Endothelial Nitric Oxide Synthase Is Regulated by Tyrosine Phosphorylation and Interacts with Caveolin-1

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The regulation of endothelial nitric oxide synthase (eNOS) by phosphorylation is poorly understood. Here, we demonstrate that eNOS is tyrosine-phosphorylated in bovine aortic endothelial cells (BAEC) using 32P metabolic labeling followed by phosphoamino acid analysis and by phosphotyrosine specific Western blotting. Treatment of BAEC with hydrogen peroxide and the protein tyrosine phosphatase inhibitor, sodium orthovanadate, increases eNOS tyrosine phosphorylation. Utilizing a novel immunoNOS assay, the increase in tyrosine phosphorylation is associated with a 50% decrease in the specific activity of the enzyme. Because eNOS is localized in plasmalemma caveolae, we examined if tyrosine phosphorylated eNOS interacts with caveolin-1, the coat protein of caveolae. Immunoprecipitation of eNOS from bovine lung microvascular endothelial cells resulted in the co-precipitation of caveolin-1. Conversely, immunoprecipitation of caveolin-1 resulted in the co-precipitation of tyrosine-phosphorylated eNOS. Thus, tyrosine phosphorylation is a novel regulatory mechanism for eNOS and caveolin-1 is the first eNOS-associated protein. Collectively, these observations provide a novel regulatory mechanism for eNOS and suggest that tyrosine phosphorylation may influence its activity, subcellular trafficking, and interaction with other caveolin-interacting proteins in caveolae.

Post-translational modification of proteins is a widely used mechanism for transmitting biological signals and regulating the activities, biosynthesis, and degradation of enzymes. One of these modifications, protein phosphorylation, plays a central role in signal transduction pathways regulating many biological processes, including cellular proliferation, migration, and differentiation. More specifically, phosphorylation of tyrosine residues is an essential step in cellular activation by many external signals, including growth factors, cytokines, and cellular stress (1, 2).

In the cardiovascular and nervous systems, activation of cell surface receptors triggers the immediate synthesis of nitric oxide (NO).1 In the cardiovascular system, NO is derived from one of the chemically equivalent guanidino nitrogens of L-arginine, in a reaction catalyzed by endothelial NO synthase (eNOS or NOS 3). In intact blood vessels and in cultured endothelial cells (EC), eNOS is rapidly activated by agonists that mobilize intracellular calcium. Increases in intracellular calcium facilitate interactions with calmodulin and the activated calcium-calmodulin complex can stimulate NADPH-dependent electron flux through eNOS to produce NO (3). Recent evidence suggests that eNOS can also be activated in a calcium-independent manner by fluid shear stress (4–6) and insulin-like growth factor (7), presumably mediated through a tyrosine kinase cascade, since inhibitors of tyrosine kinases, but not chelation of intracellular calcium, inhibits NO release. Whether eNOS is directly tyrosine-phosphorylated or indirectly linked to a tyrosine kinase cascade is not known. Once NO is produced by either mechanism, it rapidly diffuses and mediates endothelium-dependent relaxation of blood vessels, inhibition of platelet aggregation and leukocyte adhesion, and vascular smooth muscle growth (8).

eNOS has been shown to reside in plasmalemmal caveolae (9, 10). Recent data suggests that plasmalemmal caveolae are cellular signal processing centers. Activation of fibroblasts with platelet-derived growth factor or epidermal growth factor results in the rapid recruitment of signal transducing proteins (Syp, Shc, Ras/Raf-1, and mitogen-activated protein kinase) into caveolae microdomains (11, 12). The direct interaction between eNOS-1, the major coat protein of caveolae, with inactive Ga and Ras supports the concept that not only the recruitment, but the inactivation of signaling molecules, occurs in caveolae (13, 14). The presence of eNOS in caveolae suggests that resident caveolar proteins can potentially interact with eNOS and that NO can influence signal transduction through caveolae.

Here, we report that eNOS is tyrosine-phosphorylated in EC. Treatment of EC with hydrogen peroxide (H2O2) or inhibition of endogenous protein tyrosine phosphatases increases eNOS tyrosine phosphorylation and hyperphosphorylation is associated with a decrease in NOS specific activity. Additionally, tyrosine-phosphorylated eNOS interacts with the caveolar coat protein, caveolin-1, based on co-immunoprecipitation assays. These findings demonstrate a novel regulatory mechanism for eNOS and suggest that tyrosine phosphorylation may influence its activity, subcellular trafficking, and interaction with other caveolin-interacting proteins in caveolae.

¹ The abbreviations used are: NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; EC, endothelial cells; BLMVEC, bovine lung microvascular endothelial cells; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; mAb, monoclonal antibody; Ab, antibody; L-NMMA, Nω-monomethyl-L-arginine.
EXPERIMENTAL PROCEDURES

Materials and Antibodies—Tissue culture reagents were from Life Technologies, Inc., and chemicals were from Sigma. n-Octyl-β-D-glucopyranoside was from Calbiochem. [32P]Orthophosphoric acid was from New England Nuclear. eNOS monoclonal antibody (H32) and anti-phosphotyrosine antibody (4G10) were kindly provided by J. S. Pollock (Medical College of Georgia), and the caveolin-1 polyclonal antibody and horseradish peroxidase conjugate were from Transduction Laboratories. Bovine lung microvascular endothelial cells (BLMVEC, 10 cell doublings) were provided by Peter Del Vecchio. Stock solutions of sodium orthovanadate (vanadate, assuming a hydration number of 10) were prepared in sterile distilled water and adjusted to pH 10.

Preparation of Cells—Cell Culture—Bovine aortic endothelial cells (BAEC) or BLMVEC were isolated and cultured in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal bovine serum, penicillin, streptomycin, and l-glutamine as described previously (15, 16). BAEC were used for these experiments between passages 2 and 4. In some experiments, BAEC were incubated in complete medium with vanadate (1 mM) for 2 h or H2O2 (0.1–10 mM) for 20 min and processed for immunoprecipitation. These treatments were not associated with EC toxicity, presumably due to the presence of 10% serum, as assessed by overall cell morphology.

Labeling and Phosphoamino Acid Analysis—Confluent 100-mm dishes of BAEC were incubated in phosphate-free Dulbecco’s minimum essential medium with 80 μCi/ml of [32P]Orthophosphoric acid for 4 h, harvested, and immunoprecipitated as described below. Proteins were resolved by SDS-PAGE (7.5% acrylamide) transferred to PVDF, and identified by autoradiography. For two-dimensional phosphoamino acid analysis, proteins were blotted on PVDF membranes, and the eNOS band was isolated after autoradiography. In brief, PVDF-blotted eNOS was hydrolyzed in 6 N HCl for 45 min at 105°C. The aqueous phase was lyophilized, extensively washed, and resuspended in electrophoresis buffer as described previously (17).

Immunoprecipitations and Western Blotting—Cells were washed twice with TBS (50 mM Tris-HCl, 150 mM NaCl), lysed on ice in modified RIPA buffer (100 mM Tris-HCl, pH 7.4, 1%, v/v, Nonidet P-40 10 mM NaF, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM vanadate), and eNOS activity was determined by measuring the conversion of l-[3H]arginine into l-[3H]citrulline as described previously (18). Brieﬂy, beads were incubated (total volume 100 μl) in assay buffer containing 1 mM NADPH, 3 μM tetrahydrobiopterin, 100 mM calmodulin, 2.5 mM CaCl2, and 10 μg l-arginine, and l-[3H]arginine (0.2 μCi, 55 Ci/mmol) for 30 min at 37°C. After the incubation period, the reaction was quenched by the addition of 1 ml of cold stop buffer (20 mM HEPEs, pH 5.5, containing 2 mM or each metalion, 1 mM methyl-L-arginine (L-NMMA, 1 mM), and NOS activity isolated from vanadate treated BAEC was expressed as a percent of control (vehicle-treated BAEC). Immunoprecipitated NOS activity was in the range of 0.1–2.0 pmol of l-citrulline generated/min (n = 5 independent experiments). In all experiments, similar amounts of enzyme and the phosphorylated state of NOS in each incubation was verified by eNOS and phosphotyrosine blotting, respectively.

RESULTS AND DISCUSSION

As demonstrated previously, biosynthetic labeling of BAEC with [32P]Orthophosphate resulted in the incorporation of the label into immunoprecipitated eNOS (Fig. 1A, Refs. 19–21). To identify the phosphorylated residues, immunoprecipitated [32P]-labeled eNOS was isolated from BAEC, hydrolyzed under acidic conditions, and phosphoamino acids analyzed by two-dimensional TLC electrophoresis. As seen in Fig. 1B, a majority of the label was incorporated into phosphoserine as described previously (19), but clearly detectable amounts of phosphotyrosine were also found. Acid hydrolysis of labeled eNOS for longer times resulted in the reduced ability to detect phosphotyrosine (data not shown), consistent with the known acid lability of phosphotyrosyl residues (22). In addition to the qualitative nature of phosphoamino acid analysis, the stoichiometry of eNOS tyrosine phosphorylation is not yet known.

To corroborate the presence of tyrosine phosphorylated re-
lysophosphatidylcholine (1–100 µM) enhances tyrosine phosphorylation, and did not incubate with hydrogen peroxide (H₂O₂) for 20 min, and eNOS was immunoprecipitated and Western blotted with eNOS and phosphotyrosine Abs. BAEC were treated with vanadate (1 mM for 2 hr), and eNOS was immunoprecipitated and Western blotted with phosphotyrosine (B) and eNOS Abs (C). The band at approximately 55 kDa is the Ig heavy chain. The identity of lower molecular weight band in B is not known. Similar results were obtained in several experiments (n > 5). IP denotes immunoprecipitating Ab (α-eNOS) and WB denotes Western blotting Abs (α-eNOS and α-pTyr).

Some signal transducing proteins are basally tyrosine phosphorylated (i.e. Src or mitogen-activated protein kinase), whereas others are phosphorylated in response to growth factors (i.e. platelet-derived growth factor receptor (1)) or integrin-extracellular matrix engagement (i.e. focal adhesion kinase (23)). Serum activation of serum-starved BAEC did not increase eNOS tyrosine phosphorylation, nor did incubation with lysophosphatidylcholine (1–100 µM), bradykinin (10 µM), ionomycin (1 µM), angiotensin II (1 µM), or tumor necrosis factor α (100 units/ml). Additionally, BAEC in suspension and cells plated on fibronectin or poly-L-lysine had similar amounts of eNOS, but not phosho-serine or threonine, completely prevented the immunoreactivity with eNOS. Identical results were obtained using another phosphotyrosine Ab, 4G10 (data not shown), and was also seen in human umbilical vein endothelial cells and in HEK 293 cells stably transfected with the eNOS cDNA. Thus, eNOS is tyrosine-phosphorylated as determined by two independent biochemical methods.

To examine if increases in tyrosine phosphorylation induced by blockade of protein tyrosine phosphatases regulate NOS activity, we developed an immunoprecipitation-NOS assay (immunoNOS) to assess the amount of eNOS, its tyrosine phosphorylation state, and the corresponding activity of the enzyme from the same sample isolated from BAEC. Fig. 3 demonstrated that vanadate pretreatment of BAEC increased the phosphotyrosine content of eNOS (A) with equal amounts of eNOS present in control and treated cells (B). ImmunoNOS assay of an equal proportion of the immunoprecipitated protein in A and B showed that enhanced tyrosine phosphorylation of eNOS was associated with a 50% decrease in NOS activity (n = 5 different experiments; similar results were obtained in total cell lysates). The NOS activity isolated from both control and vanadate-treated BAEC was attenuated with the NOS inhibitor, L-NMMA. Since vanadate was also present in the lysis buffer of control and treated cells to preserve phosphotyrosine during the immunoprecipitation, the inhibition of NOS activity in untreated cells was most likely not due to a direct inhibitory effect of the drug on NOS per se. The mechanism by which vanadate increases eNOS phosphorylation is presumably due to inhibition of endogenous eNOS-specific phosphotyrosine phosphatases, however, we cannot rule out the possibility that vanadate is indirectly activating cellular tyrosine kinases, thereby increasing the tyrosine phosphorylation of eNOS. Thus, increases in eNOS tyrosine phosphorylation in BAEC decrease NOS catalytic activity.

eNOS has been shown to reside in the plasmalemmal microdomain, caveolae (9, 10). Caveolae may play a role in the organiz-
tion of signal processing centers suggesting that eNOS has the propensity to interact with the caveolar coat protein caveolin-1, as recently described for the G-protein α subunits Gαs, Gαi1, and Ras (13, 14). To examine if tyrosine-phosphorylated eNOS interacts with caveolin-1, we performed co-immunoprecipitation experiments with eNOS and caveolin antisera. Immunoprecipitation of eNOS from BLMVEC resulted in the co-precipitation of caveolin-1 (Fig. 4A). The amount of caveolin-1 co-precipitated with eNOS antisera was not stoichiometric relative to the total pool of cellular caveolin (data not shown). Conversely, immunoprecipitation of caveolin-1 resulted in the co-precipitation of tyrosine-phosphorylated eNOS (Fig. 4B). As seen above, the amount of eNOS that co-precipitated with caveolin-1 was not stoichiometric. Immunoprecipitation with non-immune antisera did not result in the detection of either eNOS or caveolin-1 (data not shown). The data are consistent with our previous findings in BLMVEC, demonstrating that a pool of eNOS co-localizes with caveolin-1 based on purification of plasmalemma caveole and confocal microscopy (9). These results provide a novel mechanism to regulate the catalytic activity and potentially the subcellular trafficking of eNOS. In general, oxidative stress induced by pro-inflammatory cytokines or oxidants activates tyrosine kinase signaling pathways. Increases in oxidative stress brought about by H₂O₂ or by inhibition of endogenous protein-tyrosine phosphatases increase eNOS tyrosine phosphorylation, and in the latter case, reduce NO activity. Interestingly, several cardiovascular diseases with diverse etiologies, such as atherosclerosis, vascular complications of diabetes, ischemia-reperfusion injury, and hypertension are associated with the common hallmarks of increased oxidative stress and endothelial cell dysfunction (24). Dysfunction is manifested by the inability to vasodilate in response to endothelium-dependent vasodilators, in the face of normal or increased expression of eNOS. Perhaps, increased eNOS tyrosine phosphorylation can contribute to dysfunction in these disease processes.

The co-immunoprecipitation of caveolin-1 with eNOS raises several interesting possibilities relevant to the trafficking of both proteins and to the activation and phosphorylation of eNOS. Both caveolin-1 and eNOS localize on the Golgi complex and in caveolae (9, 15, 26). However, the mechanisms of anterograde traffic-