Ca$^{2+}$-Independent Activation of the Endothelial Nitric Oxide Synthase in Response to Tyrosine Phosphatase Inhibitors and Fluid Shear Stress

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Abstract—Fluid shear stress enhances NO formation via a Ca$^{2+}$-independent tyrosine kinase inhibitor–sensitive pathway. In the present study, we investigated the effects of the protein tyrosine phosphatase inhibitor phenylarsine oxide and of fluid shear stress on endothelial NO production as well as on the membrane association and phosphorylation of the NO synthase (NOS) III. Phenylarsine oxide (10 μmol/L) induced an immediate and maintained NO-mediated relaxation of isolated rabbit carotid arteries, which was insensitive to the removal of extracellular Ca$^{2+}$ and the calmodulin antagonist calmidazolium. This phenylarsine oxide–induced vasodilatation was unaffected by genistein but abrogated by the tyrosine kinase inhibitor erbstatin A. Incubation of native or cultured endothelial cells with phenylarsine oxide resulted in a time-dependent tyrosine phosphorylation of mainly Triton X-100–insoluble (cytoskeletal) proteins, along with a parallel change in the detergent solubility of NOS III, such that the enzyme was recovered in the cytoskeletal fraction. A similar, though slightly delayed, phenomenon was also observed after the application of fluid shear stress but not in response to any receptor-dependent agonist. Although Ca$^{2+}$-independent NO formation was sensitive to erbstatin A, phenylarsine oxide-induced treatment was associated with the tyrosine dephosphorylation of NOS III rather than its hyperphosphorylation. Proteins that also underwent redistribution in response to the tyrosine phosphatase inhibitor included paxillin, phospholipase C-γ₁, mitogen-activated protein kinase, and the tyrosine kinases Src and Fyn. We envisage that fluid shear stress and tyrosine phosphatase inhibitors may alter the conformation and/or protein coupling of NOS III, facilitating its interaction with specific phospholipids, proteins, and/or protein kinases that enhance/maintain its Ca$^{2+}$-independent activation. (Circ Res. 1998;82:686-695.)

Key Words: tyrosine kinase • shear stress • nitric oxide • cytoskeleton

The endothelial NO synthase (NOS III) is classified as a constitutive and strictly Ca$^{2+}$/calmodulin-dependent enzyme.¹ An increase in [Ca$^{2+}$ i] as observed after receptor-dependent and -independent agonist stimulation, enhances endothelial NO production and elicits vasodilatation in isolated arterial segments (for review see Reference 2). Both the agonist-induced NO formation and subsequent vasodilatation are abolished by the removal of Ca$^{2+}$ from the extracellular fluid.³,⁴ However, a basal enzyme activity, which is sensitive to the NOS inhibitor (L-NNA), is evident in native endothelial cells at Ca$^{2+}$ i concentrations as low as 10 nmol/L, indicating that a significant portion of the NO produced by unstimulated endothelial cells may be formed via a Ca$^{2+}$-independent pathway.⁵ Little physiological relevance was attributed to this phenomenon, and the identification of a calmodulin-binding domain in the primary structure of NOS III⁶–⁸ together with the finding that calmodulin binding proteins inhibited enzyme activity¹ strengthened the hypothesis that the binding of a Ca$^{2+}$/calmodulin complex is essential to activate the constitutive enzyme.

Maintained exposure of native endothelial cells to fluid shear stress results in the sustained production of NO,² a phenomenon that is directly at odds with the transient, shear stress–induced increase in [Ca$^{2+}$ i].¹⁰–¹² Such observations imply that shear stress elicits the production of NO via a pathway that is independent of a maintained increase in [Ca$^{2+}$ i]. Indeed, we² and others¹³,¹⁴ have recently found that the mechanical stimulation of native and cultured endothelial cells by fluid shear stress results in the formation of NO via a pathway that is unaffected by either the removal of extracellular Ca$^{2+}$ or the application of calmodulin antagonists. Although alterations in pH i may contribute to the sustained Ca$^{2+}$-independent activation of NOS III,⁶,⁹ it is likely that additional signaling pathways, protein-protein interactions, and/or posttranslational modification of the NOS III protein are involved in the regulation of endothelial NO production. The effects of cell stimulation on the phosphorylation of NOS III and caveolin/NOS III/calmodulin interactions as well as the subsequent effects on the production of NO by endothelial cells have been investigated by several
groups.\textsuperscript{12-15} Although such interactions may have an impact on the regulation of agonist-induced NOS III activation, the signaling cascade resulting in the Ca\textsuperscript{2+}-independent activation of NOS III is not necessarily associated with an identical signal transduction pathway.\textsuperscript{9} Therefore, we determined the effect of the signal transduction pathway. Recent findings have demonstrated that the tyrosine phosphorylation of NOS III is not necessarily associated with an identical enzyme activation pattern.\textsuperscript{17} Therefore, we determined the effect of enhancing cellular levels of phosphoryroside on endothelial NO production. In the present study, we report that tyrosine phosphatase inhibitors, like fluid shear stress, activate NOS III in a Ca\textsuperscript{2+}-independent manner sensitive to erbstatin A and herbimycin A. This effect could not be attributed to an enhanced tyrosine phosphorylation of NOS III but was associated with a change in the detergent solubility of the enzyme, such that the enzyme was “redistributed” from the Triton X-100–soluble to the –insoluble cell fraction.

Materials and Methods

Materials

Genistein and Pansorbin were obtained from Calbiochem-Novabiochem GmbH; erbstatin A, from Biomol; and BAFTA-AM, from Molecular Probes. HEPES and L-NNA (free acid) were from Serva; M-199 medium, geldanamycin, and herbimycin A, from Gibco; penicillin, streptomycin, L-glutamine, glutathione, and β-mercaptoethanol (Biotect protection medium), from Biochrom; and heat-inactivated FCS, from Vitromex. The antibodies to NOS III, Lck, and anti-Fyn were from Santa Cruz Biotechnology, Inc; the focal adhesion kinase (p125\textsuperscript{FAK}), NOS III, and P-xin antibodies, from Transduction Laboratories; and the phosphotyrosine, PLC-γ, and MAP kinase antibodies, from Upstate Biotechnology Inc. Recombinant bovine superoxide dismutase was provided by Grünenthal. Phenylarsine oxide, 2,3-dimercaptopropanol, IBMX, orthovanadate, sodium nitroprusside, Zn-potase inhibitors as described in “Results.” Thereafter, cells were washed with ice-cold HEPES buffer containing NaF (10 mmol/L), Na,PO\textsubscript{4} (15 mmol/L), and Na,VO\textsubscript{3} (2 mmol/L) and harvested by scraping.

Determination of cGMP Concentration

The concentration of cGMP in confluent cultured human or porcine endothelial cells under resting conditions and after stimulation in the presence of the phosphodiesterase inhibitor IBMX (0.1 mmol/L) was determined by a specific radioimmunooassay. In the case of shear stress, IBMX was present only during the last 30 minutes of stimulation. Because of the variation in basal cGMP content between cell batches (from 1.5±0.16 to 25.5±3.6 pmol/mg protein), the results are expressed as percentage of control cGMP content.

Immunoblotting

Endothelial cell suspensions were centrifuged at 13 000g for 60 seconds; cells contained in the pellet were then lysed in buffer containing leupeptin (2 μg/mL), pepstatin A (2 μg/mL), trypsin inhibitor (10 μg/mL), PMSF (44 μmol/L), and Triton X-100 (1% [vol/vol]), left on ice for 10 minutes, and centrifuged at 10 000g for 10 minutes. Proteins in the supernatant or from the Triton X-100–insoluble fraction were eluted by heating with SDS-PAGE sample buffer and were separated by 10% or 7% SDS-PAGE, as described previously.\textsuperscript{21} Proteins were detected by using their respective antibodies as described in “Results” and were visualized by enhanced chemiluminescence using a commercially available kit (Amersham). Prestained molecular weight marker proteins (Bio-Rad) were used as standards for the SDS-PAGE.

Isolation of Caveolin-Rich Membrane Domains and Determination of NOS III Activity

Freshly isolated porcine aortae were slit longitudinally, mounted in an open chamber, and washed twice in HEPES-modified Tyrode’s solution (mmol/L: NaCl 132, KCl 4, CaCl\textsubscript{2} 1, MgCl\textsubscript{2} 0.5, HEPES 9.5, and glucose 5), and the exposed endothelial layer was incubated at 37°C in the presence and absence of agonists as indicated in “Results.” Thereafter, the incubation was stopped by exchanging the incubation medium with ice-cold HEPES buffer, and the cells were harvested by scraping.

Preparation of Native Porcine Endothelial Cells

Freshly isolated porcine aortae were slit longitudinally, mounted in an open chamber, and washed twice in HEPES-modified Tyrode’s solution (mmol/L: NaCl 132, KCl 4, CaCl\textsubscript{2} 1, MgCl\textsubscript{2} 0.5, HEPES 9.5, and glucose 5), and the exposed endothelial layer was incubated at 37°C in the presence and absence of agonists as indicated in “Results.” Thereafter, the incubation was stopped by exchanging the incubation medium with ice-cold HEPES buffer, and the cells were harvested by scraping.

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(10 μmol/L, 10 minutes). Thereafter, the incubation was stopped by exchanging the incubation medium with ice-cold HEPES buffer, the cells were harvested by scraping, the cytosolic cell fraction and caveolin-rich membrane domains were prepared by detergent-free sequential centrifugation as described, and the presence of NOS III in each fraction was determined by Western blotting after separation of proteins by SDS-PAGE. NOS activity in aliquots (4 μg protein) of the cytosolic and caveolar cell fractions was assayed by the ability of the samples to stimulate purified soluble guanylyl cyclase as previously described.

32P Labeling, NOS III Immunoprecipitation, and Phosphoamino Acid Analysis
Subconfluent cultured porcine endothelial cells were loaded with 32P (H3PO4, 30 μmol/L) in 6 hours in phosphate-free Tyrode’s solution. After stimulation, cells were harvested and lysed in ice-cold homogenization buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% [vol/vol] Triton X-100, 25 mmol/L NaF, 1 mmol/L Na3VO4, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 10 μg/ml trypsin inhibitor, and 1% [vol/vol] Triton X-100). Lysates were boiled in sample buffer and separated by SDS-PAGE. NOS III antibody/protein complexes were recovered after incubation with Pansorbin and washed three times with homogenization buffer. The immunoprecipitates were boiled in sample buffer and separated by SDS-PAGE. In some experiments, proteins were transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore), and after autoradiography, the labeled NOS III protein band was excised, hydrolyzed by heating to 110°C in HCl (6N) for 45 minutes, and subsequently dried in a vacuum concentrator. The phosphoamino acids were extracted by boiling the samples to stimulate purified soluble guanylyl cyclase as previously described.

Statistical Analysis
Unless otherwise indicated, data are expressed as mean±SEM. Statistical evaluation was performed using Student’s t test for unpaired data, one-way ANOVA followed by a Bonferroni t test, or ANOVA for repeated measures where appropriate. Values of P<.05 were considered statistically significant.

Results
Effects of Phenylarsine Oxide on NO Production and Intracellular cGMP in Native and Cultured Endothelial Cells
In the isolated, perfused, phenylephrine-constricted rabbit carotid artery, repetitive stimulation with ACh (0.3 μmol/L) resulted in a highly reproducible vasodilatation (~80% of the preconstriction, Fig 1A). The protein tyrosine phosphatase inhibitor phenylarsine oxide (10 μmol/L) also induced an endothelium-dependent vasodilatation, which consisted of an initial increase followed by a sustained plateau phase (Fig 1A). A similar endothelium-dependent vasodilatation was observed in response to pervanadate (30 μmol/L, data not shown). After the removal of extracellular Ca2+ from the luminal perfusate ([Ca2+]o=25±6 mmol/L), repetitive stimulation with ACh (0.3 μmol/L) resulted in a gradual attenuation of the ACh-induced vasodilator response indicative of the depletion of endothelial Ca2+ stores. The loss of responsiveness to ACh was not due to receptor desensitization, since the same protocol performed in the presence of extracellular Ca2+ resulted in reproducible responses (Fig 1A). The subsequent application of phenylarsine oxide (10 μmol/L) induced a marked and maintained increase in vessel diameter. Dilation of rabbit carotid arteries to phenylarsine oxide was 55±6.8% in the presence of extracellular Ca2+ compared with 48.0±7.5% in the absence of Ca2+ (P=NS, n=7).

Pretreatment of the segments with the NOS inhibitor L-NNA abolished the Ca2+-independent vasodilator elicited by the phosphatase inhibitor (Fig 1C). The phenylarsine oxide–induced vasodilatation was unaffected by the calmodulin antagonist calmidazolium (10 μmol/L), which abrogated the NO-mediated vasodilator response to ACh (Fig 2A).

To determine the role of tyrosine kinases in modulating NO-mediated vasodilatation after the application of phenylarsine oxide, we investigated the effect of tyrosine kinase inhibitors. Under Ca2+-free conditions, the tyrosine kinase inhibitor erbstatin A (30 μmol/L) abolished the dilator response of carotid artery segments to phenylarsine oxide (Fig 2B); identical effects were observed in the presence of extracellular Ca2+ (not shown). The tyrosine kinase inhibitor herbimycin A (5 μmol/L, not shown) also inhibited the phenylarsine oxide–elicited, Ca2+-independent, NO-mediated vasodilatation, whereas genistein (100 μmol/L) was without effect (Fig 2B).

As a further index of NO production, phenylarsine oxide–induced changes in the intracellular concentration of cGMP...
were monitored in cultured human endothelial cells. Phenylarsine oxide (10 μmol/L) induced a time-dependent increase in intracellular cGMP that peaked 2 minutes after its application. Although the removal of extracellular Ca^{2+} decreased basal levels of the cyclic nucleotide (cGMP was 10.8 ± 1.35 pmol cGMP/mg protein in the presence of extracellular Ca^{2+} compared with 4.25 ± 1.24 pmol cGMP/mg protein in the absence of extracellular Ca^{2+}, P < .01, n = 4), phenylarsine oxide still induced an increase in intracellular cGMP comparable to that observed in the presence of Ca^{2+} (Fig 3A). The phenylarsine oxide–induced increase in cGMP, in both the presence and absence of extracellular Ca^{2+}, was abolished in cells treated with either the tyrosine kinase inhibitor erbstatin A (30 μmol/L) (Fig 3B) or the NOS inhibitor L-NNA (0.3 mmol/L, not shown).

Phenylarsine Oxide–Induced Alteration in the Detergent Solubility of NOS III

As reported previously,22 phenylarsine oxide induced a time-dependent increase in tyrosine phosphorylation of a series of mainly cytoskeletal proteins isolated from primary cultures of human endothelial cells. Erbstatin A (30 μmol/L) did not prevent an increase in the tyrosine phosphorylation of cytoskeletal proteins after the application of phenylarsine oxide but modified the pattern of tyrosine phosphorylation observed (Fig 4A).

In cultured human and porcine endothelial cells under resting conditions, NOS III was recovered mainly (87 ± 5%) in the Triton X-100–soluble fraction, as determined by densitometric analysis of fractions obtained from 20 different primary cultures of human and porcine endothelial cells. Only a small proportion of the total NOS III protein could be detected in the Triton-insoluble (cytoskeletal) fraction (11 ± 2%). The application of phenylarsine oxide (10 μmol/L) to cultured endothelial cells was associated with a time-dependent decrease in NOS III recovered in the Triton X-100–soluble fraction and concomitant increase in the Triton-insoluble fraction (Fig 4B). Similar findings were also obtained using native porcine and rat aortic endothelial cells (not shown).

The phenylarsine oxide–induced alteration in the detergent solubility of NOS III was unaltered by the removal of extracellular Ca^{2+} or by pretreatment of cells with the intracellular Ca^{2+} chelator BAPTA (10 μmol/L) (Fig 5A).

Erbstatin A attenuated, but did not prevent, the phenylarsine oxide–induced alteration in the detergent solubility of NOS III.
oxide–induced alteration in NOS III detergent solubility (Fig 5B), whereas genistein (100 μmol/L) was without effect (not shown). No effect on the distribution of NOS III was observed after application of either the receptor-dependent agonist bradykinin (100 nmol/L), the Ca\(^{2+}\)-ATPase inhibitor thapsigargin (1 μmol/L), or the sulfhydryl reagent thimerosal (5 μmol/L) (data not shown), all of which activate NOS III in a Ca\(^{2+}\)-dependent manner.

The assay of NOS III activity in the Triton X-100–insoluble cell fraction was hindered by the need to use denaturing conditions to resolubilize the enzyme. To circumvent this problem, NOS III activity was assayed using a specific NOS III antibody as described in the text. The results presented are representative of experiments performed using four different primary cultures of human endothelial cells.

Shear Stress–Induced NO Production and Alteration of the Detergent Solubility of NOS III

Exposure of primary cultured human or porcine endothelial cells to shear stress (45 dynes/cm\(^2\)) in a cone-plate viscometer was associated with an immediate and maintained increase in the L-NNA–sensitive production of cGMP (Fig 6A). In BAPTA (10 μmol/L–loaded endothelial cells, basal levels of intracellular cGMP were ~8-fold lower (P<.05) than cGMP levels in untreated endothelial cells; however, the application of shear stress induced an increase in cGMP that was biphasic, with an initial peak being apparent within 5 minutes of the application of shear stress. Thereafter, cGMP levels returned to baseline levels but began to increase after an additional 30 minutes to 1 hour and were maintained at a constant level (Fig 6A). This shear–stress–induced increase in cGMP, in both the presence and absence of BAPTA, was abolished in cells pretreated with the tyrosine kinase inhibitors erbstatin A (30 μmol/L) or herbimycin A (5 μmol/L), with cGMP levels remaining at 80% of control (not shown).

Given the similarities in the Ca\(^{2+}\)-independence of NO production after application of phenylarsine oxide or shear stress, we investigated the effects of shear stress on the detergent solubility of NOS III. Shear stress resulted in the redistribution of NOS III from the Triton X-100–soluble to the TX-insoluble fraction (Fig 6B and 6C). This shear stress–induced change in the detergent solubility of NOS III was attenuated in cells pretreated with the tyrosine kinase inhibitors erbstatin A (30 μmol/L) or herbimycin A (5 μmol/L), and genistein (100 μmol/L), all of which activate NOS III in a Ca\(^{2+}\)-independent manner.

The phenylarsine oxide (PAO)-induced redistribution of NOS III to the cytoskeletal fraction is insensitive to the chelation of either extracellular or intracellular Ca\(^{2+}\) but sensitive to erbstatin A (Erb A). A, Western blot analyses showing the effect of solvent (CTL) and PAO (10 μmol/L, 15 minutes) on the recovery of NOS III from the Triton X-100–soluble (TX-sol) and Triton X-100–insoluble (TX-insol) fraction of cells incubated in the presence or absence of extracellular Ca\(^{2+}\) or in the presence of the intracellular Ca\(^{2+}\)-chelating compound BAPTA (10 μmol/L, 30 minutes). B, Western blot showing the effect of Erb A (30 μmol/L) on the PAO-induced redistribution of NOS III to the TX-insol fraction. After cell stimulation, TX-sol and TX-insol cell fractions were prepared, separated by SDS-PAGE, and probed using a specific NOS III antibody as described in the text. The results presented are representative of experiments performed using four different primary cultures of human endothelial cells.
Phenylarsine Oxide Induces the Redistribution of Cytoskeleton-Associated Proteins and Tyrosine Kinases

Proteins that underwent a similar redistribution in response to the tyrosine phosphatase inhibitor included paxillin, PLC-γ1, the tyrosine kinases Src and Fyn, and the 42- and 44-kD isoforms of the MAP kinase. The phenylarsine oxide–induced alteration in detergent insolubility was not a generalized phenomenon, since no effect was observed on any of the other proteins investigated (eg, FAK, Lck; Fig 8). The caveolar marker protein, caveolin (21 kD), was detected only in the Triton X-100–insoluble cell fraction and was unaffected by phenylarsine oxide (not shown).

NOS III Phosphorylation

Of the proteins time-dependently tyrosine-phosphorylated after the application of phenylarsine oxide (see Fig 4) was a faint band of ≈135 kD, suggesting that NOS III may be tyrosine-phosphorylated. Immunoprecipitation of NOS III was performed using lysates prepared from native porcine aortic endothelial cells. Under these conditions, phosphotyrosine blotting revealed one major (135 kD) and three minor (≈97, 115, and 150 kD; Fig 9A) tyrosine-phosphorylated proteins. The same four proteins were immunoprecipitated using two different NOS III antibodies, but not an unrelated antibody. The 135-kD protein was identified as NOS III by reprobing the same membrane with a specific antibody. After the application of phenylarsine oxide (10 μmol/L, 15 minutes), there was a marked decline in the tyrosine phosphorylation of NOS III (Fig 9A). In separate experiments, immunoprecipitation of NOS III from the detergent-soluble fraction confirmed its basal tyrosine phosphorylation; however, immunoprecipitation of NOS III from the detergent-insoluble cell fraction after stimulation with phenylarsine oxide failed to result in the recovery of a tyrosine-phosphorylated 135-kD protein.

Figure 6. Effect of fluid shear stress on the activation of NOS III and its detergent solubility. A, Cultured endothelial cells were exposed to shear stress (45 dynes/cm²) in a cone-plate viscometer for 2 to 120 minutes in the absence (○) and presence (●) of the intracellular Ca²⁺ chelator BAPTA (10 μmol/L), and intracellular levels of cGMP were determined as described. The results are presented as the mean±SEM from 14 different primary cultures of human endothelial cells. *P<.05. B and C, Endothelial cells were exposed to shear stress (45 dynes/cm²) for 30 seconds to 8 hours. After cell stimulation, Triton X-100–soluble (Tx-sol) and Triton X-100–insoluble (Tx-insol) cell fractions were prepared, separated by SDS-PAGE, and probed using a specific NOS III antibody as described in the text. The results presented are representative of experiments performed using seven different primary cultures of human endothelial cells.

Figure 7. Effect of the tyrosine kinase inhibitors herbimycin A and geldanamycin on the shear stress–induced redistribution of NOS III to the Triton X-100–insoluble (Tx-insol) cell fraction. Western blot analyses show the effect of shear stress (45 dynes/cm²) on the recovery of NOS III from the Tx-insol cell fraction. Experiments were performed in the absence and presence of herbimycin A (5 μmol/L) and geldanamycin (1.8 μmol/L). After cell stimulation, Triton X-100–soluble (Tx-sol) and Tx-insol cell fractions were prepared, separated by SDS-PAGE, and probed using a specific NOS III antibody as described in the text. The results presented are representative of experiments performed using four different primary cultures of human endothelial cells.

Figure 8. Effect of phenylarsine oxide (PAO) on the redistribution of signal proteins to the cytoskeletal fraction in endothelial cells. Western blot analysis of the Triton X-100–insoluble fraction from cells pretreated with either solvent (CTL) or PAO (10 μmol/L, 15 minutes). Membranes were probed with monoclonal antibodies to paxillin, Fyn, Src, Lck, FAK, PLC-γ1, and MAP kinase. The results presented are representative of experiments performed using three different primary cultures of human endothelial cells.
Ca\textsuperscript{2+}-Independent NO Formation

**Figure 9.** Effects of phenylarsine oxide (PAO) on the phosphorylation of NOS III. A, Western blot analysis of NOS III immunoprecipitated from total cell lysates prepared from native porcine aortic endothelial cells. Cells were stimulated with either solvent (CTL, 0.01% dimethyl sulfoxide) or PAO (10 μmol/L, 15 minutes), and NOS III was immunoprecipitated as described. Precipitates were separated by SDS-PAGE and immunoblotted with an anti-phosphotyrosine antibody. Membranes were subsequently reprobed with a monoclonal anti-NOS III antibody. The results presented are representative of experiments performed using three different preparations. P. Tyr indicates phosphorytrosine. B, Phosphoamino acid analysis of the NOS III immunoprecipitated from unstimulated primary cultures of porcine aortic endothelial cells. Two-dimensional thin-layer electrophoresis (at pH 1.9 and then at pH 3.5) was performed as described in the text, and the data shown are representative of eight additional experiments. Peptide indicates nonhydrolyzed peptides; P-Y, weak phosphothreonine and phosphotyrosine signals being detected (Fig 9B). The qualitative nature of this method, however, renders it impossible to comment further on stoichiometry of NOS III phosphorylation.

**Discussion**

In the present study, we have demonstrated that the tyrosine phosphatase inhibitor phenylarsine oxide activates NOS III and induces vasodilatation via a Ca\textsuperscript{2+}-independent pathway, induced a similar but slightly delayed redistribution of NOS III, which was attenuated by the tyrosine kinase inhibitors erbstatin A, herbimycin A, and geldanamycin. No effect on the distribution of NOS III was observed after application of Ca\textsuperscript{2+}-elevating receptor-dependent and -independent agonists. Since phenylarsine oxide treatment induced a decrease in the tyrosine phosphorylation of NOS III, rather than hyperphosphorylation, our results suggest that the Ca\textsuperscript{2+}/calmodulin-independent activation of NOS III is related to the activation of a tyrosine kinase pathway, which indirectly regulates NOS III activity.

Cellular levels of phenophosphorytosine appear to have a marked impact on signaling in endothelial cells. For example, tyrosine kinase inhibitors have been shown to selectively attenuate Ca\textsuperscript{2+} influx into agonist-stimulated endothelial cells, whereas the tyrosine phosphatase inhibitors phenylarsine oxide and vanadate are reported to activate transmembraneous Ca\textsuperscript{2+} influx via an inositol 1,4,5-trisphosphate-independent mechanism. However, there do appear to be marked differences in the role played by phosphotyrosine in mediating the endothelial response to receptor-dependent and receptor-independent stimulation. For example, erbstatin A, which abolished the phenylarsine oxide–induced vasodilatation as well as the shear stress–induced production of NO, only slightly attenuated the vasodilator response to acetylcholine in carotid arteries. Although it was initially presumed that the concomitant effects of phenylarsine oxide on [Ca\textsuperscript{2+}], and endothelial NO production were related, the results of the present study demonstrate that phenylarsine oxide also stimulates NOS III via a Ca\textsuperscript{2+}-independent mechanism involving tyrosine kinase activation. The endothelial response elicited by phenylarsine oxide therefore exhibits characteristics identical to those thought to be exclusive to the shear stress–induced production of NO. The demonstration that both phenylarsine oxide and shear stress, but not receptor-dependent agonists such as bradykinin, which activate NOS III in a Ca\textsuperscript{2+}-dependent manner, induce the redistribution of NOS III to the cytoskeletal fraction supports the hypothesis that both stimuli elicit the Ca\textsuperscript{2+}-independent activation of NOS III by the same signaling pathway. The lack of effect of bradykinin on the detergent solubility of NOS III contrasts with results obtained by one other group. No obvious difference in experimental protocol can account for the contradictory results obtained nor can the responsiveness of the endothelial cells used: in our hands, bradykinin induces the time-dependent tyrosine phosphorylation of Triton X-100–insoluble proteins without affecting the recovery of NOS III from this cell fraction.

Although phenylarsine oxide and pervanadate may exhibit actions unrelated to tyrosine phosphatase inhibition, both agents, at concentrations that we have shown to inhibit tyrosine phosphatase activity in endothelial cells, enhanced tyrosine phosphorylation and elicited the concomitant Ca\textsuperscript{2+}-independent formation of NO. This NOS III activation was sensitive to both erbstatin A and herbimycin A but was insensitive to genistein, indicating that the Ca\textsuperscript{2+}-independent activation of NOS III is regulated by one or more tyrosine kinases with differential sensitivity to the inhibitors used. A
similar lack of effect of genistein on the tyrosine phosphorylation of cellular proteins after the application of shear stress has also been reported by other groups.29,30

Several consensus sequence sites for phosphorylation by protein kinase A, protein kinase C, and calmodulin kinase II are found in all of the cloned NOS isoforms; therefore, it is likely that the phosphorylation of NOS III may regulate its activity. Indeed, NOS III is reported to be serine-phosphorylated after endothelial cell stimulation by receptor-dependent and -independent agonists31,32 as well as by fluid shear stress,33 but until recently, NOS III was thought not to be phosphorylated on tyrosine residues.28,32,33 Although the enhanced serine phosphorylation of NOS III observed after the application of shear stress was almost immediate, the enhanced phosphorylation of NOS III after endothelial stimulation with high concentrations of bradykinin was a delayed phenomenon observed after translocation of the enzyme from the membrane to the cytosol32 and thus may represent an inactivation mechanism. Although we were able to demonstrate the tyrosine phosphorylation of NOS III in native and primary cultures of endothelial cells, the level of NOS III tyrosine phosphorylation markedly decreased after the first passage (authors’ unpublished data, 1997). Since the tyrosine phosphorylation of NOS III has been observed by groups using only primary cultures or low passages (up to passage 4) of endothelial cells, it appears that the intracellular mechanisms involved in regulating the tyrosine phosphorylation of NOS III are rapidly lost in culture.

Although the NOS III isolated from native porcine endothelial cells was phosphorylated on tyrosine residues, the phenylarsine oxide–induced Ca2+-independent activation of this enzyme was consistently associated with its tyrosine dephosphorylation. This latter finding would seem to contradict reports of hyperphosphorylation on tyrosine residues after endothelial stimulation with tyrosine phosphatase inhibitors.16 However, the enhanced tyrosine phosphorylation of NOS III in the latter study was observed only after prolonged incubation of endothelial cells with high concentrations of sodium orthovanadate or H2O2. In native endothelial cells, however, low concentrations of sodium orthovanadate are able to elicit the immediate release of NO and completely decrease, rather than an increase, in enzyme activity (authors’ unpublished data, 1997). Thus, it is conceivable that a slowly developing tyrosine hyperphosphorylation, such as that reported using multipassaged endothelial cells, inhibits rather than stimulates NOS III. Despite efforts to determine the effect of shear stress on the tyrosine dephosphorylation of NOS III, no quantifiable data could be obtained because of the weak NOS III/phosphotyrosine signal in cultured endothelial cells. However, fluid shear stress clearly did not induce hyperphosphorylation of NOS III on tyrosine residues. On the other hand, we did observe that the application of shear stress to endothelial cells increased the serine phosphorylation of NOS III (not shown) as described previously by Corson et al.33

The localization of NOS III within the caveolin-rich membrane domain,34,35 a subcompartment of the plasma membrane in which several key signal transducing complexes are concentrated (eg, heterotrimeric and small G proteins and Src-family tyrosine kinases16), is likely to have a profound repercussion on enzyme activity as well as on its sensitivity to activation by signal transduction cascades other than those resulting in [Ca2+]i. Triton insolubility alone, however, cannot be taken as evidence of caveolar localization. For example, the NOS III isolated from porcine aortic and rat pulmonary microvascular endothelial cells is mostly Triton X-100 soluble,34 although it is concentrated in caveolin-rich membrane domains and is reportedly associated with the caveolar marker protein caveolin.16 Since we were unable to discern a physical translocation of NOS III using confocal microscopy (not shown), we investigated whether the phenylarsine oxide–and shear stress–enhanced production of NO was associated with an increase in the amount of NOS III detected in caveolin-rich membrane domains. However, the application of shear stress to primary cultures of human endothelial cells did not alter the distribution of NOS III between the various membrane domains, although the enzyme was activated Ca2+-independently and became insoluble in Triton X-100. Whether such a change in the detergent solubility of NOS III represents a general activation process remains to be determined. However, such a possibility seems unlikely, since shear stress, which initiates an immediate increase in NO production, induces only a relatively slow change in the Triton solubility of the enzyme. However, a correlation between the Ca2+-independent activation of NOS III and a change in its detergent solubility may exist, since in BAPTA-treated cells a maintained increase in cGMP was observed only after a delay of 30 to 60 minutes after the application of shear stress.

A change in detergent solubility is frequently indicative of the formation of a protein complex. For example, TNF stimulation of adherent neutrophils causes the redistribution of the cytosolic proteins p91-phox, p22-phox, p47-phox, and p67-phox (four components whose assembly constitutes an active NADPH oxidase) to a Triton-insoluble membrane fraction and is associated with an enhanced production of superoxide anions.35 It is tempting to speculate that fluid shear stress and tyrosine phosphatase inhibitors may alter the conformation and/or protein coupling of NOS III, facilitating its interaction with specific phospholipids, proteins, and/or protein kinases that enhance/maintain its activation. Indeed, we found that the direct application of phenylarsine oxide to isolated preparations of NOS III was associated with a decrease, rather than an increase, in enzyme activity (authors’ unpublished data, 1997). This finding tends to suggest that the tyrosine phosphatase inhibitor–induced activation of NOS III requires cell integrity and possibly interaction of the enzyme with a modulator/adapter protein. The concept that NOS III may complex proteins that determine cellular targetting or regulate its activity is somewhat analogous to the situation described for the neuronal NOS, which associates with α1-syntrophin,39 the postsynaptic density proteins (PSD-95 and PSD-93),39 and a small dimer-destabilizing protein.40 Apart from calmodulin and caveolin-1,16,17,18 no additional NOS III–associated proteins have been characterized. In the present study, we observed that a group of tyrosine-phosphorylated proteins were coprecipitated with
NOS III, suggesting that the enzyme may exist as part of a multimolecular complex and that its activity may be regulated by one or more NOS III–associated/adapter proteins. Recently, it was reported that NOS III isolated from cultured bovine endothelial cells is associated with a 90-kD tyrosine-phosphorylated protein. This latter protein may well prove to be identical to one of the associated proteins detected here in native endothelial cells. Additional candidate proteins could include paxillin, PLC-γ, Fyn, and Src, which also underwent an acute phenylarsine oxide–induced alteration in detergent solubility.

In summary, we have demonstrated for the first time that tyrosine phosphatase inhibitors and fluid shear stress elicit the production of endothelium-derived NO via a pathway completely different from the classical signaling cascade, which results in enhanced NO formation after endothelial stimulation with receptor-dependent and -independent agonists. This novel activation pathway is independent of Ca²⁺/calmodulin, is sensitive to erbstatin A and herbimycin A, and is associated with the delayed redistribution of NOS III to a detergent-insoluble cell fraction rich in tyrosine kinases and cytoskeletal-associated proteins. Moreover, although the Ca²⁺-independent production of NO is highly sensitive to tyrosine kinase inhibitors, NOS III appears to be acutely dephosphorylated, rather than hyperphosphorylated, on tyrosine residues after stimulation with phenylarsine oxide. These observations suggest that protein tyrosine kinases are unlikely to directly regulate NOS III activity after cell stimulation with phenylarsine oxide but that Ca²⁺/calmodulin–independent activation of NOS III may be regulated by an associated tyrosine-phosphorylated protein.

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