Stimulation of Receptor-Mediated Nitric Oxide Production by Vanadate

Gary R. Hellermann, Brenda R. Flam, Duane C. Eichler, Larry P. Solomonson

Abstract—Nitric oxide (NO) production by endothelial cells in response to bradykinin (Bk) treatment was markedly and synergistically enhanced by cotreatment with sodium orthovanadate (vanadate), a phosphotyrosine phosphatase inhibitor. This enhancement was blocked by tyrosine kinase inhibitors. Calcium ionophore- (A23187) activated production of NO was also enhanced by cotreatment with vanadate. No significant changes were found in total endothelial NO synthase (eNOS) protein or in eNOS distribution between membrane (caveolae) and cytosolic fractions in response to the various treatments. Vanadate had no direct effect on eNOS activity, and lysates prepared from cells treated with vanadate showed little change in specific activity of eNOS. Western blots of immunoprecipitated eNOS showed the presence of a major tyrosine-phosphorylated protein band at a mass corresponding to \( \approx 125 \) kDa and 2 minor bands corresponding to \( \approx 105 \) and 75 kDa after treatment with vanadate/Bk. No tyrosine phosphorylation of eNOS after treatment with vanadate/Bk was observed. Geldanamycin, an inhibitor of heat shock protein 90, also inhibited the enhancement of NO production by vanadate/Bk or vanadate/A23187, and there was an increase in the amount of heat shock protein 90 that coimmunoprecipitated with eNOS after treatment with vanadate/Bk. These results show that there is a clear link between tyrosine phosphorylation and stimulation of eNO production, which does not appear to involve direct modification of eNOS, changes in eNOS levels, or compartmentation, but rather appears to be due to changes in proteins associating with eNOS, thereby enhancing the state of activation of eNOS. (Arterioscler Thromb Vasc Biol. 2000;20:2045-2050.)

Key Words: nitric oxide ■ endothelial ■ vanadate ■ bradykinin ■ tyrosine phosphorylation

Endothelial nitric oxide synthase (eNOS) plays a key role in vasoregulation through the highly regulated production of NO, which diffuses to the smooth muscle layer, causing relaxation. Several studies have suggested a linkage between phosphorylation/dephosphorylation events and eNO production. An early study by Michel et al demonstrated that short-term incubation of endothelial cells with bradykinin (Bk) or calcium ionophore A23187 led to serine phosphorylation of eNOS, which appeared to be associated with intracellular translocation of eNOS. More recent studies have demonstrated that Akt (protein kinase B) activates eNOS by phosphorylation of a specific serine residue. The involvement of tyrosine phosphorylation in eNOS regulation has also been studied. Exposure of endothelial cells to vanadate resulted in a low level of tyrosine phosphorylation, compared with a much greater level of serine phosphorylation. The in vitro activity measured on immunoprecipitates of eNOS suggested that tyrosine phosphorylation reduced the activity of eNOS by about one half. Fleming et al found a low level of basal tyrosine phosphorylation of eNOS, which was lost after treatment of endothelial cells with a phosphotyrosine phosphatase inhibitor (phenylarsine oxide). Venema et al observed neither basal nor Bk-mediated stimulation of tyrosine phosphorylation of eNOS but did find that Bk activation of eNOS was accompanied by tyrosine phosphorylation of an eNOS-associated protein (ENAP-1). Activation of eNOS by flow-mediated shear stress appears to involve tyrosine phosphorylation, the activation being reduced by tyrosine kinase inhibitors and stimulated by inhibitors of phosphotyrosine phosphatases. Corson et al proposed that shear stress–mediated eNOS activity might be regulated by phosphorylation of the enzyme. They found an increase in serine/threonine phosphorylation, but not tyrosine phosphorylation, of eNOS after application of shear stress. Thus, the precise role of tyrosine phosphorylation in the regulation of eNOS activity is still uncertain.

In the present study, we examined the effect of phosphotyrosine phosphatase inhibition by sodium orthovanadate (vanadate) on eNO production and found an unexpectedly large and sustained increase in the amount of eNO produced after Bk- or calcium ionophore–mediated activation of eNOS. Although eNOS does not appear to be directly tyrosine-phosphorylated under these conditions, changes in the level of certain eNOS-associated phosphotyrosine-containing and other proteins suggest a role for tyrosine phosphorylation/dephosphorylation in the physiological regulation of eNOS activity.
Methods

Cell Culture

Endothelial cells used in this study were isolated from bovine aortas (BAECs) according to a standard method\(^1\) and verified as endothelial by positive immunostaining for von Willebrand factor, negative staining for muscle actin, and morphological examination by electron microscopy.\(^2\) Cells were routinely seeded in 12-well culture plates (Corning/Costar) at dilutions of 1:3 to 1:5 and grown to confluency in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U penicillin, 100 μg streptomycin, 0.25 μg amphotericin B, and 50 μg gentamycin per milliliter at 37°C in an atmosphere of 95% air/5% CO\(_2\).

Treatment Conditions

Cells were used 2 to 3 days after reaching confluence at a density of 1.5 to 1.7×10\(^5\) cells/cm\(^2\) and >98% viability by the trypan blue dye-exclusion test. Cell monolayers in 12-well cluster dishes were rinsed 3 times with standard PBS at 37°C, followed by the addition of 0.7 mL of Dulbecco’s modified Eagle’s medium containing antibiotics but no serum to each well. The NOS inhibitor L-NAME was added, where indicated, to a final concentration of 1 mmol/L and incubated for 30 minutes at 37°C before addition of the test compounds. Phosphotase or kinase inhibitors were added 5 minutes before NOS activation with Bk, A23187, vanadate, vanadate/Bk, or vanadate/A23187. Incubations were carried out at 37°C and test compounds were present throughout the incubation period. Solutions of sodium orthovanadate at a concentration of 1 mmol/L were prepared by heating at 95°C for 10 minutes, and actual concentrations of orthovanadate were determined by spectrophotometric measurement at 260 nm by using a millimolar extinction coefficient of 3.55.\(^3\)

Assay for NO as Nitrite in Culture Medium

The method for measuring nitrite, a stable reaction product of NO, involves the conversion of 2,3-diaminonaphthalene to the highly fluorescent 1-(\(^2\)H)naphtotriazole through specific reaction with nitrite under acidic conditions.\(^4\) Cell counts were performed on a hemocytometer. For determination of eNOS protein levels, aliquots of cell lysates were sufficient to lyse membrane and cytosolic fractions by 15 minutes of centrifugation at 100000g in a TL-100 ultracentrifuge with a fixed-angle rotor. Protein determinations were done using the bicinchoninic acid protein assay kit (Pierce) with bovine serum albumin as the standard.

Immunodetection of Proteins and Immunoprecipitation

For determination of eNOS protein levels, aliquots of cell lysates or fractions were either directly adsorbed to nitrocellulose membranes with a dot-blot apparatus (Bio-Rad) or separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes. For immunoprecipitations, cell lysates (~100 μg protein) were incubated with a specific antibody (~1 μg) for 1 hour at 4°C, followed by 1 hour at 4°C with protein A/G Sepharose (Sigma). Precipitated proteins were solubilized with SDS-PAGE sample buffer and electrophoretically separated. Blots were blocked in 1% gelatin or 5% nonfat dry milk in 10 mmol/L Tris · HCl (pH 7.5), 0.1 mol/L NaCl, and 0.2% Tween 20 and then incubated, where indicated, with 1:1000 dilutions of monoclonal antibodies specific for eNOS (Transduction Labs), caveolin-1 (Transduction Labs), protein tyrosine phosphate (clone PY20, Zymed Labs), or heat shock protein 90 (hs90; StressGen Labs). Horseradish peroxidase–conjugated IgG (Transduction Labs) was used to detect the respective primary monoclonal antibody by means of a horseradish peroxidase–activated chemiluminescent substrate (enhanced chemiluminescence, Amersham). Densitometric measurements on scanned images were completed by using ImageQuaNT software (Molecular Dynamics).

In Vitro Assay for eNOS Activity

The possible direct effect of sodium orthovanadate on eNOS activity in lysates of endothelial cells was tested by using an eNOS assay based on the conversion of \([^{\text{3}}\text{H}]\)-arginine to \([^{\text{3}}\text{H}]\)-citrulline.\(^5\) Assay mixtures contained 25 mmol/L HEPES (pH 7.4), 3 μmol/L tetrahydrodiproterin, 1 μmol/L FAD, 1 μmol/L flavin mononucleotide, 1 mmol/L NADPH, 0.6 mmol/L CaCl\(_2\), 0.1 mmol/L calmodulin, and 20 μC/mL \([^{\text{3}}\text{H}]\)-arginine (60 μCi/mmol). Assays were run for 30 and 60 minutes in the presence or absence of 1 mmol/L sodium orthovanadate at 37°C, and activity was compared with background levels in the absence of calcium or in the presence of the NOS inhibitor L-NAME. Lysates from cells treated with Bk and/or vanadate were also assayed for eNOS activity to determine whether the treatments resulted in any change in specific activity of eNOS.

Statistical Analysis

Data are expressed as mean±SEM. At least triplicate determinations were performed for quantitative analyses. Student’s t test was used for evaluating significance.

Results

Vanadate-Stimulated eNOS Production

Endothelial cells were incubated with the protein phosphotyrosine phosphatase inhibitor \(^6\) sodium orthovanadate, and the rate of eNOS production was measured during continuous exposure to either Bk or calcium ionophore A23187. A small, continuous basal level of eNOS was produced in the absence of vanadate, Bk, or A23187. Bk stimulated eNOS production to a level about double that of the untreated controls (Figure 1A), whereas A23187, which acts in a receptor-independent manner to directly raise the intracellular calcium concentration, caused a 4- to 5-fold increase in NO production (Figure 1B). Vanadate alone stimulated the formation of eNOS in a time-dependent manner even in the absence of added Bk or A23187. When Bk or A23187 was added to endothelial cells in the presence of vanadate, however, there was a dramatic and sustained enhancement of eNOS formation to levels 10 to 20 times that of controls (Figure 1). The amount of eNOS
generated by treatment with a combination of vanadate and either Bk or A23187 was 2-fold greater than the sum of eNO generated by treatment with the individual components, indicating a synergistic effect of vanadate and Bk or A23187 (Figure 1, summation curves).

We also examined the effect of phosphotyrosine phosphatase inhibition by vanadate over a range of Bk or A23187 concentrations. When cells were incubated with Bk (Figure 2A), the enhancing effects of 50 μmol/L vanadate were most apparent at the lower concentrations and reached a plateau as Bk activation peaked at ~10 μmol/L. Incubation of endothelial cells with A23187 led to a rapid and sustained rise in eNOS activity due to calcium influx, and vanadate further stimulated eNO production. This enhancement was most striking at A23187 concentrations, 5 μmol/L, but enhancement of eNO production by vanadate occurred even under conditions of apparent calcium saturation (Figure 2B). Incubation of cells in medium lacking calcium or containing EGTA completely abolished the sustained generation of eNO, indicating the necessity of a continuous influx of calcium from outside the cells for extended eNOS activity. Likewise, removal of L-arginine from the medium prevented the sustained generation of eNO (data not shown).

**Effects of Protein Kinase and Other Inhibitors on Vanadate-Stimulated NO Production**

The specific involvement of tyrosine phosphorylation in the enhancement of eNO production is shown in Figure 3. Cells were pretreated with the protein tyrosine kinase inhibitors genistein or tyrphostin ST638 before activation with either Bk (Figure 3A) or A23187 (Figure 3B), and the effects of vanadate were abrogated. Phenylarsine oxide, which inhibits a class of phosphotyrosine phosphatases that contain vicinal sulfhydryls at the active site, in contrast to vanadate, which mimics the transition state of phosphotyrosine phosphatase–catalyzed reactions, did not cause enhancement of eNO generation (Figure 3A), indicating that the class of phosphotyrosine phosphatases inhibited by phenylarsine oxide was not involved in the regulation of eNO production under these conditions. Pretreatment of endothelial cells with the NOS inhibitor L-NAME blocked eNO production, whereas co-treatment with dexamethasone, which blocks induction of the inducible isoform of NOS, had little effect (Figure 3B). Treatment with the protein synthesis inhibitor cycloheximide did not alter the vanadate response over the first 2 hours, suggesting that the enhancement most likely involved a constitutive protein (data not shown). Okadaic acid, a potent inhibitor of the phosphoserine/phosphothreonine phosphatases pp1 and pp2a, reduced the vanadate enhancement of eNO production in Bk-treated cells (Figure 3A). When endothelial cells were activated by A23187 in the presence of okadaic acid, however, there was no effect on the vanadate response, indicating that involvement of a putative serine kinase was specific for the Bk-mediated signaling pathway (Figure 3B). The same basic experiments were repeated with primary cultures of BAECs and human umbilical vein endothelial cells, with essentially the same results (data not shown).
Effect of Vanadate on eNOS Compartmentation and Expression

To determine whether vanadate affects the intracellular location of eNOS, the relative levels of eNOS protein were measured in cell lysates and subcellular fractions by immunoblotting and densitometry. After centrifugation of endothelial cell lysates at 100,000 g, 95% of total eNOS protein was associated with the particulate fraction, and vanadate treatment, in the presence or absence of Bk, caused little or no change in this distribution. Also, there was no significant difference in total eNOS protein under the various treatment conditions compared with controls. Treatment of endothelial cells with Bk, vanadate, or vanadate/Bk had no significant effect on the subcellular distribution of eNOS, as indicated by isopycnic centrifugation of cell lysates through a sucrose gradient under conditions designed to separate caveolae from denser membrane fractions.16 Most of the eNOS protein was associated with the caveolar peak fraction (fraction 3) under all treatment conditions (Figure 4). This fraction corresponded to the interface between 15% and 25% sucrose. Noncaveolar membrane fractions were distributed in denser regions of the gradient (fractions 4 to 8).16 The presence of eNOS and caveolin-1 in the peak fractions shown in Figure 4 was confirmed by Western blotting (data not shown). Because the samples were loaded from the bottom of the gradients, cytosolic and cytoskeletal proteins would remain in the 45% sucrose sample zone (fractions 8 to 10).

Effect of Vanadate on eNOS Activity Measured In Vitro

To determine whether vanadate affected eNOS directly, lysates of untreated endothelial cells were assayed for eNOS activity in the presence or absence of vanadate. No effect of vanadate, at a concentration of 1 mmol/L, was observed (data not shown), indicating that vanadate had no direct effect on eNOS. Lysates prepared from cells that had been incubated with vanadate or vanadate/Bk for 4 hours showed a slight increase in eNOS activity compared with controls (31.2 ± 2.4 versus 25.3 ± 2.0 pmol citrulline produced per minute per milligram protein), but this possible increase in eNOS activity measured in vitro does not account for the striking increase in NO production by endothelial cells after treatment with vanadate/Bk.

Vanadate-Induced Changes in Tyrosine-Phosphorylated Proteins

The state of tyrosine phosphorylation of eNOS and other protein components after the various treatment conditions was analyzed by Western blotting, after immunoprecipitation with anti-eNOS, by using an antibody probe to protein tyrosine phosphate. A tyrosine-phosphorylated protein with an apparent molecular mass of 125 kDa, along with lesser amounts of tyrosine-phosphorylated proteins with apparent molecular masses of 105 and 75 kDa, appeared to be specifically associated with eNOS after vanadate/Bk treatment, as indicated by coimmunoprecipitation with anti-eNOS (Figure 5). The apparent molecular mass of eNOS run under identical conditions was 140 kDa. Little or no tyrosine phosphorylation of eNOS was observed under these conditions.

Possible Role of hsp90 on Vanadate Stimulation of eNO Production

hsp90 is a molecular “chaperone” with a molecular mass of 90 kDa that functions, in part, to facilitate the folding of...
Results may be due to different experimental conditions. We observed a certain signal-transducing proteins.\textsuperscript{25,26} hsp90 has been shown to associate with and stimulate eNOS activity.\textsuperscript{27} Geldanamycin, an inhibitor of hsp90,\textsuperscript{25,26} inhibited the vanadate/Bk and vanadate/A23187 stimulation of eNO production with an apparent IC\textsubscript{50} of \approx 6 mol/L (data not shown). Direct involvement of hsp90 in the enhancement of eNOS activity under these conditions was further supported by the finding that an increased amount of hsp90 coimmunoprecipitated with eNOS after vanadate/Bk treatment (Figure 6).

**Figure 5.** Effect of vanadate on phosphotyrosine-containing proteins in Bk-stimulated endothelial cells. BAECs were incubated for 4 hours with 2 \mu mol/L Bk in the presence or absence of 50 \mu mol/L vanadate (V). Cell lysates were prepared, immunoprecipitated with anti-eNOS monoclonal antibody, and run on SDS PAGE. Blots were probed with an anti-phosphotyrosine monoclonal antibody (left) and anti-eNOS monoclonal antibody (right). C indicates control.

**Figure 6.** Immunoprecipitation of Bk-treated endothelial cell lysates with anti-eNOS monoclonal antibody. BAECs were incubated for 4 hours with 2 \mu mol/L Bk in the presence or absence of 50 \mu mol/L vanadate (V), followed by preparation of cell lysates and immunoprecipitation with an anti-eNOS monoclonal antibody. Immunoprecipitated proteins were separated by SDS PAGE and immunoblotted with the indicated monoclonal antibodies. C indicates control.

Discussion

Early studies with purified NOS showed that the enzyme could be a substrate for a number of protein kinases.\textsuperscript{28} Although the bulk of in vivo eNOS phosphorylation occurs on serine/threonine, there have been reports of tyrosine phosphorylation of eNOS itself.\textsuperscript{6,7} Garcia-Cardena et al\textsuperscript{6} found that treating endothelial cells with high levels (1 mmol/L) of vanadate caused an increase in basal tyrosine phosphorylation of eNOS and reduced eNOS activity by \approx 50\% in an in vitro immunoprecipitation-complex assay. Our results, in contrast, indicate that lower levels (50 \mu mol/L) of vanadate enhanced the Bk- and calcium ionophore–mediated production of eNO. This enhancement was apparently indirect, because there was no change in eNOS activity measured in vitro and no tyrosine phosphorylation of eNOS was observed. These apparently contradictory results may be due to different experimental conditions. We used a significantly lower concentration of vanadate (50 \mu mol/L versus 1 mmol/L) and focused on receptor-mediated production of eNO. It is unlikely that vanadate at the low concentration used in this study (50 \mu mol/L) could cause such an enhancement of eNOS activity through a nonspecific cellular effect. Venema et al\textsuperscript{8} also reported that binding of Bk to endothelial cells did not elicit tyrosine phosphorylation of eNOS, but rather of a 90-kDa ENAP-1, which appeared to lead to the translocation of eNOS to a detergent-insoluble fraction. The change in eNOS localization, however, did not result in a change in enzyme activity. Inhibition of tyrosine kinases blocked phosphorylation of ENAP-1 and also prevented the translocation of eNOS. We did not observe an alteration in the overall pattern of eNOS intracellular distribution under our experimental conditions. It should be noted, however, that our results represent a "steady-state" condition, because eNO production was monitored over several hours under the various conditions. Therefore, transient changes would not be detected in phosphorylation states or in intracellular location that have been reported by other investigators.\textsuperscript{2}

We found that continuous treatment of endothelial cells with Bk for up to 6 hours stimulated eNO production to about twice the basal level, whereas cotreatment with vanadate caused a much greater (\approx 20-fold) and synergistic agonist response. The response to vanadate was abolished by the tyrosine kinase inhibitors genistein\textsuperscript{18} and tyrphostin,\textsuperscript{19} thus demonstrating the specific involvement of tyrosine phosphorylation in this enhancement. We also observed a synergistic enhancement by vanadate of A23187-mediated activation of eNO production, suggesting that the observed vanadate effect was not mediated by Bk.

The tyrosine phosphorylation–associated enhancement of eNO production cannot be explained through an increase in the relative amounts of eNOS because Western blots showed no significant change in the distribution or in total eNOS levels in either particulate or cytosolic fractions after vanadate treatment, consistent with the lack of effect of cycloheximide on enhancement of eNO production by vanadate. The observed vanadate enhancement appears to be indirect, because vanadate had no effect on the specific activity of eNOS when included in an NOS assay of lysates from untreated cells. There was also little difference in the specific activity of eNOS from lysates of cells treated with Bk, vanadate, or Bk/vanadate.

Activation of eNOS may occur within the plasma membrane subcompartments known as caveolae, which are cholesterol-rich structures consisting of a scaffolding protein, caveolin-1, and an array of receptors and signal transduction factors. Caveolin-1 overexpression was found to inhibit eNOS activity in lysates from COS-7 cells coexpressing eNOS, and the effect was reversed by addition of calmodulin.\textsuperscript{29} It has also been reported that eNOS undergoes a flow-mediated dissociation from caveolin and a reassociation with calcium-calmodulin,\textsuperscript{30} which could serve to potentiate eNOS activity through maintaining the active dimeric form of the enzyme.\textsuperscript{31} Other proteins have been shown to bind to and modify eNOS activity, including ENAP-1,\textsuperscript{2} the Bk B2 receptor,\textsuperscript{32,33} and hsp90.\textsuperscript{27,34} We found that a 125-kDa tyrosine-phosphorylated protein, and possibly 105- and 75-kDa tyrosine-phosphorylated proteins, were associated with eNOS...
after treatment with Bk/vanadate. It was previously reported that eNOS coimmunoprecipitated with hsp90. Binding of hsp90 is associated with stimulation of eNOS activity, and enhanced binding of hsp90 to eNOS also appears to be involved in the estrogen receptor–mediated activation of eNOS. We found that hsp90 was apparently involved in the vanadate/Bk stimulation of eNOS production, on the basis of the inhibition of this stimulation by geldanamycin, a specific inhibitor of hsp90, and on the apparent increase in hsp90 that coimmunoprecipitated with eNOS after exposure to vanadate. It should be noted that hsp90 and ENAP-1 are of the same apparent size and may even be the same protein (R.C. Venema, personal communication, 2000). Thus, the increase in tyrosine phosphorylation of certain proteins appears to trigger a marked enhancement of the activation state of eNOS through association with specific proteins that are presumably involved in regulating eNOS activity in vivo.

Clearly, there are different pathways that may affect eNOS activity, depending on the relative types and levels of phosphorylation/dephosphorylation in a system. Through this regulated interaction of enzymes and accessory proteins, a fluid complex is formed that promotes efficient coupling of the components of the signaling pathway. Our results support the view that protein tyrosine phosphorylation plays a prominent role in the complex signaling network regulating eNOS activity in vivo. This tyrosine phosphorylation–mediated modulation of eNOS activity appears to be due, in large part, to association/dissociation of “modulator” proteins, rather than to a direct modification of tyrosine residues of eNOS.

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