Agonist-Stimulated Endothelial Nitric Oxide Synthase Activation and Vascular Relaxation
Role of eNOS Phosphorylation at Tyr83

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Abstract—Tyr83 in endothelial nitric oxide synthase (eNOS) has been identified previously as a site of Src kinase–mediated phosphorylation of eNOS in bovine aortic endothelial cells (BAECs) that is phosphorylated in response to oxidant stress. In the present study, we have used a phospho-specific antibody to show that Tyr83 in eNOS is also phosphorylated in both BAECs and intact blood vessel segments in response to treatment with a variety of different eNOS-activating agonists, including thapsigargin, vascular endothelial growth factor, bradykinin, ATP, sphingosine-1-phosphate, estrogen, angiopoietin, and acetylcholine. Agonist stimulation of eNOS Tyr83 phosphorylation as well as agonist stimulation of endothelial NO release in BAECs is blocked by Src kinase inhibition by either 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d] pyrimidine (PP2) or by dominant negative Src. Mutation of Tyr83 to a nonphosphorylatable Phe blocks agonist stimulation of NO release from eNOS-reconstituted eNOS knockdown endothelial cells. Mutation of Tyr83 also attenuates agonist-induced relaxation of eNOS-reconstituted aortic rings from eNOS knockout mice. Phosphorylation of eNOS at Tyr83 thus appears to be a common covalent modification that is induced, not only by oxidant stress but also by other physiologically relevant extracellular signals known to be important in regulation of eNOS activity in vivo. Moreover, our results demonstrate an important role for Src-mediated phosphorylation of eNOS at Tyr83 in agonist stimulation of eNOS activation and vascular relaxation. (Circ Res. 2008;102:497-504.)

Key Words: Src kinase ■ nitric oxide synthase ■ endothelium

Basal and agonist-stimulated synthesis of nitric oxide (NO) by the endothelial NO synthase (eNOS) plays a key role in control of cardiovascular homeostasis. eNOS is posttranslationally regulated in endothelial cells primarily through protein–protein interactions and multisite phosphorylation. In particular, regulation by serine/threonine phosphorylation at Ser116, Thr497, Ser617, Ser635, and Ser1179 (residue numbers are for the bovine sequence, equivalent to Ser114, Thr495, Ser615, Ser633, and Ser1177 in the human sequence) has been well documented.1–8 Phospho-specific antibodies that recognize eNOS only when it is phosphorylated at one of these specific sites are now commercially available and have been widely used in many recent studies of eNOS regulation. Evidence also exists for regulation of eNOS by tyrosine phosphorylation. García-Cardena et al first reported that treatment of endothelial cells with high concentrations of the oxidant, hydrogen peroxide (H₂O₂) stimulates eNOS tyrosine phosphorylation at an unknown site.9 Recently, we have also shown that treatment of bovine aortic endothelial cells (BAECs) with high concentrations of either H₂O₂ or the potent synthetic oxidant and tyrosine phosphatase inhibitor pervanadate (PV) stimulates eNOS tyrosine phosphorylation.10 Oxidant-induced phosphorylation appears to be directly mediated by Src kinase. Domain mapping and site-directed mutagenesis studies in COS-7 cells transfected with either eNOS alone and then treated with PV or cotransfected with eNOS and constitutively active Src identifies Tyr83 in bovine eNOS (equivalent to Tyr81 in the human sequence) as the major tyrosine phosphorylation site. Tyr83 phosphorylation is associated with a 3-fold increase in basal NO release from cotransfected cells.

An important unanswered question regarding eNOS phosphorylation at Tyr83 is whether it only occurs in response to high levels of oxidant stress or whether phosphorylation of this residue is involved in the eNOS activation process induced by agonist stimulation. To answer this question, in the present study, we have raised and characterized a phospho-specific antibody directed against Tyr83-phosphorylated eNOS and used it to examine agonist-stimulated eNOS Tyr83 phosphorylation in cultured endothelial cells and intact blood vessel segments. We have also examined the role of Tyr83 phosphorylation in agonist stimulation of NO release from endothelial cells and in agonist modulation of blood vessel relaxation.
**Materials and Methods**

Anti-eNOS antibody was obtained from BD Biosciences. Anti-actin antibody was purchased from Sigma Chemical. Escherichia coli BJS183 cells were purchased from Stratagene, and mouse IgG Trueblot horseradish peroxidase-conjugated secondary antibody was purchased from eBioscience. Protein A/G-agarose came from Santa Cruz Biotechnology.

**Cell Culture**

Primary cultures of BAECs were purchased from VEC Technologies Inc and were used in experiments during passages 2 to 6. Cultures were maintained in medium 199 supplemented with 10% FBS, 5% iron-supplemented calf serum, 0.6 μg/mL thymidine, 2.2 mg/mL sodium bicarbonate, 500 IU/mL penicillin, and 500 μg/mL streptomycin. HEK293 cells were obtained from Stratagene. HEK293 cells and COS-7 cells were maintained in DMEM supplemented with 10% FBS, 500 IU/mL penicillin, and 500 μg/mL streptomycin.

**Generation of a Polyclonal Antibody Specific for Tyr83-Phosphorylated eNOS**

Rabbits were immunized with a synthetic phosphopeptide (GSITYp-DTL) containing the eNOS Tyr83 phosphorylation site (Phosphosolutions, Aurora, Colo). Anti–phospho-Tyr83 eNOS antibody was purified from immunized rabbit serum by phosphopeptide affinity chromatography after preclearing by dephosphopeptide affinity chromatography.

**Agonist Stimulation of BAECs**

Confluent BAECs in 100 mmol/L culture dishes were serum-starved overnight and then either not treated or treated with TG (100 nmol/L), VEGF (20 ng/mL), BK (1 μmol/L), ATP (10 μmol/L), S-1-P (100 nmol/L), estrogen (10 ng/mL), or angiopoietin (50 nmol/L) for various times at 37°C. Incubations were terminated by incubation of BAECs with S-1-P (100 nmol/L), estrogen (10 ng/mL), or angiopoietin (50 nmol/L), VEGF (20 ng/mL), BK (1 μmol/L), Ach, and 4-amo-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine (PP2) were obtained from Sigma Chemical.

**Immunoprecipitation and Immunoblotting**

Immunoprecipitation and immunoblotting was performed as described previously.10

**Measurement of NO Release**

NO release from BAECs was measured by the bioassay reporter cell procedure of Ishii et al11 with the modifications described below. COS-7 cells were transduced with adenoviruses expressing the α and β subunits of soluble guanylate cyclase (kindly provided by A. Papapetrououlos, University of Athens, Greece) and then used as reporter cells. BAECs were transfected to Locke’s buffer containing 20 U/mL superoxide dismutase and 0.3 mmol/L 3-isobutyl-1-methylxanthine and then treated or not treated with agonists for 10 minutes. Conditioned medium was transferred to reporter cells which were incubated for 3 minutes and then extracted with 0.1 mol/L HCl. cGMP concentrations were quantified using an enzyme immunoassay kit (Cayman Chemical Co). cGMP production by the reporter cells was completely blocked by incubation of BAECs with 1 mmol/L Nω-nitro-1-arginine methyl 1 ester (L-NAME), confirming that it was authentic NO that was being measured.

**eNOS Knockdown BAECs**

Knockdown BAECs, in which endogenous eNOS expression is reduced by ~90% by stable transfection with a retrovirus that encodes an eNOS interfering RNA, were generated as described previously.12

**Construction and Purification of Recombinant eNOS Adenoviruses**

Adenoviruses expressing wild-type (WT) and Y83F forms of bovine eNOS were generated by the procedure of He et al.13 Coding sequences for WT and Y83F eNOS were subcloned into a pAd-Track-CMV shuttle vector. In addition to the nonmutated WT and Y83F eNOS constructs, additional mutant WT and Y83F eNOS constructs were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the instructions of the manufacturer. Mutant constructs contained several silent mutations, which did not change the amino acid sequence but made them impervious to knockdown by interfering RNA. Primers used to create the mutations were as follows: sense, 5'-GCCAGCATATGAAACAGGAAGGGCCTGCAGGCCCCTATG-3'; antisense, 5'-CATGGGCGGGCAGTCAGCCTGGTTTCTGATACTGTCG-3'.

**Transduction of Regular BAECs, eNOS Knockdown BAECs, and Mouse Aortic Endothelium with Recombinant Adenoviruses**

Confluent BAECs were transduced with adenoviruses expressing β-galactosidase (β-gal) (negative control), a dominant negative (DN) Src (K296R/Y528F), WT eNOS, or Y83F eNOS overnight at a multiplicity of infection of 100. Congenic eNOS−/− mice were transduced with adenoviruses expressing WT and Y83F forms of eNOS according to the procedure described previously by Luo et al.16 Briefly, mice were anesthetized (40 mg/kg pentobarbital, IP) and exanguinated by transection of the abdominal aorta and perfusion of saline through the left ventricle. Heart and lungs were removed to expose the thoracic aorta. The aorta was then infused with a small volume of high titer adenovirus (5 to 7×1011 particles/mL), and each end of the aorta was tied off with tight sutures. The virus-filled vessel was then incubated in situ at 37°C for 2 hours. Following the 2-hour in situ incubation, the aorta was dissected free from the surrounding tissue and rinsed in saline before overnight incubation of the tissue in culture medium at 37°C with 95% O2/5% CO2. Efficient gene transfer primarily into the endothelium using this method was confirmed by staining for β-gal in mouse aorta transduced with an adenovirus encoding β-gal.

**Vascular Reactivity Experiments**

Adenoviral-transduced mouse aortae were cut into cylindrical segments of 2-mm length and mounted on small stainless-steel holders in an organ bath for isometric force recording using a Multi- myograph with PowerLab software. Organ baths were filled with physiological salt solution containing 130 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L KH2PO4, 1.17 mmol/L MgSO4, 1.6 mmol/L CaCl2, 14.9 mmol/L NaHCO3, 5.5 mmol/L dextrose, and 0.03 mmol/L Na2 EDTA and were aerated with 95% O2/5% CO2 at 37°C. The rings were stretched to an optimal resting tension (3 g) and allowed to stabilize for 1 hour. A dose–response curve to phenylephrine was then constructed to determine the ED50 for contraction to phenylephrine. After phenylephrine had been washed out, rings were preconstricted with this ED50 concentration of phenylephrine. When contraction reached a plateau phase, vessels were treated with various concentrations of Ach ranging from 10−8 to 10−4 mol/L and percentage relaxation was recorded.

**Argon-Stimulated Tyr83 Phosphorylation of eNOS in Rat Aortic Rings**

Male Sprague-Dawley rats (200 to 215 g) were purchased from Charles River Laboratories Inc and anesthetized with pentobarbital (40 mg/kg, IP). Thoracic aortae were dissected from anesthetized rats, cleaned of adherent fat and connective tissue, and cut into 4-mm segments. Ring segments were incubated overnight in serum-free medium 199 at 37°C with 95% O2/5% CO2. Rings were then incubated for 10 minutes with or without Ach (1 μmol/L) or BK (1 μmol/L). Rings were snap-frozen in liquid nitrogen and pulv-
ized into powder followed by homogenization in 50 mmol/L Tris–HCl, pH 7.5, containing 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.1% SDS, 0.1% deoxycholic acid, 1% Triton X-100, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 μmol/L pepstatin A, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium orthovanadate. Homogenates were sonicated with 3 bursts of 3 seconds each. Equal amounts of homogenate protein from each condition were then used to partially purify eNOS by 2', 5'-ADP-Sepharose affinity chromatography followed by immunoblotting with anti–phospho-Tyr83 eNOS antibody and nonphospho-specific anti-eNOS antibody.

**Statistical Analysis**

NO release data and vascular reactivity are expressed as means±SEM. Overall differences between groups were analyzed using a 2-way ANOVA with Student–Newman–Keuls post hoc analysis for determining differences between the means when more than 2 groups were compared. Differences were considered as significant at P<0.05.

**Results**

**Generation and Characterization of an Antibody Specific for Tyr83-Phosphorylated eNOS**

In our previous study of PV- and H2O2-stimulated phosphorylation of eNOS at Tyr83, we attempted to detect tyrosine phosphorylation of eNOS in BAECs in response to stimulation with various agonists. Using a protocol of overnight immunoprecipitation of eNOS from stimulated cell lysates, followed by immunoblotting of immunoprecipitates with anti-phosphotyrosine antibody, we were unable to detect agonist-induced eNOS tyrosine phosphorylation.10 In the present study, therefore, we have taken a different approach and have prepared a highly sensitive phospho-specific antibody that recognizes eNOS only when it is phosphorylated at Tyr83. This antibody allows direct detection of eNOS Tyr83 phosphorylation in cell lysates and bypasses the need for overnight immunoprecipitation. Rabbits were immunized with a synthetic phosphopeptide (GSITYpDTL) corresponding to bovine eNOS residues 79 to 86 containing a phosphotyrosine at position 83. Anti–phospho-Tyr83 eNOS antibody was purified from immunized rabbit serum by phosphopeptide affinity chromatography. To evaluate the performance of the purified antibody, COS-7 cells were transfected with either WT eNOS or a nonphosphorylatable Tyr83 form of eNOS (Y83F)10 and then either not stimulated or stimulated with PV for 10 minutes. Cells were then lysed and eNOS was immunoprecipitated (IP) with an anti-eNOS antibody. Immunoprecipitates were immunoblotted (IB) with anti–phospho-Tyr83 eNOS antibody (A) or anti-eNOS antibody (B). Results shown are representative of at least 3 separate experiments.

As shown in Figure 1A and 1C, immunoblotting of equal amounts BAEC lysate protein with the anti–phospho-Tyr83 eNOS antibody detected only a single band on the blots that was of the appropriate molecular weight (130 KDa) and that was time-dependently phosphorylated (and subsequently dephosphorylated) in response to both TG and VEGF stimulation. Probing of blots with an anti-eNOS antibody that recognizes both phosphorylated and nonphosphorylated eNOS detected equal amounts of total eNOS in each condition (Figure 2B and 2D).

To determine whether phosphorylation of eNOS at Tyr83 might be involved in activation of the enzyme by other agonists, we examined the effects of several other known eNOS-activating agonists, including BK, ATP, S-1-P, estrogen, and angiopoietin. BAECs were either not treated or treated for 10 minutes with BK (1 μmol/L), ATP (10 μmol/L), S-1-P (100 nmol/L), estrogen (10 ng/mL), or angiopoietin-stimulated phosphorylation of eNOS at Tyr83, we performed additional immunoblotting experiments with BAEC lysates. BAEC lysates were prepared from cells that were treated for 0, 1, 5, 10, 20, 40, or 60 minutes with the intracellular calcium-elevating agent TG (100 nmol/L) or VEGF (20 ng/mL), 2 stimuli that we have shown previously to be implicated in eNOS activation by a Src-mediated pathway.10 As shown in Figure 2A and 2C, immunoblotting of equal amounts BAEC lysate protein with the anti–phospho-Tyr83 eNOS antibody detected only a single band on the blots that was of the appropriate molecular weight (130 KDa) and that was time-dependently phosphorylated (and subsequently dephosphorylated) in response to both TG and VEGF stimulation.

**Figure 1.** Characterization of an antibody specific for Tyr83-phosphorylated eNOS. COS-7 cells were transfected with plasmids encoding WT or Y83F forms of eNOS. After 24 hours, cells were either not stimulated or stimulated with 200 μmol/L PV for 10 minutes. Cells were then lysed and eNOS was immunoprecipitated (IP) with an anti-eNOS antibody. Immunoprecipitates were immunoblotted (IB) with anti–phospho-Tyr83 eNOS antibody (A) or anti-eNOS antibody (B). Results shown are representative of at least 3 separate experiments.

**Figure 2.** Time course analysis for determining differences between the means when more than 2 groups were compared. Differences were considered as significant at P<0.05.

**Agonist-Stimulated Phosphorylation of eNOS at Tyr83 in Endothelial Cells**

To further confirm the specificity of the antibody and to determine whether the antibody could detect agonist-
Src Kinase Activity is Required for Agonist-Stimulated eNOS Tyr83 Phosphorylation and NO Release

We next sought to obtain evidence that agonist-stimulated eNOS phosphorylation at Tyr83 is specifically mediated by Src kinase and further that this phosphorylation event has an important role in agonist stimulation of endothelial NO release. BAECs were incubated for 10 minutes with or without BK (1 μmol/L) or VEGF (20 ng/mL) after preincubation for 30 minutes with or without the Src family kinase inhibitor PP2 (1 μmol/L). Lysates were prepared and equal amounts of lysate protein were immunoblotted with the anti–phospho-Tyr83 eNOS antibody as well as with nonphospho-specific anti-eNOS antibody. As shown in Figure 4B, BK- and VEGF-stimulated phosphorylation of Tyr83 was completely blocked by PP2 pretreatment implicating c-Src or a Src family kinase as being responsible for agonist-stimulated Tyr83 phosphorylation of eNOS in intact endothelial cells. In a parallel set of experiments, the effects of Src kinase inhibition on agonist-stimulated NO release from BAECs was also determined. Cells were either not treated or treated with BK (1 μmol/L) or VEGF (20 ng/mL) for 10 minutes after preincubation for 30 in the presence or absence of PP2. Relative amounts of NO release at the end of the 10-minute treatment period were quantified using a cGMP reporter cell assay, an assay that is based on differences in cGMP production in reporter cells being directly proportional to differences in the amounts of NO release from the BAECs. As compared with unstimulated cells, conditioned medium from BK- and VEGF-stimulated cells elicited large increases in cGMP production in reporter cells that was almost completely blocked by PP2 (Figure 4A). These data demonstrate that Src kinase activity is required for agonist stimulation of NO release from endothelial cells, presumably because of direct Src-mediated phosphorylation of eNOS at Tyr83.

The involvement of Src-mediated phosphorylation of Tyr83 in agonist-stimulated eNOS activation was further confirmed using a nonpharmacological approach. BAECs were transduced with either a negative control β-gal adeno-virus or with an adeno-virus that expresses a dominant negative, kinase-inactive Src (DN Src). Cells were then either not treated or treated with BK (1 μmol/L) or VEGF (20 ng/mL) (A), ATP (10 μmol/L) (B), S-1-P (100 nmol/L) (C), estrogen (10 ng/mL) (D), or angiopoietin (50 ng/mL) (E). Cells were lysed and equal amounts of protein in lysates were immunoblotted (IB) with anti–phospho-Tyr83 eNOS antibody (A and C) or anti-eNOS antibody (B and D). Similar results were obtained in at least 3 separate experiments.

Phosphorylation and NO Release

Agonist-Stimulated eNOS Tyr83 Src Kinase Activity is Required for NO release from endothelial cells, presumably because of

Figure 2. Time-dependent phosphorylation of eNOS at Tyr83 in BAECs stimulated with TG and VEGF. BAECs were treated with TG (100 nmol/L) or VEGF (20 ng/mL) for the times indicated. Cell lysates were prepared and equal quantities of lysate protein were immunoblotted (IB) with anti–phospho-Tyr83 eNOS antibody as well as with anti–phospho-Tyr83 eNOS antibody as well as with

Figure 3. Agonist-stimulated phosphorylation of eNOS at Tyr83 in BAECs. BAECs were either not treated or treated for 10 minutes with BK (1 μmol/L) (A), ATP (10 μmol/L) (B), S-1-P (100 nmol/L) (C), estrogen (10 ng/mL) (D), or angiopoietin (50 ng/mL) (E). Cells were lysed and equal amounts of protein in lysates were immunoblotted (IB) with anti–phospho-Tyr83 eNOS antibody. Similar results were obtained in at least 3 separate experiments.
ng/mL) for 10 minutes. Lysates were prepared and equal amounts of lysate protein were immunoblotted with the anti–phospho-Tyr83 eNOS antibody and the nonphospho-specific anti-eNOS antibody. DN Src transduction dramatically increased total Src expression levels and completely blocked BK- and VEGF-stimulated Tyr83 phosphorylation. Total eNOS expression was unaffected (Figure 4D). In parallel experiments, agonist-stimulated NO release was also determined, BAECs were transduced with adenoviruses expressing either β-gal or DN Src and then either not treated or treated with BK (1 μmol/L) or VEGF (20 ng/mL) for 10 minutes. NO release after 10 minutes was measured by cGMP reporter cell assay. As shown in Figure 4C, BK- and VEGF-stimulated NO release were almost completely blocked by the DN Src adenovirus (Figure 4C).

Figure 4. Inhibition of agonist-stimulated Tyr83 phosphorylation and NO release in BAECs by PP2 and DN Src (DN Src). BAECs were either not treated or treated for 10 minutes with BK (1 μmol/L) or VEGF (20 ng/mL) following 30 minutes of preincubation with and without PP2 or overnight infection of β-gal or DN Src adenoviruses. A, NO release with or without PP2 measured by cGMP reporter cell assay (means±SE; n=3; *P<0.05 vs without PP2, independent t test). B, immunoblotting (IB) of cell lysates with phospho-Tyr83 eNOS and nonphospho-specific anti-eNOS antibodies. Results shown are representative of at least 3 separate experiments. C, NO release with or without adenovirus infection with β-gal or DN Src adenoviruses (means±SE; n=3; *P<0.05 vs β-gal + agonist, independent t test). D, Immunoblotting of cell lysates with anti-Src and phospho-Tyr83 eNOS and nonphospho-specific anti-eNOS antibodies. Results shown are representative of at least 3 separate experiments.

Agonist-Stimulated Phosphorylation of eNOS at Tyr83 in Rat Aortic Ring Segments
Phosphorylation of eNOS at Tyr83 in response to agonist stimulation was also examined in intact blood vessel segments prepared from rat thoracic aortae. Four-millimeter ring segments were incubated in serum-free medium overnight and then either not treated or treated with Ach (1 μmol/L, Ach) or BK (1 μmol/L) for 10 minutes. Rings were snap-frozen, pulverized, homogenized, and sonicated. Equal quantities of protein from each condition were then analyzed by immunoblotting with anti–phospho-Tyr83 eNOS antibody and nonphospho-specific anti-eNOS antibody. As shown in Figure 5A, both Ach and BK stimulated an increase in Tyr83 phosphorylation of eNOS in intact blood vessel segments. Differences observed with the phospho-specific antibody,
Figure 5. Agonist-stimulated phosphorylation of eNOS at Tyr83 in rat aortic rings and effects of mutation of Tyr83 on vascular reactivity of eNOS-reconstituted aortic rings from eNOS-null mice. A and B, Rat thoracic aortic rings were incubated for 10 minutes in the absence (control) or presence of BK (1 μmol/L) or Ach (1 μmol/L) for 10 minutes. Homogenates were then prepared and equal quantities of homogenate protein from each condition were immunoblotted (IB) with anti-phospho-Tyr83 eNOS and nonphospho-specific anti-eNOS antibodies. C, eNOS knockout mice were anesthetized, and thoracic aortae were injected with WT eNOS and Y83F eNOS adenoviruses. Following 2 hours of incubation in situ, vessels were incubated in serum-free medium at 37°C overnight. Vessel segments were contracted with an ED₅₀ concentration of phenylephrine. When contraction reached a plateau, an accumulated dose response to Ach was conducted. Results shown are representative of 3 separate experiments (means±SE; *P<0.05 vs WT, independent t test). D, Equal amounts of ring homogenate protein from WT eNOS and Y83F eNOS conditions were immunoblotted with anti-eNOS and anti-actin antibodies. Similar results were obtained in at least 3 separate experiments.

Effects of Mutation of Tyr83 on Vascular Reactivity of eNOS-Reconstituted Aortic Rings From eNOS-Null Mice

To determine whether phosphorylation of eNOS at Tyr83 is required for agonist-induced relaxation of intact blood vessels, we also performed studies of vascular reactivity of aortic rings from eNOS knockout mice that were reconstituted with either WT eNOS or Y83F eNOS. eNOS⁻/⁻ mice were anesthetized and exsanguinated following perfusion with saline. The thoracic aorta was then infused with adenoviruses expressing either WT eNOS or Y83F eNOS and tied off at each end with sutures. The virus-filled vessels were incubated in situ for 2 hours and then incubated in vitro overnight. Following overnight incubation, aortic rings were prepared for isometric force recording. Rings were first precontracted with phenylephrine and then treated with various concentrations of Ach ranging from 10⁻⁹ to 10⁻⁵ mol/L, and percentage relaxation in rings was recorded. As shown in Figure 5C, rings from aortae of mice infected with the Y83F eNOS adenovirus had significantly reduced relaxant responses to Ach. This was reflected in both an increase in the ED₅₀ for Ach and a reduction in the maximal level of relaxation achieved. To confirm that the differences observed were not simply attributable to differences in levels of eNOS protein expression, equal-sized rings from each of the 2 conditions were analyzed for eNOS and actin content by immunoblotting with anti-eNOS and anti-actin antibodies. As shown in Figure 5D, the levels of expression of both proteins in WT eNOS- and Y83F eNOS-transduced rings were equivalent (130- and 42-kDa bands, respectively). Taken together, these data suggest that phosphorylation of eNOS at Tyr83 has a significant role in agonist-induced relaxation of blood vessels.

Effects of Mutation of Tyr83 on Agonist-Stimulated NO Release From eNOS-Reconstituted eNOS Knockdown Endothelial Cells

The effects of the Y83F mutation on agonist stimulation of eNOS activity was also tested in cultured endothelial cells. We have observed previously that overexpression of eNOS in regular BAECs does not result in an increase in NO release on treatment with agonists, likely because factors other than the level of eNOS expression play a dominant role in limiting the amount of NO released from BAECs in response to agonist stimulation.19 We therefore tested the effects of the Y83F mutation in a BAEC model system in which eNOS knockdown BAECs were reconstituted with WT and Y83F forms of eNOS such that the reconstituted cells had a level of eNOS expression that was exactly equivalent to that of regular BAECs. We have shown previously that endogenous eNOS expression in knockdown BAECs can be reduced by ∼90% by stable transfection by a retrovirus that encodes an eNOS interfering RNA.12 Adenoviruses were thus prepared for expression of WT and Y83F forms of eNOS containing silent mutations that changed the nucleotide sequence of the virally produced mRNAs in such a way as to make them unsusceptible to interfering RNA knockdown without changing the amino acid sequence of the proteins. Knockdown BAECs were infected with various titers of the 2 viruses to determine by immunoblotting the appropriate titers needed to achieve equivalent expression of the 2 forms of eNOS equal to the level of eNOS expression in regular BAECs. Cells were transduced with the appropriate titers of the WT and Y83F eNOS adenoviruses and then either not stimulated or stimulated with BK (1 μmol/L) or VEGF (20 ng/mL) for 10 minutes. Relative amounts of NO release after 10 minutes were measured by cGMP reporter cell assay. As shown in Figure 6A, both BK and VEGF stimulated significant increases in NO release from knockdown BAECs reconstituted with WT eNOS but not from cells reconstituted with Y83F eNOS, again implicating phosphorylation of Tyr83 as having an important role in the agonist-induced eNOS activation process. Differences in NO release were not attributable to differences in eNOS expression (Figure 6B).

Discussion

In this study, we have generated and characterized a polyclonal antibody that is highly specific for eNOS only when it
is phosphorylated at Tyr83. This conclusion is supported by the following observations. First, the antibody strongly recognizes WT eNOS when it is immunoprecipitated from PV-stimulated COS-7 cells but does not even weakly recognize a nonphosphorylatable Y83F eNOS mutant immunoprecipitated from the same PV-stimulated cells. Second, when immunoblotting of BAEC lysates is performed with the antibody, only a single band is detected on blots across the entire molecular weight range of lysate proteins. The antibody characterized here was generated against a phosphopeptide corresponding to bovine eNOS residues 79 to 86. This suggests, not only a conservation of sequence function but also that the antibody should be useful for studies in endothelial cells from many other species. Immunoblotting of endothelial cell lysates with the phospho-specific antibody shows that Tyr83 in eNOS is phosphorylated in response to treatment of cells with a variety of different eNOS-activating agonists including TG, VEGF, BK, ATP, S-1-P, estrogen, and angiopoietin. Blotting of proteins from intact blood vessel segments shows additionally that Tyr83 in eNOS is also phosphorylated in response to Ach. Phosphorylation of eNOS at Tyr83 thus appears to be a common covalent modification that is induced, not only by oxidant stress but also by a number of physiologically relevant extracellular signals known to be important in regulation of eNOS activity in vivo. Whether this residue is phosphorylated in response to all known eNOS-activating agonists is not clear. However, we have tested certain other agonists (ie, angiotensin II) and have obtained negative results.

Time course studies using the phospho-specific antibody show that agonist-stimulated phosphorylation of eNOS at Tyr83 is transient in nature and that Src-mediated phosphorylation is followed soon after by dephosphorylation by an as yet unidentified tyrosine phosphatase. It is also interesting to note that peak phosphorylation in response to TG occurs in BAECs between 5 and 10 minutes, whereas peak phosphorylation in response to VEGF is delayed until at least 20 minutes. Time course studies of NO release from BAECs show further that TG and VEGF stimulation of NO release is also transient in nature with peak levels for TG at 5 minutes (data not shown) and for VEGF at 20 minutes, exactly coincident with peak levels of eNOS Tyr83 phosphorylation. Results presented in this study show that Src kinase inhibition with PP2 or DN Src almost completely blocks BK- and VEGF-stimulated eNOS Tyr83 phosphorylation as well as BK- and VEGF-stimulated NO release from BAECs. Previous reports indicate that Src is activated in endothelial cells in response to H2O2, estrogen, and shear stress upstream from Akt-mediated phosphorylation of eNOS at Ser1179. However, our results showing a major impairment in the agonist responsiveness of Y83F eNOS indicate an additional significant role for Src-mediated eNOS phosphorylation at Tyr83. The relative roles of phosphorylation of eNOS at each of the 6 different eNOS phosphorylation sites that have thus far been identified are not yet entirely clear. Based primarily on the fact that it was the first eNOS phosphorylation site discovered, Ser1179 is generally believed to be the most important of the regulatory eNOS phosphorylation sites. However, our results showing significant block of agonist stimulation of endothelial NO release and blood vessel relaxation by either Src kinase inhibition or by Tyr83 mutation indicate that Src-mediated phosphorylation of eNOS at Tyr83 may have an equally important role in the agonist-stimulated eNOS activation process.

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Disclosures
None.

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