

# Vanadate Is a Potent Activator of Endothelial Nitric-Oxide Synthase: Evidence for the Role of the Serine/Threonine Kinase Akt and the 90-kDa Heat Shock Protein

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## ABSTRACT

We investigated the molecular mechanisms of sodium vanadate (vanadate)-induced nitric oxide (NO) production. Exposure of bovine lung microvascular cells (BLMVEC) to vanadate increased the release of biologically active NO in endothelium/smooth muscle cocultures, as measured by the accumulation of its surrogate marker, cGMP. This release was sensitive to NO synthase (NOS) inhibition and was greater than that observed with ionomycin. Although calcium chelators (BAPTA, EGTA) inhibited basal and ionomycin-induced NO production, they failed to inhibit vanadate-induced NO release. Moreover, in the absence of calcium/calmodulin, cell lysates from vanadate-treated cells exhibited greater NOS activity compared with control cells. Vanadate activates the phosphoinositide3-kinase (PI3-K)/Akt pathway, which is known to increase endothelial NOS (eNOS) activity by direct phosphorylation of Ser-1179. Treat-

ment of BLMVEC with vanadate resulted in phosphorylation of both Akt and endothelial NOS. In addition, wortmannin, a PI3-K inhibitor, blocked both the vanadate-induced phosphorylation of eNOS and the increase in cGMP accumulation. Similarly, adenovirus-mediated gene transfer of an activation deficient form of Akt (AA-Akt) blocked the release of NO brought about by vanadate. To further investigate the mechanism of action of vanadate, eNOS was immunoprecipitated and its association with proteins that alter eNOS activity was tested. Immunoblots demonstrated that the eNOS-caveolin interaction remained unaffected by vanadate, whereas vanadate promoted recruitment of the 90-kDa heat shock protein (hsp90) to eNOS. We conclude that vanadate causes NO release via a mechanism that involves Akt-induced eNOS phosphorylation and increased binding of the activator protein hsp90 to eNOS.

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Nitric oxide (NO) is a two-atom compound synthesized by different cells types and tissues in the body (Forstermann et al., 1994). Contrary to its simple chemical structure, NO has a complex role in physiology subserving a multitude of diverse phenomena. In the nervous system, it is believed to function as a neurotransmitter, whereas the immune system takes advantage of its cytotoxic properties that are apparent at higher concentrations using NO to protect organisms against infection (Forstermann et al., 1994; Moncada, 1997). In the cardiovascular system, NO was first discovered in the endothelium as a mediator of vasorelaxation. It is now known that NO does not only regulate vessel tone, platelet aggrega-

**ABBREVIATIONS:** NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; NOS, nitric-oxide synthase; hsp90, 90-kDa heat shock protein; vanadate, sodium vanadate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; AM, acetoxymethyl ester; Ab, antibody; IBMX, 3-isobutyl-1-methylxanthine; L-NAME, *N*<sup>ω</sup>-nitro-L-arginine methyl ester; BLMVEC, bovine lung microvascular endothelial cells; HBSS, Hanks' balanced salt solution; PAGE, polyacrylamide gel electrophoresis; RASM, rat aortic smooth muscle cells; β-gal, β-galactosidase; PI3-K, phosphatidylinositol-3-kinase.

tion and leukocyte adhesion, but is also involved in paracrine and autocrine pathways within the vessel wall being implicated in the control of vascular cell growth and remodelling (von der Leyen et al., 1995; Murohara et al., 1998; Rudic et al., 1998) endothelial cell apoptosis (Dimmeler and Zeiher, 1999), migration and organization (Ziche et al., 1994; Papapetropoulos et al., 1997).

Three different isoforms of the enzyme that catalyzes NO synthesis from L-arginine have been cloned (Papapetropoulos et al., 1999). Two of them, the endothelial (eNOS) and the neuronal isoforms, are activated by increases in intracellular calcium that in turn promote calmodulin binding to NOS, whereas the isoform originally isolated from the immune system has calmodulin bound to it, even at resting calcium levels, and exhibits significantly higher activity (Forstermann et al., 1994). Both eNOS and neuronal NOS are targeted, although by different mechanisms, to cell membranes (Brennan et al., 1996; Fulton et al., 2001). eNOS is present on Golgi and caveolar membranes. Targeting of eNOS to caveolae depends on co- and post-translational modifications (myristoylation, palmitoylation) and is required for optimal enzyme activity (Fulton et al., 2001).

All of the "classic" agonists used to stimulate NO release from the endothelium (i.e., acetylcholine, histamine, bradykinin) do so by increasing intracellular calcium (Moncada, 1997). Recently, a more complex mode of regulation for eNOS has evolved through protein-protein interactions and phosphorylation. eNOS is known to directly interact not only with calmodulin, but also with the 90-kDa heat shock protein (hsp90), the intracellular domain of the bradykinin B2 receptor, dynamin-2, and caveolins 1 and 3 (Fulton et al., 2001). Overexpression of hsp90 or dynamin-2 stimulates NOS enzymatic activity (Garcia-Cardena et al., 1998). In contrast, experiments with recombinant proteins demonstrated that increased interaction of eNOS with caveolin-1 or the bradykinin B2 receptor attenuates the release of NO (Garcia-Cardena et al., 1997; Ju et al., 1998). In addition, we and others have shown that bovine eNOS is phosphorylated on Ser-1179 (1177 for the human eNOS) by the Ser/Thr kinase Akt (Dimmeler et al., 1999; Fulton et al., 1999), the AMP-activated kinase (Chen et al., 1999), and cyclic nucleotide-dependent protein kinases (Butt et al., 2000); it should be noted that phosphorylation of eNOS at this residue leads to increased production of NO even at low calcium concentrations. More recently, additional phosphorylation sites affecting eNOS activity have been shown to exist (Michell et al., 2001; Bauer et al., 2003).

At least three different stimuli (insulin-like growth factor-1, estrogen, and mechanical shear stress) have been demonstrated to cause calcium-independent NO release, presumably through tyrosine phosphorylation cascades (Tsukahara et al., 1994; Corson et al., 1996; Russell et al., 2000). In addition, sodium vanadate (vanadate), a tyrosine phosphatase inhibitor, has been shown to promote endothelium-dependent relaxations that are attenuated by NOS inhibitors (Nakaike et al., 1996; Misurski et al., 2000). The structurally dissimilar inhibitor of tyrosine phosphatases, phenylarsine oxide, has also been shown to increase NO release from human endothelial cells (Fleming et al., 1996, 1998). The aim of the present study was to investigate the molecular mechanisms by which vanadate stimulates NO release from cultured endothelial cells.

## Materials and Methods

**Materials.** BLMVEC were obtained from Vec Technologies (Rensselaer, NY). Tissue culture plasticware and endothelial cell growth supplement were from Collaborative Biomedical Products (Bedford, MA). Growth medium and fetal calf serum were purchased from Invitrogen (Carlsbad, CA). BAPTA/AM was obtained from Calbiochem (San Diego, CA). Protein A Sepharose was obtained from Amersham Biosciences (Piscataway, NJ). The anti-eNOS monoclonal antibody (9D10) was obtained from Zymed Laboratories, Inc. (South San Francisco, CA). The anti-caveolin-1 was purchased from BD Transduction Laboratories (Lexington, KY). The anti-hsp90 Ab was obtained from Stress Gen Biotechnologies Corp. (Victoria, BC, Canada). The anti-Akt, anti-phospho-Akt, and anti-phospho eNOS (Ser-1179) were from Cell Signaling Technology (Beverly, MA). Anti-phospho-eNOS Ser-116, Thr-497, and Ser-635 were generated as described previously (Bauer et al., 2003). The ECL detection system and the horseradish peroxidase conjugated anti-mouse antibody and L-[2,3,4,5-<sup>3</sup>H]arginine (55 Ci/mmol) were obtained from Amersham Biosciences. Tetrahydrobiopterin was acquired from J. B. Schircks (Jona, Switzerland). Nitrocellulose membranes were purchased from Gelman Sciences (Ann Arbor, MI). X-ray film was obtained from Eastman Kodak (Rochester, NY). The <sup>125</sup>I-cGMP radioimmunoassay kit was purchased from Biomedical Technologies Inc. (Stoughton, MA). All other chemicals, including penicillin, streptomycin, bovine serum albumin, wortmannin, genistein, 3-isobutyl-1-methylxanthine (IBMX), Nonidet P-40, phenylmethylsulfonyl fluoride, aprotinin, EDTA, EGTA, *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME), ionomycin, L-arginine, calmodulin, NADPH, HEPES, Dowex AG50WX8, and others were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of sodium orthovanadate (vanadate, assuming a hydration number of 10) were prepared in sterile distilled water and adjusted to pH 10. The solution was then heated to boiling until translucent, and the pH was readjusted to 10.

**Cell Culture.** Bovine lung microvascular cells (BLMVEC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10 U/ml penicillin, and 100 μg/ml streptomycin. Cultures had typical cobblestone morphology and stained uniformly for von Willebrand factor, as assessed by indirect immunofluorescence. Smooth muscle cells were isolated from rat aortae (Papapetropoulos et al., 1994) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, antibiotics, and L-glutamine.

**Determination of cGMP Accumulation.** To determine NO production, we measured the intracellular concentration of its surrogate marker cGMP. Rat aortic smooth muscle cells were used as reporters because BLMVEC were devoid of sGC activity. BLMVEC were cultured on glass coverslips in 24-well plates and used at confluence. Endothelial cells on coverslips were exposed to 1 mM sodium vanadate for 45 min, washed with Hanks' balanced salt solution (HBSS) and transferred using the coverslip onto reporter smooth muscle cell cultures for 15 min. IBMX (1 mM) was added to the cocultures to inhibit the phosphodiesterases. Coverslips were then removed, HBSS was aspirated and 250 μl of 0.1 N HCl was added to each well to stop enzymatic reactions and to extract cGMP. Thirty minutes later, the HCl extract was collected and stored until analyzed by radioimmunoassay. To normalize cGMP values, protein content in each well was measured by the Bradford method after solubilization of the protein with 1 N NaOH. To chelate calcium, cells were incubated in calcium-free calcium HBSS in the presence of 0.25 mM EGTA or BAPTA (5 μM).

**Immunoprecipitations and Western Blotting.** BLMVEC were incubated with or without sodium orthovanadate (1 mM) for 45 min. Cells were then washed with phosphate-buffered saline and lysed in a modified radioimmunoprecipitation assay buffer [100 mM Tris-HCl, 50 mM NaCl, pH 7.4, 1% (v/v) Nonidet P-40, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. Lysates were then Dounce-homogenized, and insoluble material was pelleted by centrifugation (12,000g for 10

min at 4°C). Protein was determined by the method of Lowry (1951). Samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blotted with an anti-Akt or anti-eNOS Ab or with phosphospecific antibodies for Akt or eNOS. For immunoprecipitations, 500  $\mu\text{g}$  of protein from each group were precleared. Lysates were then incubated for 2 h with an anti-eNOS monoclonal Ab or an anti-Hsp90 Ab at 4°C. Protein A-Sepharose was then added and incubated for an additional 1 h. The immune complexes were washed three times with radioimmunoprecipitation assay buffer and boiled in SDS-PAGE sample buffer for 5 min. Immunoprecipitated samples were separated by SDS-PAGE in a 7.5% gel followed by overnight transfer of the proteins to nitrocellulose membranes. Membranes were blocked with 0.1% Tween 20 in Tris-buffered saline containing 5% nonfat dry milk for 2 h at room temperature, incubated with an anti-eNOS, anti-caveolin-1, anti-hsp90, or a phospho-specific eNOS Ab, and washed. They were then incubated with a secondary Ab before being developed with the enhanced chemiluminescence substrate (ECL).

**Assays for NOS Activity.** Endothelial cells were washed twice with phosphate-buffered saline and lysed in buffer containing 50 mM Tris-HCl, pH 7.5, 1% (v/v) Nonidet P-40, 10 mM NaF, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu\text{g}/\text{ml}$  aprotinin, and 10  $\mu\text{g}/\text{ml}$  leupeptin. Lysates were then transferred to an Eppendorf tube and rotated for 30 min at 4°C. Lysates were Dounce-homogenized (50 strokes), and insoluble material was removed by centrifugation at 12,000g for 10 min at 4°C. The protein concentration of the soluble material was determined using a Lowry assay. The conversion of L-arginine to L-citrulline was assayed as described previously (McCabe et al., 2000). Briefly, cell lysates (100  $\mu\text{g}$ ) were incubated for 3 to 10 min at 23°C in the following reaction mixture: 3 pmol of L-[ $^3\text{H}$ ]arginine (55 Ci/mmol), 10 to 300  $\mu\text{M}$  arginine, 1 mM NADPH, 120 to 200 nM calmodulin, 2 mM  $\text{CaCl}_2$ , and 30  $\mu\text{M}$   $\text{BH}_4$  in a final reaction volume of 50 to 100  $\mu\text{l}$ . The reaction was terminated by the addition of 0.5 ml of 20 mM HEPES, pH 5.5, containing 2 mM EGTA and EDTA. The reaction mixture was placed over Dowex AG50WX8, and the flow-through was counted on a liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences, Boston MA).

**Data Analysis and Statistics.** Data are presented as means  $\pm$  S.E.M. or as percentage of control of the indicated number of observations. Statistical comparisons between groups were performed using the Student's *t* test or one-way analysis of variance followed by a post hoc test (Neuman-Keuls' or Dunnett's), as appropriate. Differences among means were considered significant when  $p < 0.05$ .

## Results

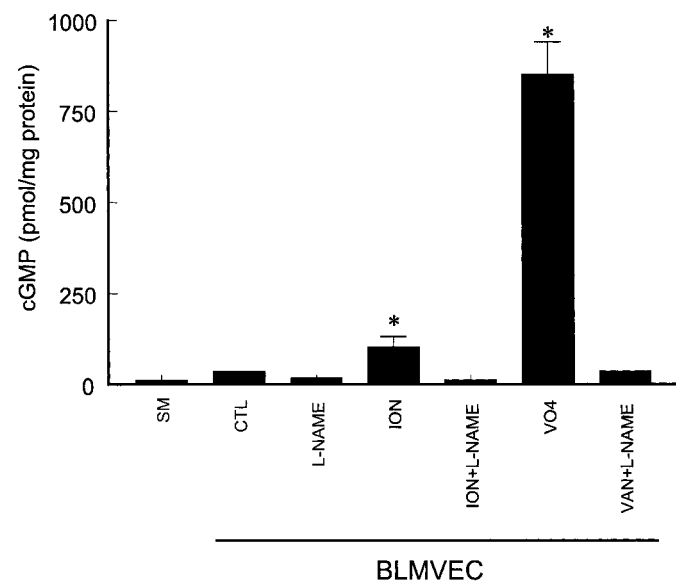
**Vanadate Induces NO-Dependent cGMP Accumulation in Cultured Cells.** To determine whether exposure of BLMVEC to vanadate leads to increased NO production, we used a bioassay system [cocultures of BLMVEC and rat aortic smooth muscle cells (RASM)]. Use of smooth muscle cells as reporters was necessary, because BLMVEC do not express soluble guanylate cyclase activity, as demonstrated by the lack of cGMP accumulation in response to sodium nitroprusside stimulation (data not shown). Incubation of RASM with BLMVEC for 15 min led to an increase in intracellular cGMP levels in the muscle cells that was inhibited by the NOS inhibitor L-NAME (Fig. 1). Addition of ionomycin (0.1  $\mu\text{M}$ ) to the medium bathing the cocultures caused a further increase in smooth muscle cGMP content. Treatment of BLMVEC with sodium vanadate (1 mM) for 45 min stimulated a far greater increase in NO production than that observed with ionomycin.

**Calcium Dependence of Nitric Oxide Release.** To determine whether the observed increase in NO release depends on increases in the levels of intracellular calcium, we

pretreated cells with BAPTA or EGTA. Removal of extracellular calcium (EGTA) or chelation of intracellular calcium (BAPTA-AM) inhibited both basal and ionomycin-stimulated NO production without affecting vanadate-induced NO release (Fig. 2A). Next, we investigated whether vanadate directly influences the calcium dependence of eNOS using NOS activity assays under  $V_{\text{max}}$  conditions. Lysates prepared from vanadate-treated endothelial cells had greater enzymatic activity than control cells and retained full activity in the absence of additional calcium/calmodulin (Fig. 2B).

**Vanadate Promotes eNOS Phosphorylation.** Endothelial NOS activity is known to be affected by phosphorylation. To determine the effect of vanadate on the phosphorylation status of eNOS, BLMVEC were pretreated with vanadate, and lysates were analyzed with phosphospecific antibodies. After exposure to vanadate, we observed an increase in total tyrosine phosphorylation and an increase in phosphorylation of Ser-116, Thr-497, Ser-635, and Ser-1197 (Fig. 3). Because the most extensively characterized phosphorylation site on eNOS is Ser-1197, we proceeded to determine the role of the Akt kinase that phosphorylates this site on vanadate-stimulated NO production.

**Vanadate-Induced NO Release Is Mediated through the PI3-K/Akt Pathway.** Inhibition of protein tyrosine phosphatases by sodium vanadate is known to activate Ser/Thr kinases. One of the kinases activated in certain cells by tyrosine phosphatase inhibition is Akt, a Ser/Thr kinase known to phosphorylate and activate eNOS on Ser-1179. To determine whether exposure of BLMVEC to vanadate activates Akt, we treated cells with vanadate and determined the phosphorylation of Akt using an antibody that specifically recognizes the Ser-473-phosphorylated form of Akt. Indeed, vanadate-treated cells exhibited increased levels of phospho-Akt (Fig. 4A). To test the involvement of PI3-K, an upstream



**Fig. 1.** Exposure of BLMVEC to sodium vanadate leads to the production of NO. BLMVEC were incubated in the presence or absence of 1 mM vanadate (VO4) for 45 min. After the pretreatment, cells were washed with HBSS and incubated with or without L-NAME (100  $\mu\text{M}$ ) for 30 min; they were then transferred to confluent RASM that served as reporters. Cocultures of BLMVEC and RASM were incubated in the presence of IBMX for 15 min. For ionomycin (ION)-stimulated cocultures, ionomycin was present during this last 15-min period at 0.1  $\mu\text{M}$ . Means  $\pm$  S.E.M.,  $n = 4$  wells; \*,  $p < 0.05$  from control. SM, smooth muscle; CTL, control.

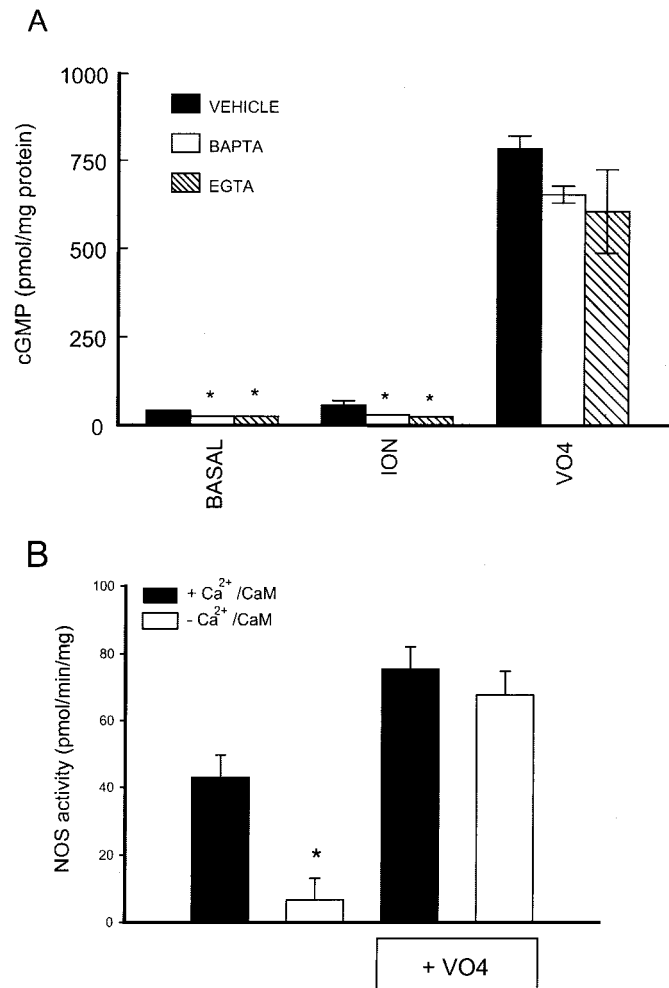
mediator of Akt activation, on Akt and eNOS phosphorylation, cells were exposed to wortmannin before stimulation with vanadate. Inhibition of PI3-K abolished vanadate-induced Akt phosphorylation and partially reduced eNOS phosphorylation on Ser-1179 (Fig. 4, A and B).

To further investigate the mechanisms involved in vanadate-induced activation of Akt and eNOS, experiments were performed in the presence and absence of the nonselective tyrosine kinase inhibitor genistein (Fig. 5). Pretreatment of cells with genistein blocked the phosphorylation of Akt on Ser-473 and eNOS on Ser-1179, suggesting that vanadate-stimulated NO release is mediated through tyrosine kinase activation.

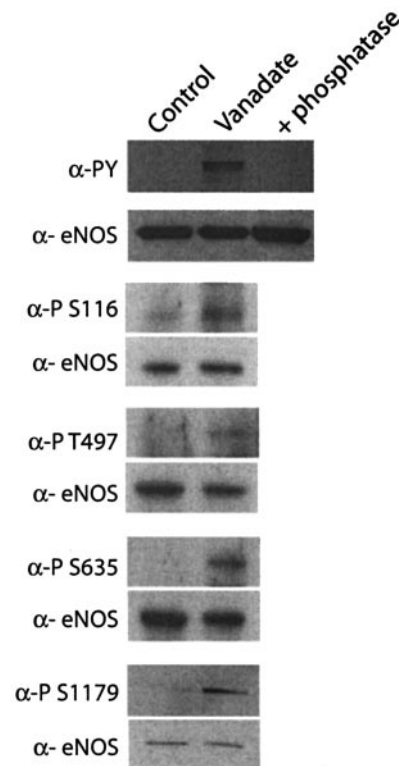
To determine the role of the PI-3K/Akt pathway in vanadate-induced NO release, BLMVEC were pretreated with

wortmannin before stimulation with vanadate. Wortmannin pretreatment abolished the release of biologically active NO ( $731 \pm 35$  pmol/mg of protein and  $162 \pm 44$  pmol/mg of protein for vanadate-treated cells and wortmannin + vanadate, respectively), suggesting that inhibition of protein tyrosine phosphatases by vanadate resulted in activation of PI3-K that ultimately led to activation of eNOS (Fig. 6A). To provide a link between vanadate-induced Akt activation and NO release, BLMVEC were infected with adenoviral vectors expressing either an activation deficient form of Akt (AA-Akt) or  $\beta$ -galactosidase ( $\beta$ -gal), and cGMP accumulation in reporter smooth muscle cells was measured (Fig. 6B). Cells infected with the  $\beta$ -gal virus showed a  $22.1 \pm 3.2$ -fold increase in the release of NO, whereas cells infected with the AA Akt virus increased their NO production by  $5.8 \pm 0.8$ -fold, suggesting that Akt, mediates, in part, the effects of vanadate.

**eNOS-Caveolin and eNOS-hsp90 Interactions in Cells Exposed to Vanadate.** To further investigate the molecular mechanisms leading to calcium-independent NO production after stimulation with sodium vanadate, eNOS was immunoprecipitated in control and vanadate-treated bovine endothelial cells and association of caveolin-1 and hsp90 was examined. As seen in Fig. 7A, the coassociation of caveolin-1 with eNOS was not altered by vanadate treatment, indicating that increased NO production does not result from withdrawal of this negative eNOS regulator from the protein complex. In contrast, greater amounts of hsp90 were recov-



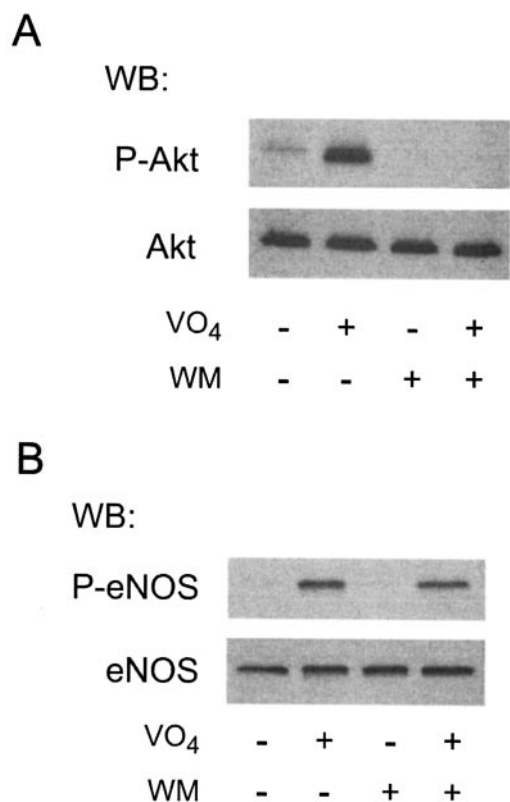
**Fig. 2.** Vanadate-induced NO release is calcium-independent. A, BLMVEC were incubated with or without 1 mM vanadate for 45 min. They were then incubated in calcium-free HBSS in the presence of vehicle (dimethyl sulfoxide), EGTA (0.25 mM), or BAPTA (5  $\mu$ M) for an additional 30 min. BLMVEC were then washed with calcium-free HBSS and transferred to confluent rat aortic smooth muscle cells that served as reporters. Cocultures of BLMVEC and RASM were incubated in the presence of IBMX for 15 min. For ionomycin-stimulated cocultures, ionomycin was present during this last 15-min period at 0.1  $\mu$ M. Means  $\pm$  S.E.M.,  $n = 4$  wells \*,  $p < 0.05$  from vehicle. B, BLMVEC were incubated with or without 1 mM vanadate (VO4) for 10 min. NOS activity was then determined in the detergent-soluble lysates of treated cells (100  $\mu$ g of protein) in the presence or absence of calcium (2 mM)/calmodulin (CaM; 120 nM). Means  $\pm$  S.E.M.,  $n = 4$ . \*,  $p < 0.05$  from Ca<sup>2+</sup>/CaM.



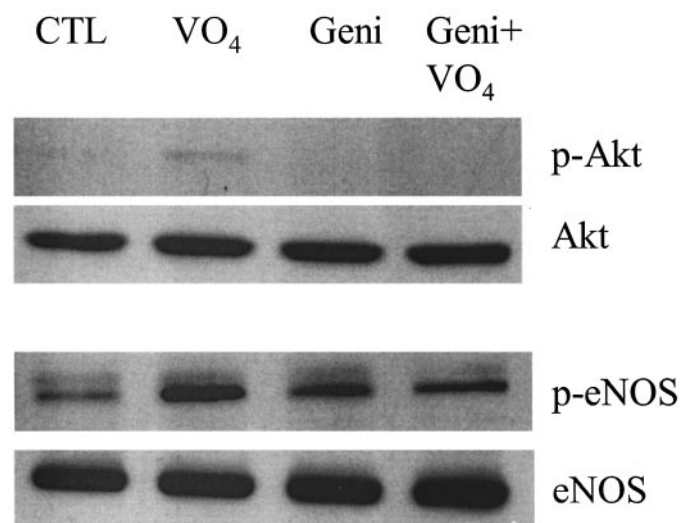
**Fig. 3.** Effects of vanadate on eNOS phosphorylation BLMVEC were treated with vanadate (1 mM for 45min). After cell lysis, samples were subjected to SDS-PAGE, and membranes were blotted with the indicated phosphospecific antibody or an eNOS Ab. For the experiment with the phosphotyrosine Ab ( $\alpha$ -PY), eNOS was immunoprecipitated and treated with calf intestinal phosphatase (60 units for 30 min at 37°C) before loading onto the gel.

ered in eNOS immunoprecipitates after vanadate treatment. Moreover, hsp90 immunoprecipitated from vanadate-treated

cells had greater amounts of Ser-1179 phospho-eNOS complexed than control cells (Fig. 7B). No change in the overall level of eNOS, hsp90, or caveolin-1 was observed after treatment with vanadate (Fig. 7C).



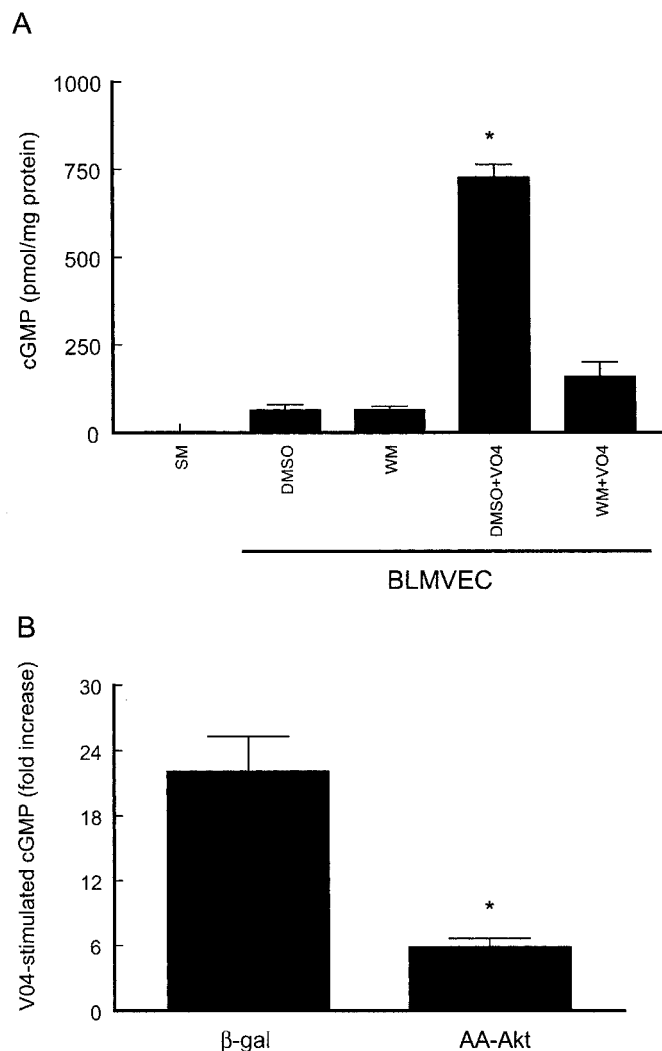
**Fig. 4.** Wortmannin (WM) inhibits vanadate (VO<sub>4</sub>)-induced Akt and eNOS phosphorylation. BLMVEC were pretreated with vehicle (dimethyl sulfoxide) or wortmannin (100 nM) for 30 min and then incubated with sodium vanadate (1 mM) for 45 min. After cell lysis, samples were subjected to SDS-PAGE and membranes were blotted with a phospho-specific anti-Akt Ab (A) or anti-eNOS Ser-1179 Ab (B), stripped, and reblotted with an anti-Akt Ab (A) or an anti-eNOS Ab (B). WB, Western blot;



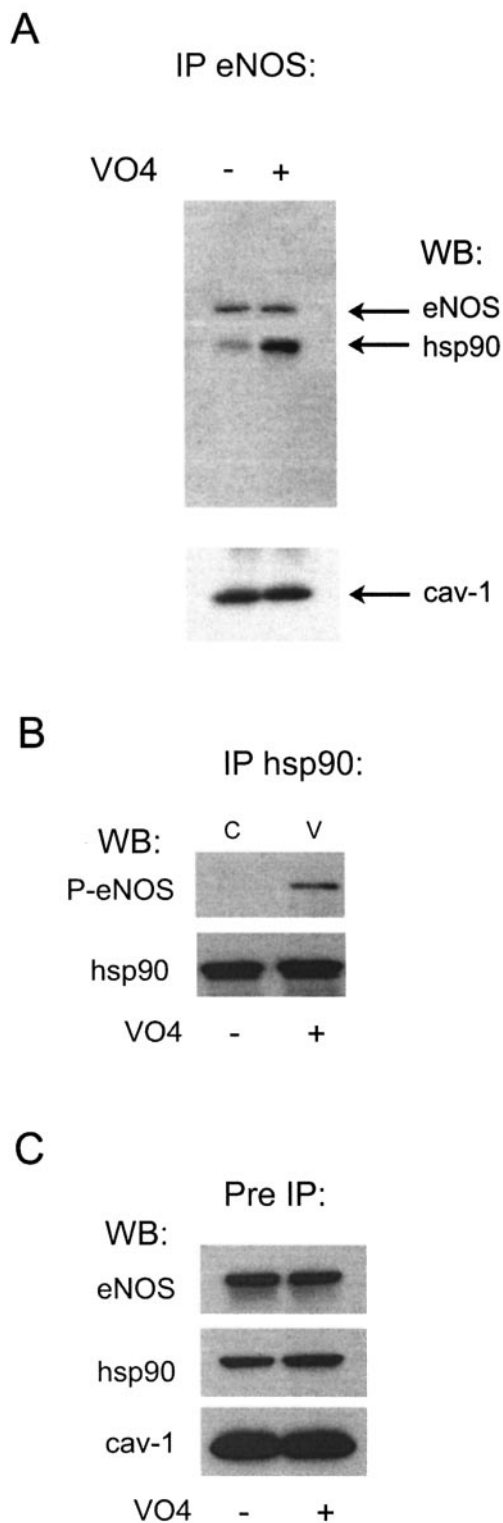
**Fig. 5.** Vanadate (VO<sub>4</sub>)-induced Akt and eNOS phosphorylation is tyrosine kinase-dependent. BLMVEC were pretreated with vehicle (EtOH) or genistein (Geni; 50 μM) for 30 min and then incubated with sodium vanadate (1 mM) for 45 min. After cell lysis, samples were subjected to SDS-PAGE and membranes were blotted with a phospho-specific anti-Akt or anti-eNOS Ser-1179 Abs, stripped, and reblotted with anti-total Akt or eNOS Abs.

### Discussion

Vanadate has been demonstrated to elicit endothelium-dependent relaxations in porcine coronary, renal, and femoral arteries and jugular veins, as well as in the perfused rat mesenteric vascular bed (Nakaike et al., 1996; Misurski et al., 2000). These relaxations are blocked by pretreatment of the vessels with the nitric-oxide synthase inhibitor, L-NAME or through removal of the endothelium. Moreover, a chemically distinct tyrosine phosphatase inhibitor, phenylarsine oxide, promotes relaxation of isolated rabbit carotid arteries



**Fig. 6.** Vanadate (VO<sub>4</sub>)-induced NO production is PI3-K/Akt-dependent. A, BLMVEC were incubated in the presence or absence of wortmannin (100 nM for 30 min). After the pretreatment, cells were exposed to 1 mM vanadate for an additional hour. BLMVEC were then transferred to confluent RASM that served as reporters. Cocultures of BLMVEC and RASM were incubated in the presence of IBMX for 15 min. Means ± S.E.M., n = 4 wells; \*, p < 0.05 from dimethyl sulfoxide. B, BLMVEC were infected with an adenovirus coding for the activation deficient form of Akt or β-gal. After 36 h, BLMVEC were transferred to smooth muscle cells that served as reporters and processed as in Fig. 1. Means ± S.E.M., n = 4 wells; \*, p < 0.05 β-gal.



**Fig. 7.** Vanadate (VO4) promotes the association of eNOS with hsp90 as well as eNOS phosphorylation. A, BLMVEC were incubated with or without sodium vanadate (1 mM) in complete medium for 45 min before cell lysis. eNOS was immunoprecipitated (IP), and complexes were separated by standard SDS-PAGE. Membranes were then blotted with an anti-hsp90 and anti-eNOS Ab, stripped, and reblotted with a caveolin-1 Ab. Bands were visualized using the enhanced chemiluminescence substrate. B, treatments for BLMVEC were performed as in A. hsp90 was immunoprecipitated and subjected to SDS-PAGE. Membranes were blotted with an anti-hsp90 or anti-phospho-eNOS Ab (Ser-1179). C, control; V, vanadate. C, cells were incubated with or without sodium vanadate as in A and cell lysates were probed for total eNOS, hsp90, and caveolin-1. WB, Western blot.

(Fleming et al., 1998) suggesting that inhibition of tyrosine phosphatases promotes the release of NO from the endothelium of intact blood vessels. In the present study, exposure of microvascular endothelial cells to the tyrosine phosphatase inhibitor vanadate increased the release of NO, as measured by its surrogate marker cGMP, as well as eNOS catalytic activity. In addition to supporting the data in intact blood vessels, our observations are in agreement with the finding that phenylarsine oxide stimulates the release of biologically active NO from human cells and that vanadate stimulates the accumulation of nitrite in bovine aortic endothelial cells (Fleming et al., 1998).

Exposure of endothelial cells to tyrosine phosphatase inhibitors causes a transient rise in free cytosolic calcium (Fleming et al., 1996). We therefore tested whether the vanadate-stimulated NO release was dependent on increased intracellular calcium by exposing cells to the calcium chelators BAPTA-AM and EGTA. Although chelation of calcium was effective in blocking both basal and ionomycin-induced NO release, it did not alter the effect of vanadate on NO release. Similarly, removal of extracellular calcium did not affect the ability of phenylarsine oxide to release biologically active NO from human umbilical vein endothelial cells or to elicit vasodilation of rabbit carotid arteries (Fleming et al., 1998). In line with these observations, Hellermann et al. (2000) reported that low concentrations of vanadate (50  $\mu$ M) potentiated bradykinin-induced accumulation of nitrite even under conditions of apparent calcium saturation. In NOS activity assays, a direct measure of enzyme catalytic function, vanadate-treated endothelial cells exhibit greater eNOS activity than control cells. The increase in NOS activity stimulated by vanadate is maintained in the absence of additional calcium/calmodulin, which contrasts the calcium/calmodulin dependent activity of control cells. These results suggest that the ability of vanadate to stimulate calcium-independent NOS activity is caused, at least in part, by direct post-translational modification of the enzyme. Although it has previously been reported that vanadate increased eNOS tyrosine phosphorylation and decreased eNOS activity in immunoprecipitated eNOS (Garcia-Cardena et al., 1996), the present data and that of others (Hellermann et al., 2000) show increased activity in cell lysates, NO release, and cGMP accumulation. The reason for this discrepancy is not known and could relate to the fact that in the immunoactivity assays, eNOS is bound to an antibody at a particular epitope that may differentially affect function and perhaps because other cytosolic factors important for activity are removed during the immunoactivity assays.

The primary sequence of eNOS reveals several putative phosphorylation sites on serine, threonine, or tyrosine residues. Ser-635 and Ser-1179 have been shown to be positive regulatory sites, in that phosphorylation at these sites increase eNOS activity both in vitro and in vivo, whereas phosphorylation of Ser-116 and Thr-497 is thought to negatively regulate NO production (Fulton et al., 2001; Michell et al., 2001). In addition, Ser-617 might play a role in controlling phosphorylation of additional sites and eNOS interactions with other proteins (Bauer et al., 2003). Pretreatment of BLVEC cells with vanadate increased the phosphorylation of Ser-116, Thr-497, Ser-635, Ser-1197, and overall tyrosine phosphorylation. Although, the individual roles of specific phosphorylation sites in response to vanadate stimulation

remain to be elucidated, the increased phosphorylation at all of the above-mentioned sites is associated with enhanced eNOS activity and calcium-independent NO release.

Ser-1179 is the best characterized phosphorylation site of eNOS. Several kinases, including AMP-activated kinase, cGMP- and cAMP-dependent protein kinases, and Akt (protein kinase B) (Fulton et al., 2001) are known to phosphorylate eNOS on this residue. Particular interest has been drawn to Akt, because many known activators of eNOS [including vascular endothelial growth factor, sphingosine-1-phosphate, bradykinin, and estrogen activate Akt (Morales-Ruiz et al., 2000; Simoncini et al., 2000; Harris et al., 2001)]. Akt is a Ser/Thr kinase that lies downstream of phosphoinositide kinase-3, mediating many of the growth promoting, antiapoptotic, and migratory effects of endothelium-specific receptor tyrosine kinases, such as vascular endothelial growth factor receptor and Tie-2 (Coffer et al., 1998; Brazil and Hemmings, 2001). Upon growth factor receptor activation, Akt is recruited from its inactive state in the cytosol to the membrane via the attraction of its lipid-binding Plekstrin homology domain to the phosphorylated products of PI3-K (Coffer et al., 1998; Brazil and Hemmings, 2001). Once at the plasma membrane, Akt is phosphorylated by 3-phosphoinositide-dependent protein kinase-1 on Thr-308; full activation of this kinase is achieved through phosphorylation of a second site at Ser-473 and more recently by phosphorylation of Tyr-474 (Conus et al., 2002). Because tyrosine phosphatase inhibition leads to phosphorylation and activation of Akt in rat adipocytes and glioma cells, presumably by attenuating the activity of the phosphatase PTEN (Wijkander et al., 1997; Chin et al., 1999) or increasing the phosphorylation of Tyr-474 (Conus et al., 2002), we sought to determine whether exposure of microvascular cells to vanadate was followed by activation of Akt. Pretreatment of cells with vanadate stimulated phosphorylation of Akt on Ser-473. This effect of vanadate was inhibited by wortmannin, suggesting that PI3-K is responsible for the vanadate-induced Akt phosphorylation. Moreover, the observation that pretreatment of cells with genistein blocks the vanadate-induced Akt phosphorylation suggests a requirement for tyrosine kinase activation in this response. Indeed, vanadate is known to activate membranous nonreceptor protein tyrosine kinases in rat adipocytes (Elberg et al., 1997).

We and others have shown that transfection of wild-type Akt, but not a kinase dead Akt mutant, leads to phosphorylation of eNOS. This phosphorylation occurs on Ser-1179 (1177 for the human eNOS) and leads to increased nitrite accumulation and endothelium-dependent vasodilation in vivo (Fulton et al., 1999; Luo et al., 2000). Moreover, adenovirus-mediated gene transfer of an activation-deficient form of Akt (AA-Akt) blocks the vascular endothelial growth factor-stimulated NO release and vasomotion. To determine whether activation of Akt by vanadate leads to phosphorylation of eNOS, endothelial cells were treated with vanadate and the levels of phospho-eNOS were determined. Indeed, exposure of cells to vanadate increased eNOS phosphorylation on Ser-1179 in a wortmannin-sensitive manner. As with the phosphorylation of Akt, inhibition of tyrosine kinase activity by genistein reduced the vanadate-stimulated eNOS phosphorylation. This observation is in line with the reported ability of genistein to attenuate vanadate-induced relaxations in porcine arteries and vanadate-induced nitrite accu-

mulation in cultured endothelial cells (Nakaike et al., 1996; Hellermann et al., 2000). Moreover, pretreatment of endothelial cells with wortmannin or infection with an adenovirus coding for AA-Akt blocked vanadate-induced cGMP accumulation, further suggesting that vanadate-induced NO production is mediated via the PI3-K/Akt pathway. The inability of AA-Akt to completely block the vanadate-induced NO release (compared with wortmannin) probably reflects the fact that this form of Akt is not a true dominant-negative protein; rather, it exhibits a low level of basal activity and lacks the ability to be stimulated through phosphorylation on Ser-473 and Thr-308 (because these two residues have been mutated to alanines). Alternatively, because vanadate alters the phosphorylation of eNOS on multiple residues inhibiting the action of Akt might not be enough to suppress NO release.

The fact that increased NO production in vanadate-treated cells is resistant to calcium chelating agents is in line with the observation that Ser-1179 phospho-eNOS is fully active at lower levels of intracellular calcium. The S1179D mutant of eNOS offers an insight to the mechanism through which phosphorylation of eNOS at Ser-1179 leads to increased activity at low calcium concentrations. In this mutant of eNOS, Ser-1179 is altered to aspartic acid to substitute for the negative charge afforded by the addition of phosphate. S1179D eNOS displays increased catalytic activity because of an increase in electron flux at the reductase domain of the enzyme (McCabe et al., 2000). In addition, dissociation of calmodulin from S1179D eNOS occurs at a slower rate than from wild-type eNOS at low calcium concentrations.

Most of the eNOS in the endothelium is associated with cellular membranes, where its activity is affected through binding to caveolin-1. Increased association of caveolin-1 with eNOS has been shown to inhibit NO production in transfected cells, and peptides from the scaffolding domain of caveolin-1 attenuate eNOS enzymatic activity (Fulton et al., 2001). Direct proof that caveolin-1 acts as a negative regulator of eNOS is offered by experiments with caveolin-1 knockout mice. Vessels from caveolin-1  $-/-$  animals exhibit enhanced NO release, contain greater amounts of cGMP, and show enhanced vasodilatory responses to acetylcholine (Drab et al., 2001; Razani et al., 2001). Alterations in the interaction between caveolin-1 and eNOS have also been shown to occur in the context of disease: impaired eNOS activity in an experimental model of cirrhosis is caused by increased caveolin-1 expression and binding to eNOS (Shah et al., 1999). To examine the contribution of caveolin-1 to eNOS activation by vanadate, eNOS was immunoprecipitated from vanadate-stimulated cells and the amount of associated caveolin-1 was determined by Western blot. Under these conditions, no change in the amount of caveolin-1 associated with eNOS was demonstrated, suggesting that increased production of NO after vanadate treatment does not result from the withdrawal of the inhibitory action of caveolin-1.

hsp90 can also interact with eNOS and positively influence function, and the formation of the hsp90/eNOS heterocomplexes correlates temporally with NO release (Garcia-Cardena et al., 1998; Brouet et al., 2001; Fontana et al., 2002). We observed that treatment of cells with vanadate leads to increased recruitment of hsp90 to the eNOS complex. Similarly, exposure of bovine aortic endothelial cells to bradykinin and vanadate increased the amount of hsp90 bound to eNOS (Hellermann et al., 2000), and pretreatment of cells

with geldamycin, an inhibitor of Hsp90, blocked the vanadate-stimulated NO production. Moreover, two groups (Brouet et al., 2001; Fontana et al., 2002) have recently shown that once hsp90 is recruited to eNOS, it facilitates the phosphorylation of eNOS by Akt. The increase in eNOS phosphorylation is achieved through the reduced dephosphorylation of Akt and through increased ability of Akt to phosphorylate hsp90-bound eNOS (Fontana et al., 2002). These observations are in agreement with our finding that increased amounts of Ser-1179-phosphorylated eNOS are present in hsp90 precipitates after vanadate treatment; in addition to increasing the phosphorylation of the eNOS complex, these increased amounts might make the enzyme less susceptible to the inhibitory action of caveolin, as suggested by the observation that hsp90-bound eNOS is less sensitive to the inhibitory action of the caveolin scaffolding domain peptide (Gratton et al., 2000).

Thus, our data support the idea that vanadate is a potent activator of eNOS. Moreover, vanadate-induced NO release is calcium-independent and is associated with increased recruitment of hsp90 to the eNOS complex. Finally, exposure of endothelial cells to vanadate promotes the phosphorylation of eNOS on Ser, Thr, and Tyr residues, altering its activity. Because vanadate-stimulated NO release is attenuated by both wortmannin and a dominant-negative, activation-deficient Akt adenovirus, it is likely that the eNOS phosphorylation site that conforms to the accepted Akt substrate motif (i.e., S1179) is the major determinant of vanadate-induced NO release. The results of the present study reinforce the notion that phosphorylation of eNOS and protein-protein interactions are important in regulating its activity.

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