Src Kinase Activates Endothelial Nitric-oxide Synthase by Phosphorylating Tyr-83*  

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The endothelial nitric-oxide synthase (eNOS), which catalyzes the conversion of L-arginine to L-citrulline and nitric oxide (NO), is post-translationally regulated by diverse protein-protein interactions and by covalent modification with fatty acylation and phosphorylation. eNOS, like the other two NOS isoforms termed neuronal NOS and inducible NOS, is a homodimer, with each of the two subunits having a bidomain structure consisting of an N-terminal oxygenase domain containing a heme moiety and binding sites for FAD, FMN, and NADPH (1). Located between the oxygenase and reductase domains is a Ca2+-calmodulin (CaM) binding sequence (2). Five serine/threonine sites of phosphorylation of bovine eNOS have been identified at Ser-116, Thr-497, Ser-617, Ser-635, and Ser-1179 (3–10). eNOS phosphorylation at Ser-1179 increases eNOS activity by increasing the rate of electron flux through the reductase domain (11). Based on mutagenesis experiments, phosphorylation at Ser-635 increases the maximal activity of eNOS by an as yet unknown mechanism, whereas phosphorylation at Ser-617 does not alter maximal activity but significantly increases the Ca2+-CaM sensitivity of the enzyme (8). In contrast, eNOS phosphorylation at Thr-497 within the CaM binding sequence reduces eNOS catalytic activity by decreasing the binding affinity for Ca2+-CaM (7). The function of eNOS phosphorylation at Ser-116 is currently not well understood (12).

Evidence exists that eNOS may also be regulated by tyrosine phosphorylation. García-Cardeña et al. (13) have reported that treatment of endothelial cells with the tyrosine phosphatase inhibitor, sodium orthovanadate, or the oxidant, hydrogen peroxide (H2O2), stimulates direct tyrosine phosphorylation of eNOS (13). Using a human breast cancer cell line Takenouchi et al. (14) found that expression of active forms of Src kinase increased eNOS phosphorylation. Earlier it was reported that treatment of endothelial cells with tyrosine kinase inhibitors attenuated NO production in response to shear stress (15, 16). Tyrosine phosphatase inhibitors have also been reported to activate eNOS in a Ca2+-independent manner (17). Given the possibility that oxidative stress may occur in vivo under certain pathophysiological conditions including those of inflammation and ischemia-reperfusion injury, we sought to identify the tyrosine kinase(s) involved and the site(s) of eNOS tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—Anti-eNOS antibody (#610297) and anti-phosphoryrosine antibody (#610000) were obtained form BD Biosciences. Anti-phospho-Thr-497 eNOS antibody (#05-811), anti-phospho-Ser-617 eNOS antibody (#07-561), anti-phospho-Ser-635 antibody (#07-562), anti-phospho-Ser-1179 eNOS antibody (#07-428), anti-Src antibody (#05-184), and normal mouse IgG (#12-371) were purchased from Upstate Biotechnology Inc. Mouse IgG TrueBlot™ horseradish peroxidase-conjugated secondary antibody was obtained from eBioscience (18-8877). Anti-V5 antibody (#R960-25) came from Invitrogen. Bovine aortic endothelial cells (BAECs) were purchased from VEC Technologies and used for experiments in passages 2–6.

Immunoprecipitation and Immunoblotting—BAECs or COS-7 cells were lysed in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaF, 15 mM Na3P2O7, 1 mM Na3VO4, 1% v/v Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, and 5 μg/ml aprotonin. Lysates were centrifuged at 10,000 × g to remove insoluble material and were precleared by incubation with protein A/G-agarose for 2 h at 4 °C with rocking. Agarose beads were then pelleted by centrifugation at 1000 × g. eNOS in precleared lysates was immunoprecipitated by incubation overnight at 4 °C with rocking after the addition of either anti-eNOS antibody (10 μl) or anti-V5 antibody (3 μl). Immunop-
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precipitated protein was eluted from the beads by boiling for 5 min in SDS sample buffer. Immunoprecipitates or cell lysates were immuno-

blotted with anti-phosphotyrosine antibody (1:1000 dilution), anti-

eNOS antibody (1:5000 dilution), anti-V5 antibody (1:10,000 dilution), anti-Src antibody (1:1000 dilution), anti-phospho-Thr-497 eNOS antibody (1:1000 dilution), anti-phospho-Ser-617 eNOS antibody (1:1000 dilution), anti-phospho-Ser-635 antibody (1:1000 dilution), or anti-

phospho-Ser-1179 eNOS antibody (1:1000 dilution).

In Vitro Phosphorylation—Recombinant wild-type bovine eNOS was expressed in Escherichia coli and purified as described previously (11). Purified eNOS (1 μg) was incubated with 200 ng of active Src (Upstate Biotechnology Inc.) for 20 min at 30°C in buffer containing 20 mM HEPES, pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, and 1 mM dithiothreitol with or without 100 μM ATP. The reaction was terminated by the addition of SDS sample buffer, and tyrosine phosphorylation was analyzed by immunoblottting with anti-phosphotyrosine antibody. Peptide substrates eNOS (73–92), VKNWELGSITDTLCAQSOQ and eNOS (73–92) VKNWELSIITDTLCAQSOQ, Gensecript Corp.) were phosphorylated in vitro by recombinant Src for 30 min at 30°C using 200 μM [γ-³²P]ATP (45 cpm/pmol). Phosphorylated peptide was separated from [γ-³²P]ATP by ion exchange chromatography. In brief, the kinase assay was terminated with 30% acetic acid, and the reaction mixture was applied to polypropylene columns containing 2 ml of anion exchange resin (AG1-X8 resin, Bio-Rad). The phosphopeptides were eluted with 30% acetic and quantified by scintillation counting of Cerenkov radiation (18).

Phosphorylation in Src-transduced Endothelial Cells—A replication-
deficient adenovirus encoding an active form of Src kinase (v-Src, Gen-

Bank™/EBI Data Bank accession number BC011566) and a dominant negative Src (K296R/Y528F) were generated using the pAdEasy system developed by He et al. (19). Confluent BAECs were transduced with adenoviruses expressing green fluorescent protein (GFP) only (negative control) or expressing GFP plus v-Src at a multiplicity of infection of 100. After 24 h cells were lysed in ice-cold lysis buffer, and eNOS was partially purified by affinity binding to 2',5'-ADP-Sepharose and immuno-

blotted with anti-phosphotyrosine antibody.

Phosphorylation in Transfected COS-7 Cells—COS-7 cells were plated in 60-mm dishes and transfected with various forms of eNOS with or without v-Src cotransfection using Lipofectamine 2000 according to the manufacturer’s instructions. Varying concentrations of the empty expression vector, pcDNA3, were used to balance the amount of DNA used per transfection. After overnight transfection, cells were lysed, and eNOS was affinity-purified with 2',5'-ADP-Sepharose and immuno-

blotted with anti-phosphotyrosine antibody.

Domain Mapping of Tyrosine Phosphorylation Sites in eNOS—Fragment-

mens of bovine eNOS containing potential tyrosine phosphorylation sites were generated by PCR amplification. eNOS (1–616) was generated using the following primers: sense, 5'-GCGGCCGCAGGCGGCAGCAACAGCGTCTG-3' and antisense, 5'-GAATTCCTGGCATCACCAACACTCTGCAGC-3'. Primers for the 200–300 fragment were sense, 5'-GCGGCCGCCCAGGACCTGCAGTC-3' and antisense, 5'-GAATTCCTCTGTCGTTGGGGGACTCAC-3' and antisense, 5'-GAATTCCTGGCATCACCAACACTCTGCAGC-3'. Primers for the 500–616 fragment were sense, 5'-GCGGCCGCGTGGAGATCAACCTGGCGGAAC-3' and antisense, 5'-GAATTCCTGGCATCACCAACACTCTGCAGC-3'. Primers for the 750–900 fragment were sense, 5'-GCGGCCGCGTGGAGATCAACCTGGCGGAAC-3' and antisense, 5'-GAATTCCTGGCATCACCAACACTCTGCAGC-3'. All sequences of bovine eNOS control constructs were confirmed by automated DNA sequencing in the Genomics Core Facility of the Medical College of Georgia.

Nitric Oxide Release—COS-7 cells were transiently transfected with cDNAs encoding wild-type and mutant (183F, T1367F, Y156F, and S1179A) eNOS constructs with or without transfection with dominant negative c-Src or active v-Src, and NO release was determined as described previously (9). In brief, cells were incubated in serum-free medium for 45 min and then either not stimulated or stimulated with thapsigargin (100 μM), H₂O₂ (10 mM), or pervanadate (200 μM) for 25 min. Basal and stimulated NO release was determined via NO-specific chemiluminescence using a Sievers 280i NO analyzer (Inonics Instru-

ments, Boulder, CO). Confluent BAECs were transduced with adenovi-

ruses encoding red fluorescent protein (RFP) (control), v-Src, and dominant negative (DN) Src at a multiplicity of infection of 100. Twenty-

four hours later cells were analyzed for basal and stimulated NO release as described above.

Measurement of eNOS Enzymatic Activity—Purified eNOS (1 μg) was phosphorylated by purified Src as described above or was subjected to identical treatment with heat-denatured Src and then assayed for eNOS activity by monitoring the rate of formation of L-[14C]-citrulline from L-[¹⁴C]arginine (100 μM) in the presence of excess cofactors including Ca²⁺ (2 mM), calmodulin (200 units), NADPH (1 mM), and tetrahydrobiopterin (40 μM). Product was sepa-

rated from substrate on Bio-Rad AG 50W-X8 cation exchange col-

umn.

Ca²⁺ Measurements Using Fluo-3—COS-7 cells transfected with either active Src or empty vector (pcDNA3) were incubated with a load-

ing solution consisting of physiological saline (120 mM NaCl, 4.75 mM KCl, 1.44 mM MgSO₄, 11 mM glucose, 25 mM HEPES, 1.5 mM Ca²⁺, pH 7.3) supplemented with 2 μM Fluo-3/AM, 0.02% pluronic F-127, and 1 mg/ml bovine serum albumin for 1 h, then incubated in the loading solution without Fluo-3 for 30 min to allow de-esterification of the probe. Loading solution was then replaced with standard physiological
saline, and cells were placed in a Fluorimeter (Fluostar) and subjected to the appropriate treatment protocols. Fluorescence intensity ($F$) was converted into intracellular Ca$^{2+}$ by using the equation

$$[Ca^{2+}] \text{(nM)} = K_d \left( \frac{F - F_{\text{min}}}{F_{\text{max}} - F} \right) \text{(Eq. 1)}$$

where $F_{\text{max}}$ was the fluorescence intensity in the presence of 0.1% Triton X-100 and 5 mM CaCl$_2$, $F_{\text{min}}$ was the fluorescence intensity in the presence of 0.1% Triton X-100 and 1 mM EGTA, and $K_d$ was the dissociation constant for Fluo-3 (390 nM).

RESULTS

Garçia-Cardeña et al. (13) have shown previously that eNOS in BAECs is tyrosine-phosphorylated in response to H$_2$O$_2$-induced oxidant stress (13). To determine whether oxidant stress might stimulate eNOS phosphorylation by a member of the Src family of tyrosine kinases, we treated BAECs for 10 min with H$_2$O$_2$ (10 mM) or pervanadate (PV, 200 mM) after either no pretreatment or pretreatment for 30 min with PP2 (1 μM). Cells were lysed, lysates were immunoprecipitated (IP) with anti-eNOS antibody, and immunoprecipitates were immunoblotted (IB) with either anti-phosphotyrosine antibody (A) or anti-eNOS antibody (B). Similar results were obtained in at least three experiments.

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FIGURE 3. Tyrosine phosphorylation of eNOS under different in vitro conditions. A, purified eNOS was incubated with purified c-Src in the presence and absence of MgATP for 20 min at 30 °C and then immunoblotted (IB) with either anti-phosphotyrosine (PY) or anti-eNOS antibody. B, COS-7 cells were transfected with eNOS and increasing amounts of v-Src. eNOS was partially purified by affinity binding to 2′,5′-ADP-Sepharose and immunoblotted with anti-phosphotyrosine and anti-eNOS antibodies. C, BAECs were infected overnight with adenoviruses encoding either GFP only or encoding both GFP and v-Src. eNOS was then partially purified by affinity binding to 2′,5′-ADP-Sepharose and immunoblotted with either anti-phosphotyrosine antibody or anti-eNOS antibody. Results were similar in three separate experiments.

FIGURE 4. Tyrosine phosphorylation of wild-type, 1–616, and Δ76–615 forms of eNOS in eNOS-transfected COS-7 cells treated with PV or cotransfected with v-Src. A, schematic diagram of three different forms of eNOS used for transfection. B, cells were transfected overnight with wild-type, 1–616, and Δ76–615 forms of eNOS and subjected to either no further treatment (control), stimulated with PV (200 μM for 10 min), or cotransfected with v-Src. Cels were lysed, and eNOS was immunoprecipitated (IP) with either anti-eNOS or anti-V5 antibodies and immunoblotted (IB) with anti-phosphotyrosine (PY) antibody. C, immunoblotting of samples was also performed with the same antibody used for the immunoprecipitation. Results are representative of three experiments.

We next examined the tyrosine phosphorylation of eNOS in COS-7 cells that were transfected with either eNOS alone and then treated with PV or that were cotransfected with eNOS and v-Src. As shown in Fig. 3C, v-Src expression stimulated the tyrosine phosphorylation of eNOS without affecting the relative levels of eNOS protein expression.

We next examined the tyrosine phosphorylation of eNOS in COS-7 cells that were transfected with either eNOS alone and then treated with PV or that were cotransfected with eNOS and v-Src. In addition, we sought to determine whether Src-mediated phosphorylation might be restricted to either the oxygenase domain or reductase domain of the eNOS protein. Plasmid constructs were prepared that expressed full-length wild-type bovine eNOS (residues 1–1205), a V5 epitope-tagged eNOS oxygenase domain protein (1–616), and an eNOS reductase domain deletional mutant in which residues 76–615 were deleted (Δ76–615) (Fig. 4A). The eNOS (1–616) oxygenase domain was expressed with a V5 epitope tag to allow for immunoprecipitation of this protein with anti-V5 antibody. The wild-type and eNOS (Δ76–615) proteins, on the other hand, did not require an epitope tag because they could be immunoprecipitated by the anti-eNOS antibody that recognizes a C-terminal epitope in eNOS. All three eNOS proteins were designed to retain residues 1–75 of eNOS. This sequence does not contain any tyrosine residues (21) but does contain the Gly-2, Cys-15, and Cys-26 residues that are required for myristoylation and palmitoylation, respectively. These residues were included to ensure proper subcellular targeting of the enzyme to the plasmalemmal and Golgi membranes of transfected cells. Cells were transfected overnight with the wild-type, 1–616, and Δ76–615 forms of eNOS and subjected to either no further treatment (control), stimulated with PV (200 μM for 10 min), or...
cotransfected with v-Src. Cells were lysed, eNOS was immunoprecipitated with either anti-eNOS or anti-V5 antibodies, and immunoprecipitated proteins were immunoblotted with anti-phosphotyrosine antibody. As shown in Fig. 4B, both PV and v-Src cotransfection induced the tyrosine phosphorylation of wild-type eNOS and 1–616 eNOS. No tyrosine phosphorylation was observed for Δ76–615 eNOS, suggesting that all of the PV-stimulated and Src phosphorylation sites are located in the eNOS oxygenase domain between amino acids 76 and 615. Immunoblotting with the same antibody used for the immunoprecipitation phosphorilation of eNOS was observed for Δ76–615 eNOS, suggesting that all of the PV-stimulated and Src phosphorylation sites are located in the eNOS oxygenase domain between amino acids 76 and 615. Immunoblotting with the same antibody used for the immunoprecipitation

FIGURE 5. Tyrosine phosphorylation of wild-type, Δ201–615, Δ76–199/Δ301–615, Δ76–299/Δ401–615, Δ76–399/Δ501–615, and Δ76–499 forms of eNOS in eNOS-transfected COS-7 cells cotransfected with v-Src. A, schematic diagram of the various forms of eNOS used for transfection. B, COS-7 cells were transfected either with wild-type eNOS alone or were cotransfected with wild-type and various deletion mutants of eNOS together with v-Src. Cells were lysed, and the various forms of eNOS were immunoprecipitated (IP). Immunoprecipitated proteins were immunoblotted (IB) with anti-phosphotyrosine (PY) antibody. C, immunoprecipitated proteins were also blotted with anti-eNOS antibody. Similar results were obtained in three experiments.

FIGURE 6. Tyrosine phosphorylation of wild-type and site-directed mutants of full-length eNOS in eNOS-transfected COS-7 cells treated with PV or cotransfected with v-Src and phosphorylation of eNOS peptides by purified active Src. A, cells were transfected overnight with wild-type, Y83F, Y136F/Y7F, and Y165F forms of eNOS and either subjected to no further treatment (wild-type control) or stimulated with PV (200 μM for 10 min). Cells were lysed, eNOS was immunoprecipitated (IP), and immunoprecipitated proteins were immunoblotted (IB) with anti-phosphotyrosine (PY) antibody. B, COS-7 cells were transfected overnight with wild-type, Y83F, Y136F/Y7F, and Y165F forms of eNOS with or without cotransfection with v-Src. Cells were lysed, eNOS was immunoprecipitated (IP), and immunoprecipitated proteins were immunoblotted (IB) with anti-phosphotyrosine antibody. C and D, anti-eNOS immunoprecipitates were also immunoblotted with anti-eNOS antibody. Similar results were obtained in three experiments. E, WT and mutant peptides containing Tyr-83 or Y83F were phosphorylated in vitro by recombinant Src, and incorporated radioactivity was quantified by Cerenkov counting (means ± S.E., n = 4; *, p < 0.05).
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FIGURE 7. Effects of site-directed mutagenesis of eNOS and the Src kinase inhibitor, PP2, on basal and thapsigargin-stimulated NO release from eNOS-transfected COS-7 cells. COS-7 cells were transfected overnight with wild-type, Y83F, Y136/7F, and Y165F forms of eNOS, switched to serum-free medium for 45 min, and then either stimulated or not stimulated with thapsigargin (100 nM for 25 min). A, basal and thapsigargin-stimulated NO release during 25 min was estimated based on nitrite accumulation using a chemiluminescence NO analyzer (means ± S.E., n = 5). Relative levels of eNOS expression were estimated by immunoblotting (IB) of equal quantities of cell lysate protein from each condition. B, thapsigargin-stimulated NO release from COS-7 cells in the presence and absence of PP2 (1 μM) (means ± S.E., n = 4; * p < 0.05).

showed that equivalent amounts of eNOS were immunoprecipitated in each of the three conditions (Fig. 4C).

To more narrowly localize the subdomain within the eNOS oxygenase domain that contains the site (or sites) of Src-mediated tyrosine phosphorylation, we carried out the following deletional analysis. The Δ76–615 DNA construct was used as a backbone in which to subclone various fragments of the cDNA encoding bovine eNOS containing amino acids 75–200, 200–300, 300–400, 400–500, and 500–616 of the bovine eNOS oxygenase domain. These constructs are illustrated in Fig. 5A and are termed Δ201–615, Δ76–199/Δ301–615, Δ76–299/Δ401–615, Δ76–399/Δ501–615, and Δ76–499, respectively. These plasmids or the full-length wild-type eNOS-expressing plasmid together with the v-Src-expressing plasmid were used to cotransfect COS-7 cells overnight. Cells were also transfected with the wild-type eNOS plasmid alone as a negative control. After transfection, cells were lysed, and eNOS was immunoprecipitated with anti-eNOS antibody. Precipitated proteins were immunoblotted with anti-phosphotyrosine antibody. As shown in Fig. 5B, v-Src cotransfection stimulated the tyrosine phosphorylation of only wild-type eNOS and the Δ201–615 eNOS protein, which when considered together with the data presented in Fig. 4 indicates that the site (or sites) of Src-mediated tyrosine phosphorylation is located exclusively within the 75–200 sequence of bovine eNOS. Anti-eNOS immunoprecipitates were also immunoblotted with anti-eNOS antibody to confirm that equal quantities of the various eNOS proteins were immunoprecipitated in each condition (Fig. 5C).

The bovine eNOS sequence contains four tyrosines between amino acids 75 and 200 located at positions 83, 136, 137, and 165. To determine which site(s) among these four is phosphorylated by Src protein tyrosine kinase, we used site-directed mutagenesis to prepare mutant forms of full-length eNOS in which the various tyrosine residues were changed to phenylalanine residues. COS-7 cells were then transfected with wild-type, Y83F, Y136/7F, or Y165F forms of eNOS before stimulation of cells with PV (200 μM for 10 min). Cells were lysed, eNOS was immunoprecipitated, and immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody. As shown in Fig. 6A, PV stimulated equivalent levels of tyrosine phosphorylation of wild-type, Y136/7F, and Y165F forms of eNOS but did not stimulate the tyrosine phosphorylation of Y83F. Experiments were also carried out in which COS-7 cells were transfected with either wild-type eNOS only or were cotransfected with wild-type, Y83F, Y136/7F, and Y165F forms of eNOS together with v-Src. Consistent with the results of the experiments with PV stimulation, Src transfection induced the tyrosine phosphorylation of the wild-type, Y136/7F, and Y165F forms of eNOS but did not induce phosphorylation of the Y83F form of eNOS (Fig. 6B). Immunoblotting of anti-eNOS immunoprecipitates with anti-eNOS antibody confirmed that the differences observed in both types of experiments were not due to differences in total amounts of eNOS protein in the various conditions (Fig. 6, C and D). Thus far we have been unable to detect either the dephosphorylated or phosphorylated form of the eNOS tryptic peptide containing Tyr-83 by mass spectrometry, which has prevented us from using mass spectrometry to monitor Tyr-83 phosphorylation. We, therefore, tested whether Src can indeed phosphorylate Tyr-83 in in vitro kinase assays using recombinant Src and peptides containing Tyr-83 plus flanking sequence (wild-type bovine eNOS (73–92) or a corresponding Y83F mutant sequence. As shown in Fig. 6E, Src efficiently phosphorylated only the wild-type peptide with a stoichiometry approaching 0.5 mol of phosphate/mol of peptide after incubation for 30 min at 30 °C.

To assess whether phosphorylation of tyrosine 83 in eNOS might influence eNOS enzymatic activity and subsequent NO release, we measured basal and thapsigargin-stimulated NO release from COS-7 cells transfected with the wild-type, Y83F, Y136/7F, and Y165F forms of eNOS. Cells were transfected with eNOS overnight and then switched to serum-free medium for 45 min. Relative levels of basal and thapsigargin-stimulated NO release were measured by chemiluminescence NO analyzer (means ± S.E., n = 5; * p < 0.05).

FIGURE 8. The effects of PP2 on thapsigargin-stimulated NO release from eNOS-transfected COS-7 cells. COS-7 cells were transfected overnight with wild-type, Y83F, Y136/7F, and Y165F forms of eNOS, switched to serum-free medium for 45 min, and then either stimulated or not stimulated with thapsigargin (100 nM for 25 min). A and B, basal and thapsigargin-stimulated NO release during 25 min was estimated based on nitrite accumulation using a chemiluminescence NO analyzer (means ± S.E., n = 5). Relative levels of eNOS expression were estimated by immunoblotting (IB) of equal quantities of cell lysate protein from each condition. C, thapsigargin-stimulated NO release from COS-7 cells in the presence and absence of PP2 (1 μM) (means ± S.E., n = 4; * p < 0.05).
at Thr-497, Ser-617, Ser-635, and Ser-1179 were also examined by immunoblotting of anti-eNOS immunoprecipitates with phospho-specific antibodies that recognize eNOS only when it is phosphorylated at these particular sites. Tyrosine phosphorylation was examined in parallel using the anti-phosphotyrosine antibody. The results of this analysis showed that the Thr-497, Ser-617, Ser-635, and Ser-1179 sites of eNOS were phosphorylated under basal conditions in eNOS-transfected COS-7 cells and that the extent of phosphorylation at these sites was unaffected by cotransfection with either DN Src or v-Src (Fig. 8A). Tyrosine phosphorylation of eNOS, by comparison, was only observed in the case of v-Src cotransfection (Fig. 8A). Basal NO release from cells was increased by at least 3-fold (Fig. 8B). NO release stimulated by thapsigargin (100 nM) was also investigated again.
in COS-7 cells transfected with eNOS either without or with cotransfection with DN Src or active v-Src. As shown in Fig. 8C, thapsigargin-stimulated NO release was markedly reduced in cells cotransfected with DN Src. Interestingly, whereas basal NO release was much greater in v-Src-cotransfected cells, stimulation of these cells with thapsigargin resulted in no greater NO synthesis than in thapsigargin-stimulated cells transfected with eNOS alone. To further address the contribution of Tyr-83 to v-Src-stimulated NO release, we transfected COS-7 cells with wild-type (WT) and Y83F eNOS with or without v-Src and measured basal NO as described above. Mutation of Tyr-83 significantly inhibited the ability of v-Src to stimulate basal NO release (Fig. 8D). The relative contribution of Tyr-83 and Ser-1179 to hydrogen peroxide-, pervanadate-, and Src-stimulated NO release was determined as shown in Fig. 8E. Hydrogen peroxide-stimulated NO release was significantly reduced in cells expressing the Y83F eNOS mutant compared with WT and the S1179A mutant. The ability of pervanadate to stimulate NO release was also reduced in cells expressing the Y83F mutant compared with WT. However, pervanadate-stimulated NO release was reduced to an even greater extent in cells expressing the S1179A eNOS mutant. The ability of hydrogen peroxide to stimulate NO release was also reduced in cells expressing the S1179A mutant but to a lesser extent than in cells expressing the Tyr-83 mutant. There was no difference in the level of eNOS expression (Fig. 8E, lower panel). Basal NO release was greatly suppressed in the S1179A mutant, and co-expression of v-Src did not activate eNOS to the level of the WT enzyme (data not shown).

To evaluate the effect of Src on NO release from endothelial cells, BAECs were transduced with control adenovirus (RFP), active v-Src, or dominant negative (K296R/Y528F) Src. As shown in Fig. 9A, active Src stimulated robust basal NO release compared with the control or dominant negative Src-treated cells without affecting eNOS protein levels. In thapsigargin-treated cells, v-Src increased NO release consistent with the data obtained in COS cells, whereas the dominant negative Src significantly reduced NO release (Fig. 9B). Interestingly, vascular endothelial growth factor-stimulated NO release was greatly potentiated in the presence of v-Src and also inhibited by the dominant negative (Fig. 9C).

To address the possibility that v-Src influences calcium homeostasis, we measured the levels of intracellular calcium in the presence and
Phosphorylation of eNOS at tyrosine residues has been much more difficult to demonstrate or detect both in vivo and in vitro. For example, at least two previous studies have carried out phosphoamino acid analysis of eNOS isolated from BAECs and have detected only phosphoserine (3, 23), whereas phosphotyrosine has been detected by others (13, 24) as a minor species. Furthermore, García-Cardenas et al. (13) showed that treatment of BAECs with $H_2O_2$ or the tyrosine phosphatase inhibitor sodium orthovanadate increases eNOS tyrosine phosphorylation. Using an immunoprecipitation NOS assay, these authors showed further that increased tyrosine phosphorylation was associated with a 50% decrease in specific activity of eNOS. We have now identified the site of tyrosine phosphorylation, Tyr-83, and gone on to show that other oxidants such as PV (a general term for the variety of complexes formed between vanadate and hydrogen peroxide) also stimulate the tyrosine phosphorylation of eNOS. We have also identified the responsible kinase as c-Src or another Src-family tyrosine kinase in endothelial cells. The conclusion that c-Src regulates eNOS in endothelial cells is further supported by our finding that the two proteins interact in a dimeric (or perhaps multimeric) complex in BAECs. These findings are also consistent with the report that a human breast cancer cell line expressing v-Src leads to increased Tyr phosphorylation on eNOS (14). In terms of function our results indicate that tyrosine phosphorylation of eNOS contributes to an increase in eNOS activity. Active Src increased basal N0 release in both transfected COS cells and endothelial cells, whereas the dominant negative Src decreased both basal and stimulated NO release. Mutation of Tyr-83 to the non-phosphorylatable analogue phenylalanine inhibited the ability of Src to activate eNOS. Of note, whereas active Src was ineffective in modulating thapsigargin-stimulated NO release in COS-7 cells and weak at increasing thapsigargin-stimulated NO release in endothelial cells, it greatly potentiated the NO release in response to vascular endothelial growth factor.

Previous studies have shown that Src is activated in endothelial cells in response to a variety of stimuli including $H_2O_2$, estrogen, and shear stress and that this event lies upstream in a cascade that leads to activation of eNOS by Akt-mediated phosphorylation of the enzyme at Ser-1179 (25–28). Phosphorylation at Ser-1179, therefore, might contribute to the 3-fold increase in eNOS activity that we observe in v-Src-transfected COS-7 cells. However, under the conditions of the present study we have been unable to detect any effect of expression of active Src on phosphorylation of eNOS at Ser-1179. Nevertheless, we did confirm previous findings that Ser-1179 contributes to NO release in response to pervanadate (29), and in addition, we show that this site is also important in the response to $H_2O_2$. Interestingly, when comparing the relative degree of activation between these sites, we found that Tyr-83 plays a greater role in $H_2O_2$-stimulated NO release compared with pervanadate-stimulated NO release. Furthermore, our results showing reduced activity of the Y83F eNOS mutant in thapsigargin-stimulated cells suggest that phosphorylation of Tyr-83 is involved, at least in part, in the Ca$^{2+}$-stimulated eNOS activation process. These data are supported by observations that the Src kinase inhibitor, which blocks the tyrosine phosphorylation of eNOS, also reduces thapsigargin-stimulated NO release and that active Src does not elevate intracellular calcium and activate eNOS indirectly by this mechanism. 

Endothelial NOS forms homodimers with a large dimer interface in the crystal structure, and dimerization is essential for activity (30). Zinc is tetrahedrally coordinated via Cys-96 and Cys-101 from each monomer, contributing to dimer stability. Tyr-83 is located on a $\beta$ strand in the crystal structure, in proximity to the dimer interface. Phosphorylation of Tyr-83 would position the phosphate group between Arg-72 on the adjacent $\beta$ strand in one monomer with Arg-109$^+$ on the opposite
monomer. Given the large buried dimer interface surface and the Cys-96 and Cys-101 coordinating zinc, it seems unlikely that the electrostatic interaction between Tyr(P)-83 and Arg-72/Arg-109 would be sufficient to influence dimer coupling and contribute to the increased basal activity observed in cells transfected with Src and eNOS. Rather, because tyrosine phosphorylation does not seem to modulate eNOS enzymatic activity directly in vitro, it seems more reasonable that Tyr(P)-83 may function as a docking site for Src homology-containing adaptormolecules.

We have also attempted to detect tyrosine phosphorylation of eNOS in BAECs exposed to a variety of other stimuli that are known to activate both c-Src and eNOS in endothelial cells including vascular endothelial growth factor (31), angiotensin II (32), bradykinin (33), and estrogen (27). In these experiments, using the standard protocol of overnight growth factor (31), angiotensin II (32), bradykinin (33), and estrogen both c-Src and eNOS in endothelial cellsincludingvascularendothelial

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