The endothelial isoform of nitric-oxide synthase (eNOS) undergoes a complex pattern of covalent modifications, including acylation with the fatty acids myristate and palmitate as well as phosphorylation on multiple sites. eNOS acylation is a key determinant for the reversible subcellular targeting of the enzyme to plasmalemmal caveolae. We transfected a series of hemagglutinin epitope-tagged eNOS mutant cDNAs deficient in palmitoylation (palm−) and/or myristoylation (myr−) into bovine aortic endothelial cells; after treatment with the eNOS agonists sphingosine 1-phosphate or vascular endothelial growth factor, the recombinant eNOS was immunoprecipitated using an antibody directed against the epitope tag, and patterns of eNOS phosphorylation were analyzed in immunoblots probed with phosphorylation-specific fusion protein. We conclude that eNOS targeting to the membrane is mediated by dual acylation, an irreversible N-myristoylation at Gly2, and reversible thiopalmitoylation at Cys15 and Cys26. Mutagenesis of the eNOS myristoylation site blocks both myristoylation and palmitoylation and converts the membrane-targeted wild-type eNOS to a soluble protein; the myr− eNOS mutant is recovered in the soluble subcellular fraction when expressed in cells (6, 7). The palmitoylation-deficient mutant eNOS (palm−) exhibits an intermediate phenotype; the palm− eNOS mutant does undergo myristoylation, and its membrane association is only partially reduced compared with the wild-type eNOS (5). The addition of a prototypical transmembrane domain to the myr− eNOS is sufficient to promote eNOS palmitoylation as well as targeting to caveolae and also restores agonist responsiveness of the acylation-deficient mutant eNOS enzyme (9). Taken together, these results suggest that the caveolar location and functionality of eNOS are determined by structural features of the eNOS molecule that are distinct from the enzyme sites of acylation (10, 11).

Acylation is not the only covalent modification that importantly affects eNOS. Like the other NOS isoforms, eNOS is a phosphoprotein (12). There are at least three distinct eNOS phosphorylation sites: serine 116 (13, 14), threonine 497 (threonine 495 in the human sequence (15)), and serine 1179 (serine 1177 in the human sequence (13, 16, 17)). Of these phosphorylation sites, serine 116 was enhanced in the myr− eNOS mutant and was markedly attenuated in the CD8 transmembrane domain to the amino terminus of eNOS acylation-deficient mutants rescued the wild-type phenotype of robust agonist-induced serine 1179 phosphorylation. Thus, membrane targeting, but not necessarily acylation, is the critical determinant for agonist-promoted eNOS phosphorylation at serine 1179. In striking contrast to serine 1179, phosphorylation of eNOS at serine 116 was enhanced in the myr− eNOS mutant and was markedly attenuated in the CD8-eNOS membrane-targeted fusion protein. We conclude that eNOS targeting differentially affects eNOS phosphorylation at distinct sites in the protein and suggest that the interaction of eNOS acylation and phosphorylation may modulate eNOS localization and activity and thereby influence NO signaling pathways in the vessel wall.

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The abbreviations used are: eNOS, endothelial nitric-oxide synthase; S1P, sphingosine 1-phosphate; VEGF, vascular endothelial growth factor; HA, hemagglutinin; BAEC, bovine aortic endothelial cells; myr-eNOS, mutant eNOS in which the myristoylation site at glycine 2 is changed to alanine rendering the enzyme acylation-deficient; palm-eNOS, mutant eNOS in which the palmitoylation sites at cysteines 15 and 26 are changed to serine; EGFP, enhanced green fluorescent protein; MOPS, 4-morpholinepropanesulfonic acid.

The endothelial isoform of nitric-oxide synthase (eNOS) plays a major role in the control of vascular homeostasis and platelet aggregation (1, 2). In vascular endothelial cells and in cardiac myocytes, eNOS is targeted to signal-transducing microdomains in the plasma membrane termed caveolae, where eNOS interacts with the scaffolding protein caveolin (for review, see Ref. 3). Plasmalemmal caveolae serve as membrane sites of sequestration of signaling proteins including receptors, G proteins, and protein kinases as well as eNOS (for review, see Ref. 4). The targeting of eNOS to the membrane is mediated by dual acylation, an irreversible N-myristoylation at Gly2, and reversible thiopalmitoylation at Cys15 and Cys26. Mutagenesis of the eNOS myristoylation site blocks both myristoylation and palmitoylation and converts the membrane-targeted wild-type eNOS to a soluble protein; the myr− eNOS mutant is recovered in the soluble subcellular fraction when expressed in cells (6, 7). The palmitoylation-deficient mutant eNOS (palm−) exhibits an intermediate phenotype; the palm− eNOS mutant does undergo myristoylation, and its membrane association is only partially reduced compared with the wild-type eNOS (5). The addition of a prototypical transmembrane domain to the myr− eNOS is sufficient to promote eNOS palmitoylation as well as targeting to caveolae and also restores agonist responsiveness of the acylation-deficient mutant eNOS enzyme (9). Taken together, these results suggest that the caveolar location and functionality of eNOS are determined by structural features of the eNOS molecule that are distinct from the enzyme sites of acylation (10, 11). Acylation is not the only covalent modification that importantly affects eNOS. Like the other NOS isoforms, eNOS is a phosphoprotein (12). There are at least three distinct eNOS phosphorylation sites: serine 116 (13, 14), threonine 497 (threonine 495 in the human sequence (15)), and serine 1179 (serine 1177 in the human sequence (13, 16, 17)). Of these phosphorylation sites, the one most extensively characterized is serine 1179. Several distinct serine/threonine protein kinases have been implicated in the cellular activation of eNOS by promoting phosphorylation at serine 1179, including kinase Akt (16, 17) and the AMP-activated protein kinase (18) as well as protein kinase A (19). eNOS phosphorylation at serine 1179 has been observed in response to a wide variety of extracellular stimuli, including treatment of endothelial cells with vascular endothelial growth factor (VEGF) (16, 20), sphingosine-1-phos-
eNOS Targeting and Agonist-induced Phosphorylation

Materials—Fetal bovine serum was purchased from HyClone (Logan, UT). Cell culture reagents and media were from Invitrogen. FuGENE 6 transfection reagent was from Roche Molecular Biochemicals. SIP was purchased from BioMol (Plymouth Meeting, PA). VEGF and Wortmannin were from Calbiochem. Anti-phospho-Ser1179-eNOS antibody, anti-phospho-Akt antibody (serine 473), and anti-Akt antibody were from Cell Signaling Technologies (Beverly, MA). Anti-phospho-Ser1179-eNOS antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-eNOS monoclonal antibody was from Transduction Laboratories (Lexington, KY). Monoclonal antibody 12CA5 directed against the HA epitope was from Boehringer Mannheim. Anti-eNOS polyclonal antibody was from Biovision (Milpitas, CA). Anti-eNOS pantoic acid-deficient eNOS (palm εNOS; Cys3679 and Cys3680 mutated to Ser), and Akt phosphorylation-site-deficient mutant (Ser1176 mutated to Ala) were from Calbiochem. Anti-phospho-Ser116-eNOS (clone 40D11) and anti-phospho-Ser1179-eNOS antibodies using protocols provided by the suppliers. For immunoblot analyses of cell lysates, 20 μg of cellular protein were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with phospho-Akt and Akt antibodies. Densitometric analyses of the immunoblots were performed using a ChemiImager 4000 (Alpha-Innotech).

Plasmid Construction—cDNA constructs encoding the HA-tagged wild-type eNOS, myristoylation-deficient eNOS (myr εNOS, Gly2 mutated to Ala), palmitoylation-deficient eNOS (palm εNOS; Cys3679 and Cys3680 mutated to Ser), and Akt phosphorylation-site-deficient mutant (Ser1176 mutated to Ala) have been previously described (5, 26). Chimeric cDNA constructs encoding the fusion proteins CD8-myr εNOS and CD8.palm εNOS have also been described (8). The HA epitope tag was added to these various CD8.eNOS chimeras by interchanging the untranslated 3‘-terminal 1.5 kilobases of these constructs with the corresponding HA epitope-tagged eNOS cDNA, taking advantage of a propitious BglII restriction site.

For cell imaging studies, we constructed a series of eNOS fusion proteins with the fluorescent protein EGFP; our preliminary studies had revealed that the constructs tagged with the HA epitope yielded a signal-to-noise ratio that was insufficient for high resolution fluorescence imaging. The plasmid encoding the eNOS-EGFP fusion protein was made by cloning the cDNA encoding the sequence of bovine wild-type eNOS into the mammalian expression vector pEGFP-N1 (Clontech Laboratories, Inc., Palo Alto, CA) after first modifying the stop codon at codon 609 to generate a BamHI restriction site; the stop codon was then used to clone the eNOS sequence in-frame with the EGFP sequence. To produce palm εNOS-EGFP, CD8.palm εNOS-EGFP, and CD8.myr εNOS-EGFP, these cDNAs were digested with EcoRI and KpnI, and the 5‘ fragment was used to replace the corresponding region in the wild-type eNOS-EGFP plasmid that had been similarly digested to release the corresponding 5‘ fragment. To construct myr εNOS-EGFP, the CD8.myr εNOS-EGFP was first digested with ClaI, blunted with mung bean nuclease, and digested with KpnI, this cDNA fragment was subcloned into eNOS-EGFP that had been first digested with NheI, blunted with mung bean nuclease, and digested with KpnI to release the 5‘ fragment that was then replaced by the myr – sequence.

EXPERIMENTAL PROCEDURES

Preparation of Cellular Lysates, Immunoprecipitation, and Immunoblotting—After drug treatments, BAEC were washed with phosphate-buffered saline and incubated for 10 min in brefeldin a (50 μM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 2 mM Na3VO4, 1 mM NaF, 2 μg/ml leupeptin, 2 μg/ml soybean trypsin inhibitor, and 2 μg/ml lima trypsin inhibitor). Cells were harvested by scraping and centrifuged at 10,000 × g for 15 min at 4 °C. For immunoprecipitation, aliquots of cell homogenates were incubated with 12CA5 monoclonal antibody against the HA epitope (4 μg/ml) for 1 h at 4 °C. Protein G-Sepharose beads were added to the supernatant, incubated for 1 h, and washed extensively with lysis buffer. Bound immune complexes were eluted by boiling in Laemmli sample buffer, resolved by SDS-PAGE on 7.5% gels, and transferred onto nitrocellulose membranes. Immunoblots were probed with phospho-Ser1179-eNOS, phospho-Ser116-eNOS, eNOS, and HA epitope-specific antibodies provided by the suppliers. For immunoblot analyses of cell lysates, 20 μg of cellular protein were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed with phospho-Akt and Akt antibodies. Densitometric analyses of the immunoblots were performed using a ChemiImager 4000 (Alpha-Innotech).

In Vitro eNOS Phosphorylation—BAEC transfected with HA-tagged wild-type eNOS and myr εNOS were harvested, lysed, and immunoprecipitated with anti-HA monoclonal antibody as described above. The immune complexes were isolated with protein G-Sepharose and incubated with active recombinant Akt (0.1–0.5 μg) in phosphorylation assay buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM diethiothreitol) at 30 °C for 10 min. The reaction was stopped by adding 2× Laemmli sample buffer. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the phospho-Ser1179-eNOS antibody.

Results

We first studied the role of eNOS acyl modifications on agonist-induced eNOS phosphorylation at serine 1179, the putative site for agonist-induced enzyme phosphorylation by kinase Akt (13, 16, 17, 21). We compared the phosphorylation patterns of wild-type eNOS and myr εNOS and non-deficient mutants in response to two different agonists known to promote the phosphorylation of eNOS on serine 1179, the polypeptide growth factor VEGF (16, 20) and the platelet-derived sphingolipid SIP (21). BAEC were transfected with cDNA encoding wild-type eNOS, myr εNOS, or palm εNOS, as described under "Experimental Procedures." Cells were treated...
with S1P (100 nM) or VEGF (1 nM) for 5 min, and the recombinant proteins were immunoprecipitated with the anti-HA epitope antibody. eNOS phosphorylation at serine 1179 was completely abrogated, whereas the response to VEGF was reduced by 91 % (n = 3, p < 0.05) in the myr eNOS mutant, in which the putative Akt phosphorylation site was changed to alanine. As shown in Fig. 2, both the wild-type and myr eNOS served as effective substrates for in vitro phosphorylation by kinase Akt, with identical dose responses and time courses of Akt-induced phosphorylation. The control experiments shown in Fig. 4 established the specificity both of the phosphoserine 1179 antibody as well as the immunoprecipitation of recombinant eNOS using the HA epitope tag: for the S1179A eNOS mutant, in which the putative Akt phosphorylation site at serine 1179 was changed to alanine, there was no phosphorylation whatsoever when this immunoprecipitated mutant eNOS was incubated with purified kinase Akt.

To more fully understand the effects of eNOS targeting on enzyme phosphorylation, we next analyzed a series of chimeric eNOS proteins in which the transmembrane domain of the cell surface glycoprotein CD8 was fused either to the myr eNOS or...
the myr\(^{-}/\)palm\(^{-}\) eNOS (8). We have previously used biochemical approaches to explore features of the acylation and targeting of these chimeras (8). As shown in Fig. 5, we used here cellular imaging methodologies to further characterize the subcellular distribution of these CD8.eNOS chimeras by first fusing these constructs at their carboxyl termini to the green fluorescent protein EGFP and then performing laser scanning confocal microscopy on BAEC transfected with these plasmids. The subcellular distribution of these various EGFP-tagged eNOS constructs, wild-type eNOS (panel A), myr\(^{-}\) mutant eNOS (panel B), palm\(^{-}\) mutant eNOS (panel C), or the CD8 fusion constructs ligated to myr\(^{-}\) eNOS (panel D) or to myr\(^{-}/\)palm\(^{-}\) mutant eNOS (panel E), revealed distinctive patterns (Fig. 5). The wild-type eNOS-EGFP fusion protein targeted to peripheral as well as intracellular membranes. By contrast, the myr\(^{-}/\)EGFP fusion protein showed a diffuse intracellular pattern, consistent with prior reports that used biochemical (7) and imaging (28) approaches. The palmitoylation-deficient eNOS-EGFP fusion protein had a pattern of intracellular distribution similar to the wild type, but there was less uniform staining of the peripheral cell membrane in the palm\(^{-}\) mutant enzyme compared with wild-type eNOS (Fig. 5C). The addition of the CD8 transmembrane domain rescued the phenotype of the myr\(^{-}\) and palm\(^{-}\) mutants, restoring membrane targeting to yield a pattern similar to that of the wild-type eNOS-EGFP protein.

Having confirmed a similar subcellular distribution for wild-type eNOS and the CD8-eNOS chimeras, we next studied the agonist-induced phosphorylation pattern of these constructs in transfected BAEC. As shown in Fig. 6, the CD8.myr\(^{-}\) eNOS and CD8.palm\(^{-}\) eNOS chimeras showed a similar pattern of S1P- or VEGF-induced phosphorylation at serine 1179. The time course of agonist-induced serine 1179 phosphorylation of wild-type eNOS and myr\(^{-}\) eNOS by Akt. Wild-type eNOS and myr\(^{-}\) eNOS immunoprecipitated with anti-HA epitope antibody from BAEC lysates were incubated with 0.25 \(\mu\)g of recombinant Akt for the indicated times. Reaction mixtures were resolved by SDS-PAGE, and eNOS phosphorylation was analyzed in immunoblots probed with phospho-Ser1179-eNOS antibody. Equal loading was confirmed by re-probing the membranes with eNOS antibody.

FIG. 3. Time course of S1P- and VEGF-induced serine 1179 phosphorylation in wild-type and acylation-deficient eNOS. BAEC transfected with cDNA encoding wild-type (WT) eNOS and myr\(^{-}\) eNOS were treated with S1P (100 nM, upper panel) or VEGF (1 nM, lower panel) for the indicated times. Recombinant proteins were immunoprecipitated with the anti-HA epitope antibody, as noted. In parallel, cell lysates (20 \(\mu\)g/line) were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with phospho-Akt and Akt antibodies. These experiments were repeated three times with equivalent results.

FIG. 4. In vitro phosphorylation of wild-type and acylation-deficient eNOS by kinase Akt. BAEC transfected with wild-type (WT) eNOS, myr\(^{-}\) eNOS, and S1179A.eNOS were lysed, and recombinant eNOS was immunoprecipitated with anti-HA epitope antibody and analyzed in a kinase activity assay as described under “Experimental Procedures.” In panel A, immunoprecipitates were incubated with the indicated amounts of purified recombinant active Akt for 10 min, and the reaction mixture was resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with phospho-Ser1179-eNOS antibody. Equal loading of samples was confirmed by re-probing the immunoblots with eNOS antibody. These experiments were repeated twice with equivalent results. Panel B shows the time course for in vitro phosphorylation of wild-type eNOS and myr\(^{-}\) eNOS by Akt. Wild-type eNOS and myr\(^{-}\) eNOS immunoprecipitated with anti-HA epitope antibody from BAEC lysates were incubated with 0.25 \(\mu\)g of recombinant Akt for the indicated times. Reaction mixtures were resolved by SDS-PAGE, and eNOS phosphorylation was analyzed in immunoblots probed with the phospho-Ser1179-eNOS antibody. Equal loading was confirmed by re-probing the membranes with eNOS antibody.

FIG. 5. Subcellular distribution of EGFP-labeled wild-type, chimeric, and acylation-deficient eNOS constructs. The images shown are representative laser scanning confocal photomicrographs of BAEC transfected with eNOS-EGFP (A), myr\(^{-}\)eNOS-EGFP (B), palm\(^{-}\)eNOS-EGFP (C), CD8.myr\(^{-}\)eNOS-EGFP (D), or CD8.palm\(^{-}\)eNOS-EGFP (E). The images are representative of 24–100 images analyzed in 4–10 independent experiments for each sample.
tion was identical for these various constructs for both S1P (Fig. 7) and VEGF (Fig. 8). For both agonists, the time course of eNOS phosphorylation at serine 1179 coincided with the temporal pattern of agonist-induced kinase Akt phosphorylation; loading controls verified equivalent protein recovery under these different experimental treatments (Figs. 7 and 8). The phosphoinositide 3-kinase inhibitor wortmannin completely blocked the phosphorylation of kinase Akt and abrogated the agonist-induced phosphorylation of eNOS at serine 1179 (Figs. 7). In contrast to the myr mutant, the CD8 eNOS fusion proteins showed a significant reduction in serine 116 phosphorylation compared with the wild-type protein (Fig. 10). Taken together, these results suggest that, in direct contrast to the membrane-association requirement for serine 1179 phosphorylation, the phosphorylation of eNOS on serine 116 is enhanced when the enzyme is present in the cytosol and is attenuated when eNOS is membrane-targeted.

**DISCUSSION**

This study provides several new lines of evidence that agonist-induced phosphorylation of eNOS at serine 1179 by kinase Akt is largely restricted to the membrane-targeted form of the enzyme and importantly extends recent observations (14, 29).
FIG. 9. Effects of wortmannin on agonist-induced serine 1179 phosphorylation of wild-type eNOS and chimeric constructs. BAEC were transfected with wild-type eNOS, CD8.myr–eNOS, or CD8.myr−palm–eNOS, as noted. Before the addition of S1P (100 nM for 5 min) or VEGF (1 nM for 5 min), BAEC were treated with 1 μM wortmannin or vehicle for 30 min. Recombinant proteins were immuno-nprecipitated with the anti-HA epitope antibody and resolved by SDS-PAGE, and immunoblots (IB) were probed with phospho-Ser1179-eNOS antibody. Equal loading of the samples were confirmed by re-probing the immunoblots with anti-HA epitope antibody. In parallel, cell lysates (20 μg/lane) from BAEC were resolved by SDS-PAGE and probed with phospho-Akt and Akt antibodies. These results are representative of two independent experiments.

An acylation-deficient eNOS mutant (myr− eNOS) shows virtually no basal or agonist-induced phosphorylation at serine 1179 in response to either the polypeptide growth factor VEGF or the sphingolipid S1P (Fig. 1). By contrast, the wild-type eNOS shows marked phosphorylation in response to VEGF, with the effects of S1P being slightly less robust (Figs. 1–3). The wild-type and acylation-deficient eNOS mutants serve equally effectively as substrates for protein kinase Akt in vitro (Fig. 4), suggesting that it is the subcellular localization of these proteins rather than the direct effects of eNOS acylation that differentiate the wild-type and mutant proteins. We may further conclude that membrane targeting and not acylation per se represents that key determinant of eNOS phosphorylation at serine 1179; ligation of the CD8 transmembrane domain to an acylation-deficient eNOS mutant fully rescues the phenotype of the wild-type enzyme in terms of both its subcellular localization (Fig. 5) and serine 1179 phosphorylation (Figs. 6–8). The palm− eNOS mutant shows an intermediate phenotype for agonist-induced phosphorylation at serine 1179 (Fig. 1), consistent with our previous observations that the palm mutant is less effectively targeted to the membrane fraction (Ref. 5; see also Fig. 5). Palmitoylation of eNOS facilitates the targeting of the enzyme to plasmalemmal caveolae (30), and prolonged agonist treatment of endothelial cells promotes eNOS depalmitoylation (6) and translocation of the enzyme from peripheral to internal membrane fractions (31). Our finding that eNOS undergoes differential phosphorylation depending upon its subcellular targeting may thus directly relate to the control of pathways that modulate the endogenous cycle of reversible eNOS palmitoylation and translocation seen with agonist treatment. The fact that the palmitoylation-deficient eNOS shows attenuated agonist-dependent serine 1179 phosphorylation is consistent with the hypothesis that eNOS palmitoylation helps to promote deactivation of the enzyme. Interestingly, the palmitoylation-deficient mutant eNOS is indistinguishable from wild-type eNOS in its phosphorylation at serine 116. Further study will be required to discern whether the dynamic regulation of serine 116 phosphorylation is altered in the palmitoylation-deficient mutant.

The level of phosphorylation of a protein at a given residue reflects, of course, the balance of protein kinase and phosphoprotein phosphatase activities at that site. It is therefore not clear whether the enhanced phosphorylation of membrane-targeted eNOS at serine 1179 reflects more the inability of a cytosolic phosphatase to dephosphorylate the phosphoenzyme, the enhanced ability of a membrane-associated kinase to phosphorylate the enzyme, or both. The inverse may be true for phosphorylation at serine 116, but the paucity of information about the relevant kinase at the serine 116 site hinders further speculation. However, kinase Akt, which has been convincingly implicated in eNOS phosphorylation at serine 1179 (Refs. 16 and 17; see Fig. 4) is known to be principally a cytosolic enzyme (32, 33). Kinase Akt transiently targets to the membrane to be activated, and the activated kinase has important targets in the cytosol and the nucleus (32–35). Furthermore, kinase pathways upstream from kinase Akt are membrane-associated (36). Robust eNOS phosphorylation could require only that a small fraction of the total cellular Akt kinase is associated with the plasmalemmal caveolae in which eNOS resides. Alternatively, an active phosphoprotein phosphatase present in the cytosol could be responsible for the weak eNOS phosphorylation on serine 1179 that is seen with the myr− mutant eNOS. Indeed, the time course for the return of maximal levels of agonist-induced serine 1179 phosphorylation down to basal levels parallels the time course observed in BAEC for agonist-induced translocation from peripheral to internal membranes and back...
again (31, 37). It must be noted, however, that the fully acylation-deficient CD8.eNOS chimeras exhibit time courses and dose responses for agonist-induced serine 1179 phosphorylation and dephosphorylation that are identical to the wild-type enzyme (Figs. 7 and 8). The latter observations suggest that the relevant serine 1179 kinase(s) and phosphatase(s) are fully to allow membrane-associated eNOS independent of its reversible acylation.

eNOS phosphorylation at serine 116 shows striking differences from the pattern of phosphorylation at serine 1179. As shown in Fig. 10, the myr− mutant, which is restricted to the cytosol and exhibits significantly attenuated serine 1179 phosphorylation, consistently shows markedly enhanced phosphorylation at serine 116. Consistent with this observation, the CD8-eNOS fusion proteins, which are exclusively membrane-targeted, exhibit an attenuated level of serine 116 phosphorylation compared with the wild-type enzyme (Fig. 10). We have previously reported that the VEGF-induced dephosphorylation of eNOS on serine 116 appears to involve a pathway mediated by the calcium-activated phosphatase calcineurin (14), but the protein kinase involved in phosphorylation at this site has not been identified. Calcineurin is a cytosolic protein but can rapidly associate with cell membranes by virtue of its specific association with membrane-targeted signaling protein complexes (38, 39). With the available data, we cannot discern whether it is the enhanced activity of an unknown soluble kinase, the reduced activity of a membrane-targeted phosphatase, or both that are responsible for the exaggerated phosphorylation of eNOS on serine 116 when the enzyme is present in the cell cytosol. These observations do allow us to conclude that the phosphorylation of eNOS on discrete sites is differentially affected by the subcellular targeting of the enzyme and to suggest that the inter-relationships of eNOS acylation and phosphorylation may have important implications for regulation of NO-dependent signaling pathways in the vascular wall.

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