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Phosphorylation and subcellular translocation of endothelial nitric oxide synthase

(signal transduction/myristoylation/bradykinin/protein kinase/calmodulin)

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ABSTRACT **In the vascular endothelium, diverse cell surface receptors are coupled to the Ca^{2+}/calmodulin-dependent activation of nitric oxide (NO) synthase. We now report that, in intact cultured endothelial cells, several drugs and agonists are associated with increased serine phosphorylation of the endothelial NO synthase. We biosynthetically labeled bovine aortic endothelial cells with [^{32}P]orthophosphoric acid, exposed the cells to various drugs and hormones, and then immunoprecipitated the enzyme from cell extracts using a highly specific anti-peptide antibody. The marked endothelial NO synthase phosphorylation induced by bradykinin is maximal only after 5 min of agonist exposure and is stable for at least 20 min. Basal and agonist-induced phosphorylation of the NO synthase in endothelial cells is completely inhibited by the calmodulin antagonist compound W-7. We prepared subcellular fractions of endothelial cells that had been biosynthetically labeled with [^{35}S]methionine or [^{32}P]orthophosphoric acid and immunoprecipitated the endothelial NO synthase from untreated (basal) and bradykinin-treated cells. In the basal state, [^{35}S]methionine-labeled endothelial NO synthase is associated primarily with the particulate cellular fraction, but the phosphorylated enzyme is primarily cytosolic. Following exposure to bradykinin, a substantial fraction of the [^{35}S]methionine-labeled NO synthase is now found in the cytosolic fraction, associated with a marked increase in the level of cytosolic enzyme phosphorylation. We propose that agonist-induced phosphorylation of NO synthase is associated with translocation of the enzyme from membrane to cytosol and may thereby regulate the biological effects of endothelial NO synthesis *in situ*.**

Nitric oxide (NO) is a ubiquitous intercellular signaling molecule and is synthesized in diverse mammalian tissues by a family of related NO synthase enzymes. In all tissues thus far characterized, the NO synthase isoforms share a common overall catalytic scheme for the oxidation of L-arginine to form NO plus L-citrulline and appear to share similar cofactor requirements (reviewed in refs. 1–3). The different tissue-specific NO synthase isoforms subserve disparate biological functions in diverse mammalian cells and appear to be encoded by distinct genes (4).

The endothelial NO synthase plays an important role in the control of blood pressure and platelet aggregation (5). In bovine endothelial cells, the NO synthase is transiently activated by increases in intracellular calcium induced by the activation of diverse G-protein-coupled cell surface receptors, including bradykinin. Our analysis of the primary structure of the endothelial NO synthase cDNA identified consensus sequences for posttranslational modifications of the enzyme, including N-terminal myristoylation as well as phosphorylation (4). We have recently shown that the endothelial NO synthase undergoes N-terminal myristoylation, and this covalent modification is essential for the association of the enzyme with the particulate subcellular fraction (6). Some myristoylated proteins implicated in intracellular signaling pathways have been shown to regulated by reversible phosphorylation, and phosphorylation further appears to modulate their subcellular localization (reviewed in refs. 7 and 8).

The deduced primary structures of NO synthase cDNAs encoding neural (9) and endothelial (4) enzyme isoforms have been noted to contain consensus sequences for phosphorylation by diverse protein kinases. The NO synthase purified from rat brain can be phosphorylated *in vitro* by numerous protein kinases, including protein kinase C, the cyclic AMP-dependent protein kinase, as well as the calcium/calmodulin protein kinase II (10, 11). The role of phosphorylation in the regulation of intracellular NO synthase *in situ* has not yet been explored. In these studies, we report that NO synthase undergoes phosphorylation in response to diverse drugs and agonists in intact bovine aortic endothelial cells (BAECs). When the endothelial NO synthase undergoes phosphorylation in response to bradykinin, the enzyme appears to translocate from the particulate to cytosolic subcellular fractions. These findings may have implications for the regulation of endothelial NO synthase function.

MATERIALS AND METHODS

Reagents and Cell Culture. Tissue culture media and serum are from GIBCO. Radionuclides are from NEN/DuPont. Bradykinin, sodium nitroprusside, cyanogen bromide, phorbol 12-myristate 13-acetate, and other chemicals, except as noted, are from Sigma. Compounds H-7, W-7, KN-62, H-89, and ML-7 are from Seikagaku America (Rockville, MD); treatment of labeled endothelial cells with these compounds was for 2 hr. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-trypsin is from Worthington. BAECs are isolated and maintained in culture as described (4) and are used in these experiments between passages 6 and 12.

Biosynthetic Labeling, Immunoprecipitation, and Subcellular Fractionation. Confluent BAECs in six-well dishes are incubated in phosphate-free Dulbecco's minimum essential medium with 80 μCi of [^{32}P]orthophosphoric acid per ml (9000 Ci/mmol; 1 Ci = 37 GBq) for 4 hr, exposed to drugs for indicated times, harvested, and immunoprecipitated essentially as described (6). Briefly, the labeled cells are harvested by scraping in buffer B (150 mM NaCl/50 mM Tris·HCl, pH 7.4/0.1 mM EDTA/1% Triton X-100/0.1% SDS/1% sodium deoxycholate/50 mM NaF plus protease inhibitors as described in ref. 6), sonicated, and then immunoprecipitated using a polyclonal antiserum (at 1:100 dilution) directed

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Abbreviations: NO, nitric oxide; BAEC, bovine aortic endothelial cell.

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against a synthetic NO synthase oligopeptide. This polyclonal antiserum was raised in rabbits against a synthetic peptide deduced from the sequence (4) of the endothelial NO synthase cDNA (PYNSSPRPEQHKSYK-C, corresponding to amino acid residues 599–614 plus a C-terminal C residue used to couple the peptide to maleimide-activated keyhole limpet hemocyanin). We have previously shown (ref. 6) that this immune serum is highly specific for immunoprecipitation of endothelial NO synthase and that immunoprecipitation of biosynthetically labeled protein is blocked by the unlabeled peptide. Following the isolation of immune complexes with protein A-Sepharose, the proteins were eluted by heating with SDS/PAGE sample buffer. The phosphorylated proteins were analyzed by SDS/PAGE on 7% polyacrylamide gels, followed by autoradiography and exposure on Kodak XAR film or the phosphorylated proteins were quantitated using a PhosPhorImager (Molecular Dynamics). For subcellular fractionations, the cells were harvested by scraping in 50 mM Tris·HCl, pH 7.4/0.1 mM EDTA/0.1 mM EGTA/2 mM 2-mercaptoethanol/50 mM NaF plus protease inhibitors and then sonicated to yield the cell lysate, which then was centrifuged at 100,000 × *g* for 1 hr, and the pellet (particulate fraction) was resuspended in buffer B; 5 volumes of buffer B was added to the supernatant (cytosol), and the individual subcellular fractions were immunoprecipitated as above. Biosynthetic labeling and immunoprecipitation from [^{35}S]methionine-labeled endothelial cells were performed as described (6).

Phosphopeptide Mapping and Phosphoamino Acid Analysis. NO synthase immunoprecipitated from $^{32}P_i$-biosynthetically labeled BAEC was eluted from protein A-Sepharose and the samples were precipitated with 25% trichloroacetic acid and dissolved in 70% formic acid. Cyanogen bromide was added to a final concentration of 3 mg/ml, and the samples were incubated (24 hr, room temperature; ref. 12) and then lyophilized and dissolved by heating in SDS/PAGE sample buffer. The phosphopeptides were resolved on SDS/PAGE using a 15% polyacrylamide gel. Tryptic phosphopeptide mapping of the phosphorylated immunoprecipitated NO synthase was performed essentially as described (13). After oxidizing the samples with performic acid, they were digested with trypsin overnight at 37°C, spotted on thin-layer cellulose plates (Merck), electrophoresed using the Hunter thin-layer electrophoresis apparatus (kindly provided by Michael Lu at Brigham and Women's Hospital) in 50 mM ammonium bicarbonate at pH 8.9 (1000 V, 45 min), and then subjected to ascending chromatography overnight in 1-butanol/pyridine/acetic acid/H_2O (750:500:150:600), followed by autoradiography. For phosphoamino acid analysis, the NO synthase immunoprecipitated from $^{32}P_i$-labeled BAECs was incubated with 6 M HCl for 1 hr at 110°C, lyophilized, and then subjected to two-dimensional thin-layer electrophoresis exactly as described (13).

RESULTS

Agonist-Induced Phosphorylation of Endothelial NO Synthase. Fig. 1 shows the results of an experiment in which BAECs were biosynthetically labeled with $^{32}P_i$, exposed to drugs or agonists for 5 min, immunoprecipitated with an anti-NO synthase antibody, and analyzed by SDS/PAGE and autoradiography. This approach resulted in the immunoprecipitation of a 135-kDa protein by immune serum, with no specific phosphoproteins precipitated by the preimmune serum. The molecular mass of this major phosphoprotein is identical to that of the cloned (4) or purified (14) bovine endothelial NO synthase, and this protein comigrates with the [^{35}S]methionine biosynthetically labeled NO synthase from endothelial cells (data not shown) that we have previously characterized (6). There is a marked increase in the

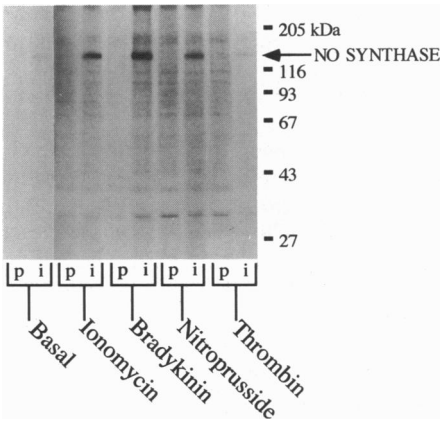

FIG. 1. Phosphorylation and immunoprecipitation of endothelial NO synthase in response to agonists: Autoradiogram of SDS/PAGE of immunoprecipitated endothelial NO synthase prepared from BAECs treated with different drugs and agonists. Confluent BAECs in six-well dishes were labeled for 4 hr with [^{32}P]orthophosphoric acid and then exposed for 5 min to ionomycin (10 μM), bradykinin (10 μM), sodium nitroprusside (10 μM), or thrombin (1 unit/ml). Immunoprecipitation was carried out as described in the text with preimmune serum (p) or immune serum (i) directed against the endothelial NO synthase; migrations of molecular mass markers are noted. The results shown above are representative of eight similar experiments.

level of basal phosphorylation of the endothelial NO synthase associated with treatment of endothelial cells with the calcium ionophore ionomycin or with the NO-liberating drug sodium nitroprusside, but the most striking increase in NO synthase phosphorylation was seen with the receptor agonist bradykinin. The agonist thrombin is without effect, consistent with prior observations that thrombin responses are lost early in the passage of cultured bovine endothelial cells (15). In most experiments, the level of basal phosphorylation is low relative to the striking increase seen with drug or agonist treatment. However, the level of basal phosphorylation (in the absence of added compounds) and the degree of stimulation with drug treatment varied somewhat from experiment to experiment; yet, in 15 experiments, bradykinin consistently increased NO synthase phosphorylation between 2- and 8-fold relative to levels of basal phosphorylation. We chose to characterize the response to bradykinin in greater detail because it has the most marked effect on NO synthase phosphorylation in BAECs and also because bradykinin appears to be an important endogenous activator of NO synthase in the vasculature (5).

Time Course of Phosphorylation. The time course of NO synthase phosphorylation by the agonist bradykinin is shown in Fig. 2. Following the addition of bradykinin (10 μM) to identically cultured $^{32}P_i$-labeled BAECs in adjacent wells of

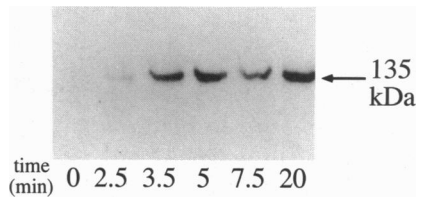

FIG. 2. Time course of bradykinin-induced phosphorylation of endothelial NO synthase. Bovine endothelial cells identically cultured and maintained in a six-well dish were labeled with $^{32}P_i$ and then exposed to bradykinin (10 μM) for the times indicated, at which time the cells were harvested. The NO synthase was immunoprecipitated and analyzed by SDS/PAGE and autoradiography; the molecular mass of 135 kDa corresponding to the NO synthase is noted. The experiment shown was repeated twice with similar results.

a six-well dish, NO synthase was immunoprecipitated at 0, 2.5, 3.5, 5, 7.5, and 20 min after agonist addition. The level of NO synthase phosphorylation was maximally increased at 5 min of bradykinin exposure, and phosphorylation persisted for at least 20 min.

Phosphopeptide Mapping of Biosynthetically Labeled NO Synthase. The bovine endothelial NO synthase contains 17 methionine residues and is predicted to yield 18 peptides ranging in size from 420 to 31,300 Da after cleavage with cyanogen bromide. NO synthase was immunoprecipitated from $^{32}P_i$-labeled BAECs following exposure to drugs or agonists (as shown in Fig. 3*A*), treated with cyanogen bromide, and analyzed by SDS/PAGE on a 15% polyacrylamide gel followed by autoradiography. Labeling of the a single ≈28-kDa cyanogen bromide phosphopeptide is found in the absence or presence of added drug; as before, the most marked increase in phosphorylation is seen following bradykinin treatment. There was no significant increase in phosphorylation seen with the adenylate cyclase activator forskolin nor with 8-bromoguanosine 3′,5′-cyclic monophosphate over the time course of these experiments. Phorbol 12-myristate 13-acetate and sodium nitroprusside each caused an increase in phosphorylation of this peptide. To more clearly delineate the site(s) of phosphorylation, two-dimensional tryptic phosphopeptide mapping (13) was performed (Fig. 3*B*) after immunoprecipitating the NO synthase from $^{32}P_i$-labeled endothelial cells exposed to different drugs and agonists. A single NO synthase tryptic phosphopeptide is seen after exposure to bradykinin, sodium nitroprusside, or calcium ionophore; the same peptide also appears to be labeled under basal conditions. Under all of these conditions, the labeled phosphoamino acid was identified as phosphoserine in analyses of acid hydrolysates of immunoprecipitated NO synthase using two-dimensional high-voltage electrophoresis using standard techniques (ref. 13; data not shown).

Effects of Protein Kinase Inhibitors. A wide variety of protein kinase inhibitors was assayed for their effects on basal and bradykinin-induced phosphorylation of the NO synthase in BAECs. Shown in Fig. 4 is the results of an experiment using the calmodulin antagonist compound W-7 (16) and the protein kinase C inhibitor H-7 (17). The autoradiogram shown is intentionally overexposed to document that W-7 treatment completely abrogates basal and bradykinin-induced phosphorylation of the NO synthase. Other protein kinase inhibitors were assayed under their optimal conditions (see refs. 16–19), including the protein kinase C inhibitors H-7 (see Fig. 4) and sphingosine (20 μM), the myosin light chain kinase inhibitor ML-9 (10 μM), the calmodulin kinase II inhibitor KN-62 (50 μM), and the cyclic AMP-dependent protein kinase inhibitor H-89 (30 μM). None of these protein kinase inhibitors had a substantive effect on basal or bradykinin-induced NO synthase phosphorylation.

NO Synthase Phosphorylation and Subcellular Translocation. We explored the effects of exposure of biosynthetically labeled BAECs to bradykinin on the subcellular distribution and phosphorylation of NO synthase. Fig. 5 *Upper* shows the results obtained with [^{35}S]methionine-labeled endothelial cells. There is no change in the total quantity of NO synthase present in the cell lysate following bradykinin treatment (10 μM, 20 min) relative to control untreated cells. In control BAECs, only a small fraction (≈10%) of the total NO synthase can be immunoprecipitated from the cytosolic fraction, as we have previously documented (6). However,

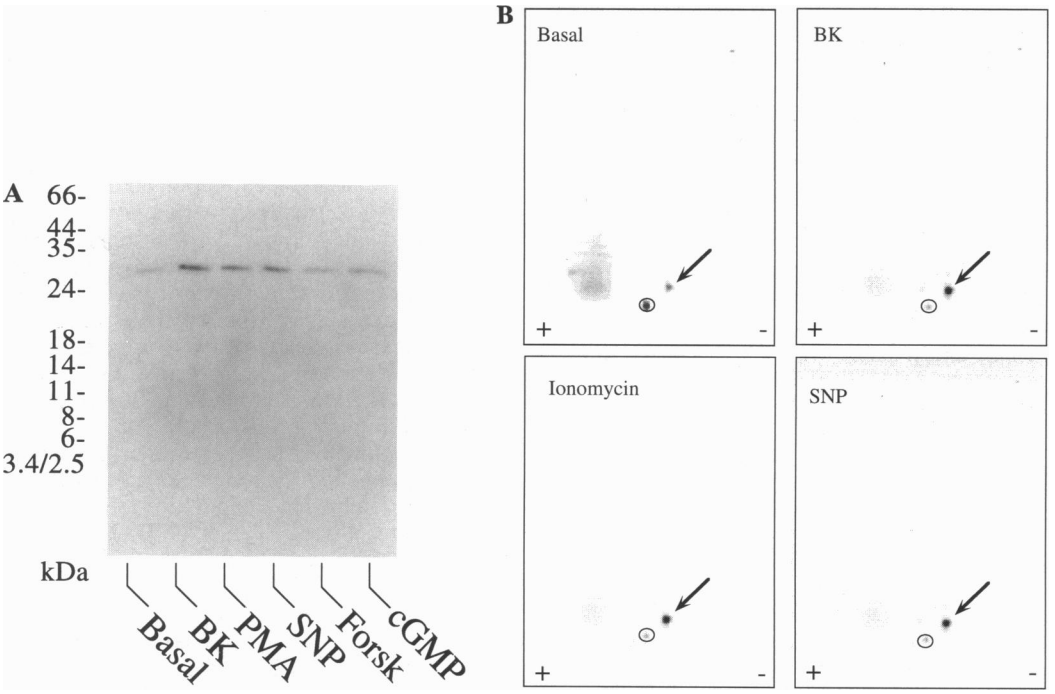

FIG. 3. Phosphopeptide mapping of NO synthase from BAECs treated with agonists. (*A*) Cyanogen bromide phosphopeptides. The immunoprecipitated NO synthase from $^{32}P_i$-labeled endothelial cells treated for 5 min with bradykinin (BK, 10 μM), phorbol 12-myristate 13-acetate (PMA, 100 nM), sodium nitroprusside (SNP, 10 μM), forskolin (Forsk, 10 μM), or 8-bromoguanosine 3′,5′-cyclic monophosphate (cGMP, 1 mM) was cleaved with cyanogen bromide, and the phosphopeptides were resolved by denaturing gel electrophoresis in 15% acrylamide, followed by autoradiography. Migration positions of molecular mass markers are noted. (*B*) Tryptic phosphopeptides. The immunoprecipitated NO synthase from $^{32}P_i$-labeled endothelial cells treated as above with bradykinin (BK), ionomycin (10 μM), sodium nitroprusside (SNP), or H_2O (Basal) was digested with trypsin and resolved by electrophoresis and chromatography on thin-layer cellulose plates. For each plate, the site where the sample was applied is circled and the single labeled phosphopeptide spot is noted by an arrow. The cathode (−) and anode (+) positions are noted for the first-dimension electrophoresis, and ascending chromatography was then performed, after which time the plate was dried and exposed to XAR film for 7–10 days using an intensifying screen. This experiment was repeated three times with identical results.

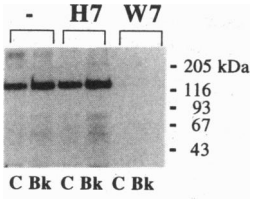

FIG. 4. Effects of H-7 and W-7 on NO synthase phosphorylation in endothelial cells: Autoradiogram of SDS/PAGE analysis of NO synthase immunoprecipitated from $^{32}P_i$-labeled endothelial cells treated for 2 hr with H-7 or W-7 (both drugs at 100 μM for 2 hr), followed immediately by a 5-min treatment with vehicle (control, C) or bradykinin (10 μM, Bk). The autoradiogram shown here is intentionally overexposed to document the complete loss of NO synthase phosphorylation with W-7 treatment. The overexposure of the film minimizes the 2-fold increase in NO synthase phosphorylation by bradykinin in the absence of W-7 quantitated by PhosPhorImager analysis and more easily seen on shorter exposures. In separate experiments (not shown), there was no change in the abundance or in the degradation rate of the endothelial NO synthase following W-7 treatment. The experiment shown is representative of three similar experiments.

following exposure to bradykinin, the fraction of NO synthase immunoprecipitated in BAEC cytosol markedly increases, to 50% of the total enzyme present. This increase in cytosolic NO synthase induced by bradykinin is not seen when the endothelial cells are treated with compound W-7 (data not shown).

A markedly different subcellular localization of the NO synthase is seen following biosynthetic labeling of BAECs with $^{32}P_i$, as shown in Fig. 5 *Lower*. There is a significant increase in NO synthase phosphorylation in BAEC lysates following bradykinin treatment, as we have documented above. Importantly, when the control $^{32}P_i$-labeled BAECs are fractionated into cytosolic and particulate fractions, the greatest proportion (80%) of the phosphorylated NO synthase is seen in the cytosol (Fig. 5 *Lower*), in inverse

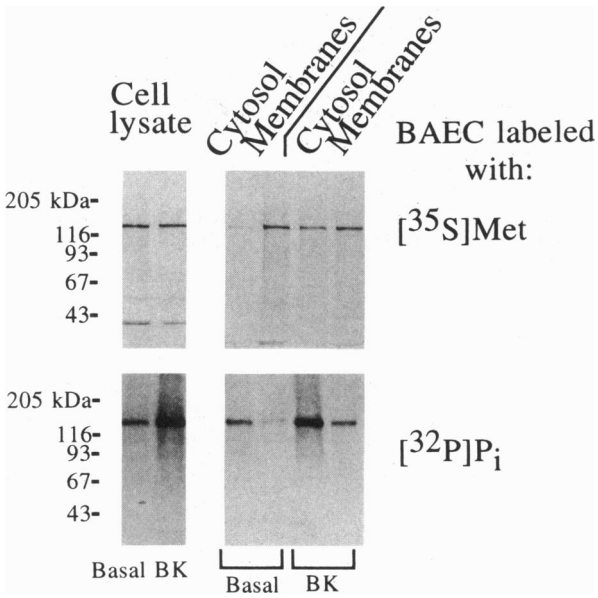

FIG. 5. Subcellular localization and translocation of phosphorylated endothelial NO synthase after exposure to bradykinin: Pattern of NO synthase expression in total cell lysates, cytosol, or membrane preparations from BAECs treated (BK) or not (Basal) with bradykinin (10 μM, 20 min). (*Upper*) Results obtained from BAECs biosynthetically labeled with [^{35}S]methionine prior to bradykinin treatment. (*Lower*) Pattern of NO synthase immunoprecipitation from $^{32}P_i$-labeled endothelial cells. The experiment shown was repeated twice with similar results.

relationship to the subcellular distribution of the total ([^{35}S]methionine-labeled) enzyme documented in Fig. 5 *Upper*. The subcellular distribution of NO synthase following bradykinin treatment of $^{32}P_i$-labeled BAECs shows a striking increase in phosphorylated, cytosolic protein, and, as before, the vast majority of the ^{32}P-labeled enzyme (85%) is present in the cytosol.

DISCUSSION

This study establishes that the endothelial NO synthase undergoes phosphorylation in response to treatment of bovine endothelial cells with diverse drugs and agonists. Agents that increase NO synthase activity are associated with increased phosphorylation of the enzyme. However, several features of NO synthase phosphorylation in endothelial cells would lead us to propose that enzyme phosphorylation is associated with deactivation of the enzyme. The phosphorylation of NO synthase induced by sodium nitroprusside, an agent that liberates NO, may suggest that the enzyme's product may lead to its phosphorylation and serve as a negative feedback to enzyme activity. Indeed, enzymatic activity of the neural NO synthase is inhibited by NO (via incompletely understood mechanisms) in cerebellar cytosol preparations (20). Most importantly, the time course of NO synthase phosphorylation documented in these studies is markedly slower than that of enzyme activation in intact endothelial cells, as has been established in several laboratories using diverse experimental approaches (5). For example, using a highly selective NO electrode, Malinski and Taha (21) have shown that bradykinin treatment of BAECs results in activation of NO synthesis within seconds, and enzyme activity returns to baseline within 5 min. In contrast, phosphorylation of NO synthase in endothelial cells in response to bradykinin is maximal only after 5 min of agonist exposure and persists for at least 20 min, long after bradykinin-induced enzyme activation has decayed. Phosphorylation of recombinant neural NO synthase in transfected cells apparently is associated with a decrease in enzyme activity (10), and *in vitro* phosphorylation of the purified brain NO synthase by diverse protein kinases has been associated with decreases in enzyme activity as reported by some laboratories (Solomon Snyder, personal communication) but not by others (11).

We found that the endothelial NO synthase is phosphorylated only on serine, and the same NO synthase tryptic phosphopeptide is identified following treatment of endothelial cells with bradykinin, sodium nitroprusside, or calcium ionophore as well as under basal conditions. This finding suggests that diverse signaling pathways may lead to the regulation of NO synthase phosphorylation at a single specific serine residue. Because of the pleiotropic effects of these different drugs, and the existence of multiple interacting signal transduction pathways within the cell, it cannot be stated with confidence that the effects of these different drugs involve activation of distinct protein kinases that share the same recognition site on the NO synthase or whether a common kinase is activated by these different drug treatments.

Our results, obtained with NO synthase phosphorylated in intact endothelial cells, contrast with the identification of distinct serine tryptic phosphopeptides (10) and the identification of phosphothreonine (11) following treatments of purified neural NO synthase by different protein kinases *in vitro*. These results may reflect intrinsic differences in the regulation by phosphorylation of different NO synthase isoforms or may be the result of the differences in experimental conditions. The challenges of obtaining sufficient quantities of pure endothelial NO synthase from cultured endothelial cells preclude the analysis of *in vitro* phosphorylation experiments for this isoform. We are hopeful that the development

of heterologous recombinant protein expression systems for production of endothelial NO synthase will permit the correlation of our observations on enzyme phosphorylation *in situ* with those obtained in purified systems and allow the definitive assignment of the specific phosphorylated serine residue.

The striking inhibition of basal and bradykinin-induced NO synthase phosphorylation seen with the calmodulin antagonist W-7 may reflect one of several mechanisms. It may be that a calmodulin-dependent protein kinase is involved in NO synthase phosphorylation, and this activation of this kinase is inhibited by W-7. An equally plausible and nonexclusive hypothesis is that it is the calmodulin-liganded form of NO synthase that comprises the substrate for phosphorylation by protein kinase, and the inhibition of calmodulin by W-7 thereby blocks the formation of the appropriately liganded NO synthase-calmodulin substrate required for effective phosphorylation. Reconstitution experiments with purified components will allow these possibilities to be distinguished. There were no marked effects on NO synthase phosphorylation using inhibitors of protein kinase C, cyclic AMP-dependent protein kinase, and calcium/calmodulin kinase II. The interpretation of these data is subject to the limitations inherent in drawing conclusions based on negative results. Nevertheless, it is tempting to speculate that the endothelial NO synthase may undergo phosphorylation by a protein kinase distinct from those listed above.

These studies suggest that the association of the endothelial NO synthase with the particulate subcellular fraction may be dynamically regulated by agonist-induced phosphorylation. There is a striking analogy with the MARCKS protein (*m*yristoylated *a*lanine-*r*ich *C*-*k*inase *s*ubstrate), a calmodulin-binding myristoylated protein that translocates from plasma membrane to cytosol upon phosphorylation by protein kinase C (8, 9). To our knowledge, the endothelial NO synthase is the only NO synthase isoform thus far characterized that undergoes N-terminal myristoylation, and this myristoylation of the endothelial isoform is essential for its association with the particulate subcellular fraction in endothelial cells (6). It seems plausible that the membrane association of the endothelial isoform may play a role in signal transduction.

The intracellular translocation of the endothelial NO synthase may have important implications for the regulation of the biological activity of the NO produced by endothelial cells. The lability of NO once it is formed and the tendency of NO to form adducts with other biomolecules (22) suggest that the intracellular compartment in which NO is synthesized may be an important determinant of the biological consequences of its synthesis. Dynamic alterations in the activity and intracellular localization of endothelial NO synthase by phosphorylation may add another level of complexity to understanding the mechanisms involved in the regulation of endothelial function in disease states.

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