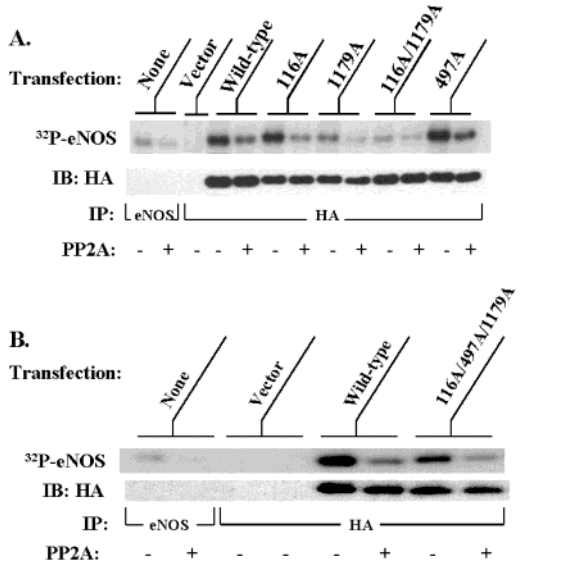

FIGURE 3: PP2A-mediated dephosphorylation of [$^{32}P_i$] biosynthetically labeled mutant eNOS. Shown are autoradiographs and immunoblots of PP2A-treatment of endogenous or transfected eNOS immunoprecipitated from [^{32}P] orthophosphate biosynthetically labeled BAEC. BAEC were transfected with the HA-tagged wild-type or mutant cDNA constructs as indicated in panels A and B, biosynthetically labeled with [$^{32}P_i$] and treated with 1 μM okadaic acid. eNOS was then immunoprecipitated with an antibody against eNOS or the HA epitope as indicated and treated with PP2A (250mU) or vehicle for 1h, 30 °C. The immunoprecipitated proteins were analyzed by SDS/PAGE and autoradiography, and then membranes were probed with an antibody directed against the HA epitope tag. The experiments shown are each representative of three independent experiments.

of L-[^{3}H] arginine. Treated cells were lysed and analyzed for NOS activity by measuring the formation of L-[^{3}H] citrulline from L-[^{3}H] arginine as described in the "Experimental Procedures" section (*9*). As shown in Figure 1B, 10 nM calyculin A or 10 nM okadaic acid markedly increases cellular eNOS activity, while 100 nM cyclosporin A has no effect. Additionally, 100 nM okadaic acid augments cellular eNOS enzymatic activity (25.3 ± 2.0 fmol L-[^{3}H] citrulline formed min^{-1} (mg protein)$^{-1}$ for 100 nM okadaic acid vs 8.7 ± 1.2 fmol L-[^{3}H] citrulline formed min^{-1} (mg protein)$^{-1}$ for vehicle; $p < 0.005$, $n = 3$ experiments).

PP2A Treatment of eNOS Immunoprecipitated from [$^{32}P_i$] Biosynthetically Labeled BAEC. Given the results using phosphatase inhibitors (Figure 1), which indicate that basal activity of PP2A and possibly PP1 as well, modulate eNOS phosphorylation and activity in BAEC, we next determined if either of these protein phosphatases might be involved in directly dephosphorylating eNOS on sites that are endogenously phosphorylated in intact endothelial cells. We treated [$^{32}P_i$] biosynthetically labeled BAEC with protein phosphatase inhibitors and immunoprecipitated eNOS. The immune complexes were treated with PP1 or PP2A and then eluted with sample buffer and analyzed by SDS−PAGE and autoradiography (Figure 2). As a positive control, we used a malachite green assay to document that the phosphatases PP1 and PP2A are each capable of dephosphorylating a model phospho-peptide substrate under conditions identical to those used in eNOS dephosphorylation experiments described above (data not shown). We found that PP2A, but not PP1, dephosphorylates eNOS isolated from [$^{32}P_i$] biosynthetically labeled BAEC (Figure 2).

Effects of PP2A on eNOS Phosphorylation-Deficient Site Mutants. In endothelial cells, eNOS is phosphorylated at serines 116 (*5*) and 1179 (*6*, *23*) as well as threonine 497 (*8*). Using the HA epitope-tagged eNOS as template for PCR, we constructed a series of phosphorylation-deficient eNOS mutants by individually changing these candidate phosphorylated residues to alanine, as described in the Experimental Procedures section. Standard molecular cloning approaches were used to combine individual eNOS phosphorylation-site mutants to create constructs deficient in phosphorylation at multiple sites. We then transfected BAEC with these various phosphorylation-deficient eNOS mutants, biosynthetically labeled the cells with [$^{32}P_i$] and immunoprecipitated the recombinant eNOS using an antibody directed against the HA epitope tag. The immune complexes were treated with PP2A, eluted with SDS sample buffer and analyzed by SDS−PAGE and autoradiography. As shown in Figure 3A, when transfected into BAEC, eNOS that is mutated at any one of the established phosphorylation sites or is mutated at both serines 116 and 1179 (116A/1179A) incorporates [$^{32}P_i$] upon biosynthetic labeling. Moreover, all of these labeled eNOS phosphoproteins are dephosphorylated by the addition of PP2A in vitro. The fact that the 116A/1179A mutant eNOS is phosphorylated in BAEC prompted us to construct and analyze an eNOS mutant with the three well-established phosphorylation sites changed to alanine (116A/497A/1179A). When transfected into BAEC, this mutant undergoes [$^{32}P_i$] biosynthetic labeling and can be almost completely dephosphorylated by in vitro PP2A treatment (Figure 3B). In these experiments, the use of the HA epitope tag permits the recombinant (mutated) eNOS construct to be selectively immunoprecipitated and thereby distinguished from the endogenous (wild-type) enzyme that is expressed in endothelial cells. Importantly, the endogenous wild-type eNOS does not co-immunoprecipitate with the HA-tagged recombinant eNOS (see Figure 4A).

Effects of Threonine 497 Phosphorylation on Serine 1179 Phosphorylation and Enzyme Activity. In endothelial cells, eNOS is constitutively phosphorylated on threonine 497 (*8*, *14*, *16*). However, as shown in Figure 3A, the 497A eNOS mutant incorporates [$^{32}P_i$] to a level comparable to that of

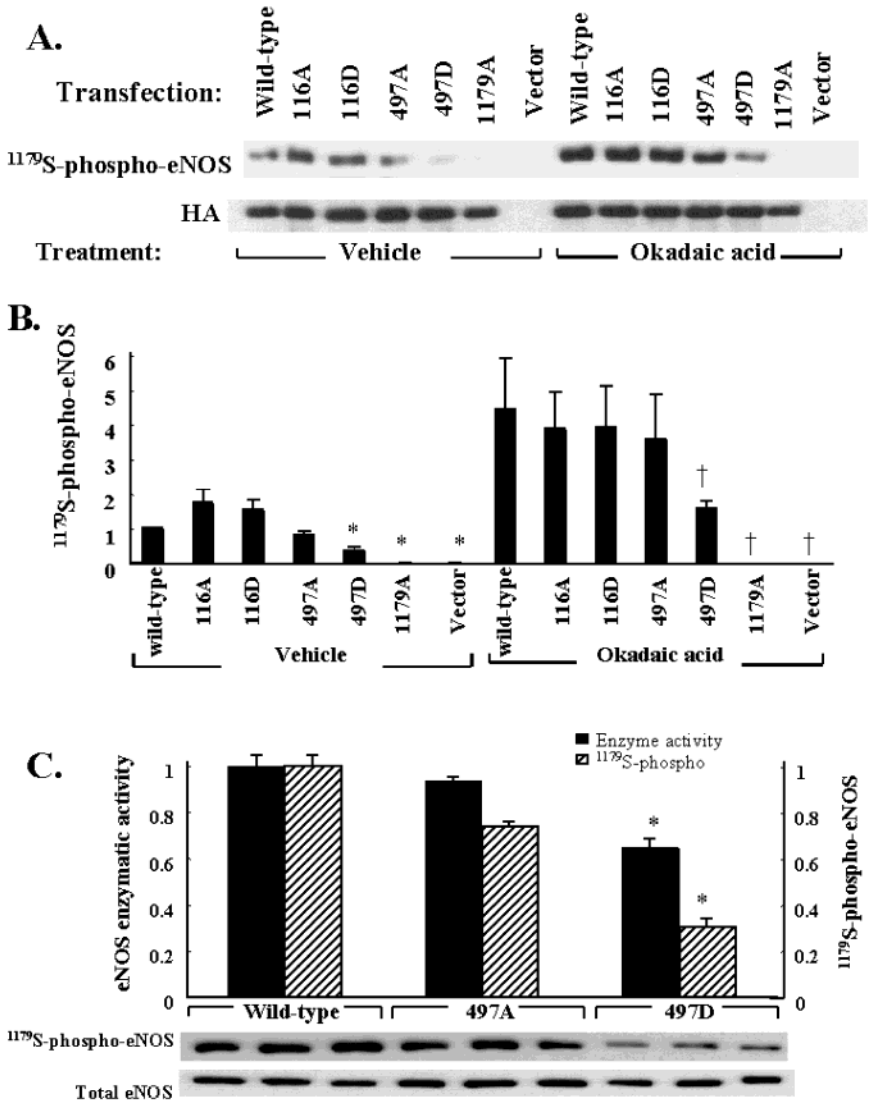

FIGURE 4: Effects of eNOS mutations on 1179 phosphorylation and enzyme activity. Panel A shows immunoblots of wild-type or mutant eNOS immunoprecipitated from transfected BAEC. BAEC were transfected with the HA-tagged wild-type or phosphorylation site mutant cDNA constructs as indicated and then treated with vehicle or 1 μM okadaic acid for 30 min. The HA-tagged proteins were immunoprecipitated with an antibody against HA and then resolved on SDS/PAGE and probed with an antibody specific for the phosphorylation of serine 1179 eNOS. Membranes were then stripped and reprobed with an anti-HA antibody. The experiments shown are representative of 3−6 independent experiments. Panel B shows the results of densitometric analysis of pooled data for serine 1179 phosphorylation of eNOS constructs transfected into BAEC and assayed by immunoprecipitation/immunoblot, plotting the fold change in serine 1179 phosphorylation relative to wild-type recombinant eNOS. * indicates $p < 0.05$ vs wild-type eNOS analyzed in vehicle-treated BAEC, and † indicates $p < 0.05$ vs wild-type eNOS analyzed in okadaic acid-treated BAEC. Panel C shows the results from eNOS activity assays as well as immunoblots analyzed in COS-7 cells transfected with wild-type, 497A or 497D mutant eNOS, as shown. Transfected cells were treated with 10 μM calcium ionophore A23187 for 10 min and NO synthase activity was measured as described in Experimental Procedures; results are normalized to the mean NOS activity determined for the wild-type eNOS (black bars), measured as 13.3 ± 1.1 fmol [^{3}H] L-citrulline formed/min/mg protein. In parallel, lysates from each plate of these same cells were resolved by SDS/PAGE and immunoblotted using an antibody against total eNOS or phosphoserine 1179 eNOS. The densitometry of these immunoblots is plotted in panel C relative to this value for wild-type eNOS (hatched bars), in each case normalizing to the total eNOS expression for each construct. Each data point represents the mean ± S.E. of three independent experiments, each performed in duplicate. * indicates $p < 0.005$ relative to wild-type eNOS transfected cells.

wild-type eNOS transfected into BAEC. These findings suggest that the stoichiometry of phosphorylation of the threonine 497 residue (or its level of phosphate turnover) may be relatively low compared to that of total eNOS phosphorylation; equally plausible is the hypothesis that the phosphorylation state of the threonine 497 residue may affect the kinetics of dephosphorylation or phosphorylation of a different eNOS phosphorylation site (e.g., serine 1179). We therefore constructed cDNAs encoding HA-tagged "phospho-null" or "phospho-mimetic" eNOS mutants in which the native threonine 497 residue is replaced by an alanine or aspartic acid, to yield constructs 497A or 497D, respectively. In a similar fashion, we also constructed 116A and 116D eNOS mutants. BAEC transfected with these cDNAs were treated with okadaic acid, and the transfected eNOS was immunoprecipitated with an antibody against the HA epitope tag. The immunoprecipitates were then resolved by SDS/PAGE and analyzed in immunoblots probed with an antibody specific for eNOS phosphorylated at the serine 1179 residue. As can be seen in Figure 4A,B, the 497D mutant shows strikingly attenuated serine 1179 phosphorylation compared to wild-type eNOS.

We next determined whether the phosphorylation of threonine 497 affects cellular eNOS enzymatic activity. COS-7 cells were transfected with 497D, 497A, or wild-type eNOS cDNA, and the cellular NO synthase activity was measured as described above. As can be seen in Figure 4C, the enzyme activity of the 497D mutant in transfected COS-7 cells is significantly less than that of recombinant wild-type eNOS at similar levels of overall enzyme expression. The NO synthase activity of COS-7 cells transfected with the 497A mutant does not differ substantively from that of cells transfected with wild-type eNOS. The phosphorylation of serine 1179 is reduced for the 497D mutant expressed in COS-7 cells (Figure 4C), as is also found for the 497D mutant expressed in BAEC (Figure 4A). Incubation of transfected cells with the NOS inhibitor L-N^{ω}-nitroarginine (*24*, *25*) produced no effect on the reduction of eNOS serine 1179 phosphorylation observed for the 497D mutant (data not shown), suggesting that differences in NOS enzyme activity do not account for the reduced serine 1179 phosphorylation characteristic of the 497D eNOS construct.

Site-Specific eNOS Dephosphorylation by PP2A. We utilized phospho-specific eNOS antibodies to explore which residues can be dephosphorylated by PP2A. eNOS was immunoprecipitated from BAEC and then treated with PP2A or PP1 (Figure 5A,B). The eluted proteins were resolved by SDS/PAGE and analyzed in an immunoblot probed with an antibody specific for eNOS phosphorylated at serine 1179. As shown in Figure 5B, PP2A promotes the dephosphorylation of eNOS phosphoserine 1179, while PP1 does not dephosphorylate eNOS at this residue. We then followed similar experimental approaches, using eNOS phosphorylation state-specific antibodies to explore the effects of PP2A on dephosphorylation of eNOS at threonine 497 or serine 116 (Figure 5C). PP2A promotes dephosphorylation of eNOS at threonine 497 and serine 1179, but not at serine 116. Additionally, treatment of BAEC with the PP2A inhibitor okadaic acid leads to a marked increase in phosphorylation of eNOS at serines 1179 and 116, as well as an increase in kinase Akt phosphorylation (Figures 6A, 6C and 6D); the calcineurin inhibitor cyclosporin A has no significant effect on eNOS serine 1179 phosphorylation (Figure 6A). We also treated endothelial cells with calyculin A, which inhibits both PP1 and PP2A, and found that this phosphatase inhibitor increases phosphorylation at both serines 116 and 1179 (Figure 6A,B,D).

VEGF promotes the robust phosphorylation of eNOS at serine 1179 (*6*, *7*), and we recently found that this agonist also induces the calcineurin-dependent dephosphorylation of eNOS at serine 116 (*15*). As shown in Figure 6B−D, the VEGF-promoted dephosphorylation of eNOS at serine 116 is not blocked by PP2A or PP1 inhibition, although basal phosphorylation at the site is markedly enhanced by okadaic acid or calyculin A treatment.

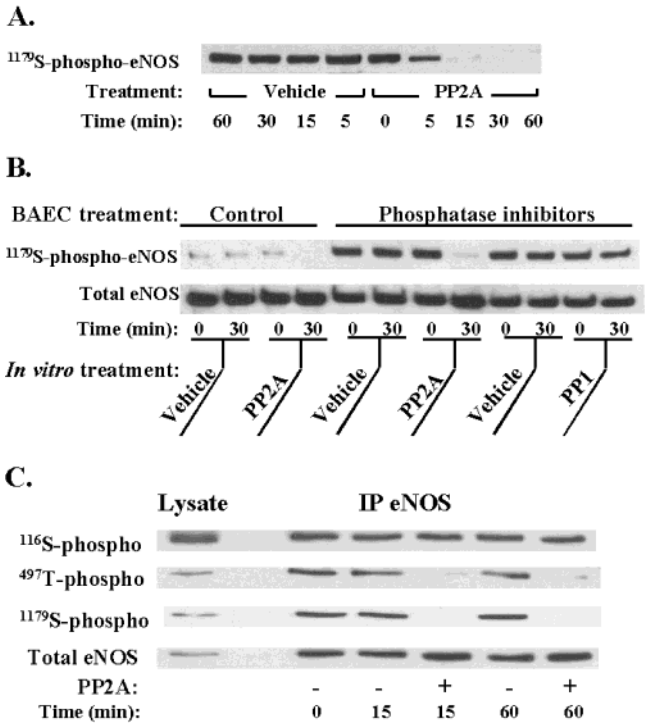

FIGURE 5: PP2A-mediated dephosphorylation of specific eNOS phospho residues. Panel A shows a time course of PP2A-mediated dephosphorylation of phosphoserine 1179 eNOS. BAEC were treated with 1 μM okadaic acid for 30 min, 37 °C, and eNOS was then immunoprecipitated. The immune complexes were treated with PP2A (400 mU) for the indicated times at 30 °C and then analyzed by SDS−PAGE and immunoblotted with an anti-phosphoserine 1179 eNOS antibody. The experiment shown is representative of three independent experiments. Panel B shows immunoblots of eNOS immunoprecipitated from BAEC incubated with or without protein phosphatase inhibitors and then treated with PP2A, PP1, or vehicle in vitro. BAEC were treated with control or 100 nM calyculin A and 100 nM okadaic acid for 30 min, 37 °C, and eNOS was immunoprecipitated. The immunoprecipitates were treated with PP2A (250 mU), PP1 (250 mU), or vehicle for 0 or 30 min at 30 °C as indicated and then resolved by SDS/PAGE and probed with an antibody to phosphoserine 1179 eNOS. Membranes were then stripped and reprobed with an anti-eNOS polyclonal antibody. The experiment shown is representative of 2−4 independent experiments. Panel C shows immunoblots of eNOS immunoprecipitated from okadaic acid-treated BAEC and then treated with PP2A or vehicle in vitro. BAEC were treated with 1 μM okadaic acid for 30 min, and eNOS was then immunoprecipitated using an anti-eNOS monoclonal antibody. The immune complexes were treated with PP2A (250 mU) or vehicle for the indicated times at 30 °C and then resolved by SDS/PAGE and probed with phosphorylation site-specific eNOS antibodies or with an anti-eNOS polyclonal antibody that detects total eNOS. As a positive control, lysates of these okadaic acid-treated BAEC are also included (first lane). The experiment shown is representative of three independent experiments.

DISCUSSION

These studies provide evidence that distinct protein phosphatase pathways differentially regulate discrete eNOS phosphorylation sites and establish a key role for PP2A in eNOS regulation. Delineating these pathways is important because the phosphorylation status of several eNOS residues has been shown to modulate eNOS enzymatic activity. Serine 1179 is the most extensively characterized phosphorylation site, and phosphorylation of this site has been shown to augment enzyme activity (*6*, *7*). We recently demonstrated that dephosphorylation of serine 116 is associated with an increase in eNOS activity (*15*), and in Figure 4 of the current manuscript, we show that phosphorylation of the threonine 497 residue is correlated with decreased cellular eNOS activity. Finally, a recent publication asserts that phosphorylation of serine 635 augments eNOS activity while phosphorylation of serine 617 increases the enzyme's sensitivity to calcium/calmodulin without altering the maxi-

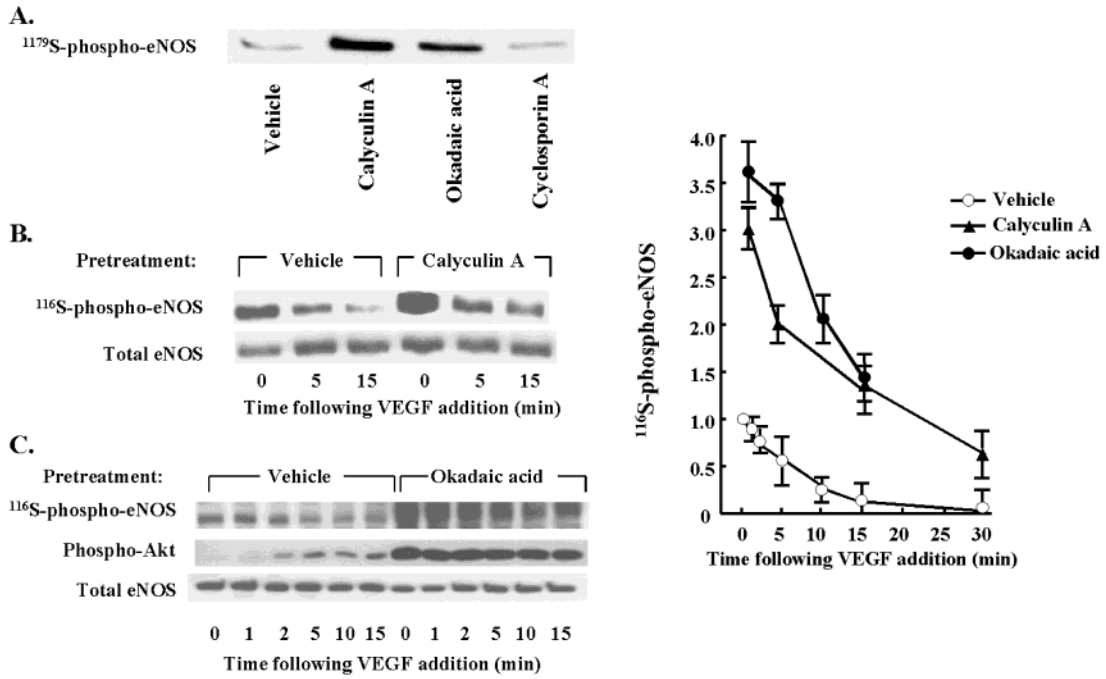

FIGURE 6: Effects of phosphatase inhibitors on phosphorylation of specific eNOS residues. Panel A shows an immunoblot of lysates from BAEC treated with individual protein phosphatase inhibitors. BAEC were treated with 100 nM calyculin A, 100 nM okadaic acid, 100 nM cyclosporin A, or vehicle for 30 min, 37 °C, and then lysed. The lysates were resolved by SDS/PAGE and probed with an antibody against eNOS phosphoserine 1179. The experiment shown is representative of three independent experiments. Panels B and C show immunoblots of lysates from BAEC treated with 100 nM calyculin A or 100 nM okadaic acid, respectively, for 30 min, 37 °C and then treated with VEGF for the indicated times. The lysates were resolved by SDS−PAGE and analyzed in an immunoblot probed with antibodies against eNOS phosphoserine 116 and Akt phosphoserine 473. Membranes were then stripped and reprobed with a monoclonal anti-eNOS antibody. The experiments shown are representative of three independent experiments. Panel D shows the results of densitometric analysis of pooled data from experiments shown in panels B and C and similar experiments, plotting the fold increase in phosphorylation of eNOS phosphoserine 116 at the indicated times, relative to this signal from the lysate of vehicle-treated cells at $t = 0$. Each data point represents the mean ± S.E. of three independent experiments. The mean phosphoserine 116 signal of the calyculin A- or okadaic acid-treated cells each differ significantly from the signal of the vehicle-treated cells ($p < 0.001$) at every time point except for 30 min.

mal activity (*29*). Hence, site-specific dephosphorylation of eNOS may represent an important mechanism modulating eNOS enzyme activity; the present studies have used several inter-related experimental approaches to explore the protein phosphatase pathways regulating eNOS dephosphorylation in endothelial cells.

We first explored the roles of protein phosphatases in modulating the phosphorylation of endogenous eNOS by studying eNOS phosphorylation in cultured endothelial cells biosynthetically labeled with [$^{32}P_i$] (Figure 1A). We found that cyclosporin A, an inhibitor of protein phosphatase calcineurin (also known as protein phosphatase 2B), has no substantive effect on overall eNOS phosphorylation in [$^{32}P_i$] biosynthetically labeled BAEC. In contrast, calyculin A (*22*), an inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), and okadaic acid (*22*), a more selective but less potent inhibitor of PP2A, each markedly potentiate eNOS phosphorylation in [$^{32}P_i$] biosynthetically labeled BAEC. Moreover, treatment of BAEC with okadaic acid or calyculin A markedly increases eNOS enzyme activity (Figure 1B). Previous studies have reported disparate effects of phosphatase inhibitors on eNOS enzyme activity (*14*, *26*), likely reflecting the fact that phosphatase inhibitors may have complex direct and indirect effects on a multisite phosphorylated protein, such as eNOS, under basal as well as agonist-stimulated conditions (Figures 5 and 6). At the least, we may infer from our data that PP2A influences the overall state of eNOS phosphorylation in endothelial cells and may importantly regulate eNOS enzyme activity.

Protein phosphatase 2A is one of the major classes of pluripotent eukaryotic protein phosphatases. PP2A is distinguished from the other major classes of phosphatases by features of its substrate specificity and by the fact that it is active in the absence of divalent cations (*27*, *28*). In contrast to the striking sequence selectivity characteristic of most protein kinases, PP2A and the other major eukaryotic phosphatases are more promiscuous in their choices of phosphoprotein substrates. However, we found that PP1 is completely unable to promote the dephosphorylation of [$^{32}P_i$] biosynthetically labeled eNOS in an in vitro assay in which the purified phosphatase was incubated with immunoprecipitated eNOS (Figure 2). In contrast, PP2A markedly dephosphorylates [$^{32}P_i$] biosynthetically labeled immunopurified eNOS. Using a series of phosphorylation state-specific eNOS antibodies, we found that PP2A rapidly and robustly dephosphorylates eNOS phosphorylated on residues serine 1179 and threonine 497 but not on serine 116 (Figure 5). This robust dephosphorylation of threonine 497 differs from the results of Mitchell et al., who reported a modest, if any, effect of PP2A treatment on threonine 497 phosphorylation (*14*). These disparate findings may be explained by differences in the methods of preparing the phospho-eNOS substrate. We utilized native eNOS phosphorylated in intact endothelial cells while Michell et al. used recombinant eNOS expressed in *E. coli* and phosphorylated in vitro with protein kinase C (*14*). As shown in Figure 4 and discussed below, our results suggest that the phosphorylation status of specific sites on eNOS may affect the susceptibility of other eNOS

phospho-residues to dephosphorylation and/or phosphorylation. Indeed, the relative phosphorylation levels of individual residues of eNOS isolated from intact cells is likely to differ greatly from that of in vitro phosphorylated eNOS and hence may explain the disparate results of these distinct approaches.

To further explore the role of PP2A in dephosphorylation of eNOS, we constructed and analyzed a series of eNOS phosphorylation mutants in which serine 1179, threonine 497, and serine 116 were individually or collectively modified. These constructs were tagged with the HA epitope to permit the immunoprecipitation and analysis of phosphorylation patterns of the recombinant proteins expressed in native endothelial cells, thereby avoiding the confounding effects of cell type-specific phosphorylation pathways. As may be seen in Figure 4, when the HA-tagged 1179A eNOS mutant (in which serine 1179 is changed to alanine) is expressed in endothelial cells and immunoprecipitated with the HA antibody, an immunoblot probed with the phospho-specific eNOS phosphoserine 1179 antibody shows no signal whatsoever. Thus, despite the fact that eNOS is a homodimeric protein, the recombinant and endogenous eNOS proteins do not co-immunoprecipitate, a finding that may simply reflect dissociation of any heterodimers during the immunoprecipitation process, or may be peculiar to HA-tagged recombinant eNOS. We favor another interpretation, in which the assembly of stable eNOS dimers is effectively a co-translational process, wherein local formation of the eNOS holoenzyme is closely coupled to protein translation and, furthermore, that free pools of monomeric eNOS do not recombine with one another or with eNOS dimers within the cell. Regardless of the explanation, this phenomenon allows one to interpret more rigorously the observation that the eNOS phosphorylation mutant in which the three well-established phosphorylation sites are inactivated (116A/497A/1179A) still undergoes labeling in [$^{32}P_i$] biosynthetically labeled BAEC and can be dephosphorylated by PP2A (Figure 3B). Since recombinant eNOS phosphorylation mutants do not co-immunoprecipitate with endogenous eNOS, we conclude that there are additional eNOS phosphorylation sites that are likely to be regulated by PP2A. Of note, a recent publication implicates eNOS serines 617 and 635 as additional eNOS phosphorylation sites *(29)*, and hence, we suggest that PP2A may dephosphorylate serine 617, serine 635 and/or another yet unidentified phosphorylated residue(s) in eNOS.

In addition to eNOS, PP2A has widespread effects on many other signaling molecules in endothelial cells (*30*, *31*). The regulation of PP2A itself, however, remains elusive. In a few cell types, the catalytic subunit of PP2A has been shown to undergo agonist-induced tyrosine phosphorylation, associated with a reduction in PP2A activity (*32*−*35*). The regulation of PP2A in endothelium is less well understood. Pretreatment of endothelial cells with okadaic acid has been shown to inhibit PMA-induced dephosphorylation of serine 1179 eNOS (*14*). Interpretation of this observation is difficult, however, because treatment of endothelial cells with okadaic acid alone robustly increases serine 1179 phosphorylation (Figure 6). Indeed, changes in eNOS phosphorylation seen in phosphatase inhibitor-treated cells do not necessarily indicate that the inhibited phosphatase directly dephosphorylates eNOS in the basal state. This point is reinforced by our observation that okadaic acid increases the level of basal phosphorylation at serine 116 in intact endothelial cells (Figure 6), while PP2A in vitro dephosphorylates eNOS phosphothreonine 497 and phosphoserine 1179, but not phosphoserine 116 (Figure 5). Thus, there are important differences between the cellular consequences of phosphatase inhibitors and the in vitro effects of purified phosphatases. In addition, okadaic acid or calyculin A treatment fails to block VEGF-induced dephosphorylation at this residue (Figure 6), a response that appears to be calcineurin-mediated (*15*). The pathways that control PP2A activity in endothelium are incompletely understood, and further exploration in this area is likely to provide additional insight into the mechanisms whereby PP2A regulates eNOS at the posttranslational as well as at the transcriptional (*36*, *37*) level in the intact cell.

In some multisite phosphorylated proteins, phosphorylation at one site can influence the phosphorylation or dephosphorylation of a nearby site, allowing for a complex pattern of enzyme regulation by kinase and phosphatase pathways (*38*). Our studies have extended this paradigm to the analysis of eNOS. We found that mutation of the threonine 497 residue to aspartate (to mimic phosphorylation) inhibits the basal phosphorylation of the 1179 residue of eNOS as well as the cellular activity of the enzyme (Figure 4), which is in agreement with the decreased in vitro enzyme activity recently demonstrated for this purified mutant (*29*). For some eNOS agonists, it appears that the effects on the phosphorylation status of threonine 497 and serine 1179 may be reciprocally linked (*8*, *12*, *16*). Hydrogen peroxide, for instance, promotes dephosphorylation of threonine 497 and phosphorylation of serine 1179 (*12*). Viewed in the context of our findings, we suggest that the phosphorylation of serine 1179 may be directly affected by the state of phosphorylation of threonine 497, and we speculate further that these residues may be in proximity at the level of eNOS tertiary structure despite their distance by primary structure.

Taken together, our results identify PP2A as a key determinant of eNOS phosphorylation and enzyme activity in cultured endothelial cells. These findings provide evidence of dynamic interactions between the different known eNOS phosphorylation sites and suggest that eNOS may contain additional PP2A-sensitive phosphorylation sites. The interactions of eNOS with PP2A-modulated signaling pathways are likely to be precisely controlled in vivo and may modulate nitric oxide bioavailability in the vasculature under basal as well as agonist-stimulated conditions. Consequently, PP2A is likely to play an important role in maintaining vascular homeostasis, and dysregulation of this protein phosphatase may contribute to the endothelial dysfunction characteristic of pathologic vascular processes, such as atherosclerosis.

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