Palmitoylation of Endothelial Nitric Oxide Synthase Is Necessary for Optimal Stimulated Release of Nitric Oxide: Implications for Caveolae Localization†

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ABSTRACT: Endothelial nitric oxide synthase (eNOS) is dually acylated by N-myristoylation and cysteine palmitoylation and resides in Golgi and caveolae membranes. N-Myristoylation is necessary for its membrane association and targeting into the Golgi complex of transfected cells whereas palmitoylation influences the targeting of eNOS into caveolae. However, the *in vivo* significance of palmitoylation, membrane association, and the corresponding caveolar localization of eNOS have not been shown. To further examine the nature of membrane association of palmitoylation-deficient forms of eNOS and to address the functional role(s) of palmitoylation in activation of eNOS *in vivo*, HEK 293 cells stably transfected with wild-type (WT) or palmitoylation-deficient mutants of eNOS were generated. Membrane association of the mutants was biochemically similar to that of the WT protein in terms of their resistance to high salt, high pH, and distribution between Triton X-114 detergent and aqueous phases, suggesting that other hydrophobic factor(s) in eNOS most likely contribute to its membrane association. Most importantly, palmitoylation-deficient mutants of eNOS released less NO from the cells than did WT enzyme, suggesting that palmitoylation plays an important role in determining the optimal release of NO from intact cells. The diminished release of NO from the palmitoylation-deficient mutants was not attributable to alterations in its catalytic properties as the purified mutant and WT enzymes were kinetically identical. Since palmitoylation is necessary for localization of eNOS in caveolae, our data suggest that such localization could regulate the frequency and magnitude of NO release in response to stimuli *in vivo*.

Endothelial cell (EC)[1] derived nitric oxide (NO) is an important regulator of cardiovascular homeostasis due to its vasodilatory, growth regulatory, and antithrombotic properties. ECs produce NO via the NADPH requiring, calcium/calmodulin activated enzyme endothelial nitric oxide synthase (eNOS, NOS 3). The absolute amount of NO produced by EC can be regulated by the nature of stimulatory signals (receptor-dependent agonists and mechanical forces), intracellular cofactors (tetrahydrobiopterin), and eNOS subcellular localization.

eNOS localizes in the Golgi complex of cultured bovine aortic EC, in human umbilical vein EC, and in intact human blood vessels (Morin & Stanboli, 1993; O'Brien et al., 1995; Sessa et al., 1995). More recently, we and others have shown eNOS localization in plasmalemma caveolae of cultured bovine lung microvascular EC and in luminal aspects of plasmalemma isolated from intact, perfused rat lungs (García-Cardeña et al., 1996; Shaul et al., 1996). The targeting of many divergent signal transducing proteins (including inositol 1,4,5-triphosphate receptors, calcium ATPase, members of the src family of nonreceptor tyrosine kinases, G proteins, and G protein-coupled membrane receptors) to caveolae suggests that protein trafficking to and from these organelles could regulate the frequency and magnitude of cellular responses (Li et al., 1995; Song et al., 1996). However, the functional significance of protein localization to plasmalemma caveolae has not been firmly elucidated, *in vivo.*

eNOS is acylated by N-myristoylation and cysteine palmitoylation (cysteines-15 and/or -26) joining a family of dually acylated proteins that reside in caveolae such as src-tyrosine kinases and G-protein α subunits (Song et al., 1996). N-Myristoylation is necessary for eNOS membrane association (Busconi & Michel, 1993; Sessa et al., 1993), compartmentalization to the Golgi complex of cells (Sessa et al., 1995), palmitoylation (Liu et al., 1995; Robinson et al., 1995), and, most importantly, efficient NO production (Sessa et al., 1995). Palmitoylation is necessary for localization of eNOS in caveolae (García-Cardeña et al., 1996; Shaul et al., 1996). However, the importance of eNOS palmitoylation in regulating the membrane association is not clear (Liu et al., 1995; Robinson & Michel, 1995), and the influence of this posttranslational modification on NO release is not known.

Therefore, the purpose of this study is to address the biochemical and functional importance of eNOS palmitoylation in HEK 293 cells stably transfected with wild-type (WT) or palmitoylation-deficient mutant forms of eNOS.

MATERIALS AND METHODS

Generation of Cell Lines Stably Transfected with WT or Palmitoylation-Deficient Mutants of eNOS. WT, C15S, C26S, and C15/26S mutant (in which cysteine-15 and/or -26 have been mutated to serine) eNOS cDNAs were constructed (Liu et al., 1995) and subcloned into the mammalian

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[1] Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; EC, endothelial cell; HEK, human embryonic kidney; WT, wild type.

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expression vector pcDNA3 (containing the neomycin-resistance gene). Human embryonic kidney cells (HEK 293, ATCC) were transfected with WT or mutant eNOS cDNAs in pcDNA3 (15 μg of plasmid DNA per 1×10^6 cells in 100 mm dishes) according to the calcium phosphate precipitation protocol (Ausubel, 1990). Transfected cells were selected for growth in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (100 IU/mL), streptomycin (100 μg/mL), and 10% (v/v) fetal calf serum (complete DMEM) in the presence of G418 (800 μg/mL). After 3 weeks, G418-resistant colonies were maintained in the complete DMEM containing G418 (250 μg/mL).

Metabolic Labeling of Cells and Hydroxylamine Treatment. Stably transfected HEK 293 cells were preincubated for 30 min with cerulenin (2 μg/mL), an inhibitor of fatty acid synthetase, in serum-free DMEM containing bovine serum albumin (10 mg/mL, fatty acid free). Cells were then labeled with 300 μCi/mL [^{3}H]palmitic acid (50 Ci/mmol, New England Nuclear) for 4 h in the same medium. The [^{3}H]palmitic acid was dried under N_2 and redissolved in DMSO so that the final concentration of DMSO in the labeling medium was 0.1%.

The labeled eNOS was partially purified by 2′,5′-ADP−Sepharose (Pharmacia). After SDS−PAGE, duplicate gels were fixed with acetic acid/methanol/water (1:2:7), rinsed with water, and then placed in either freshly prepared 1 M hydroxylamine, pH 10, or, as a control, 1 M Tris-HCl, pH 10, for 18 h with gentle shaking at room temperature (Olson et al., 1985). Proteins in the gels were then visualized by Coomassie Blue staining and followed by fluorographic analysis as directed by the manufacturer and exposed to Hyperfilm-MP (Amersham) for 2 weeks.

Salt and High pH Sensitivity of WT and Palmitoylation-Deficient Mutant Forms of eNOS. Cells were rinsed with ice-cold phosphate-buffered saline (PBS) and then harvested. Cells were homogenized in 1 mL of homogenization buffer (50 mM Tris-HCl/0.15 M NaCl/0.1 mM EDTA/0.1 mM EGTA/0.01% β-mercaptoethanol/2 μM leupeptin/1 μM pepstatin A/1 μM aprotinin/1 mM phenylmethanesulfonyl fluoride, pH 7.5) with a Dounce homogenizer at 4 °C. Cell homogenates were centrifuged at 1000*g* for 10 min to remove unbroken cells and nuclei. The supernatants were centrifuged at 100000*g* for 90 min. The pelleted membranes were resuspended in the homogenization buffer containing 1 M NaCl or 100 mM $NaHCO_3/Na_2CO_3$ buffer, pH 11, incubated at 4 °C for 30 min (Howell & Palade, 1982), and then centrifuged again to separate membrane from supernatant fractions. Samples were subjected to SDS−PAGE, electroblotted onto nitrocellulose, and probed with eNOS monoclonal antibody H32 (kindly provided by Dr. Jennifer Pollock, Medical College of Georgia).

Triton X-114 Temperature-Induced Phase Separation of Aqueous and Detergent-Soluble eNOS. Triton X-114 phase separation was carried out according to the method of Pryde (1986). Briefly, stably transfected HEK 293 cells in T-75 flasks were washed with ice-cold PBS and lysed in 1 mL of 1% Triton X-114 lysis buffer (10 mM HEPES/0.1 mM EDTA/0.1 mM EGTA/0.01% β-mercaptoethanol/150 mM NaCl/2 μM leupeptin/1 μM pepstatin A/1 μM aprotinin/1 mM phenylmethanesulfonyl fluoride, pH 7.5) for 1 h at 4 °C. The lysate, upon removal of insoluble material, was loaded over 300 μL of a sucrose cushion (6% sucrose/0.06% Triton X-114/10 mM HEPES/150 mM NaCl), incubated at 30 °C until clouding of the solution occurred (3−5 min), and then centrifuged at 13000*g* for 5 min at room temperature. The upper aqueous and bottom detergent phases were separated and added or diluted to final concentration of Triton X-114 to 1%, respectively. eNOS proteins were partially purified with ADP−Sepharose for Western blot analysis.

Measurement of Nitrogen Oxide (NO_x) Release from HEK 293 Cells. For measurement of NO_x release (the breakdown product of NO in aqueous media, representing NO_2^-, NO_3^-, and nitrosothiols) from stably transfected HEK-293 cells, cells were grown in C-6 wells, washed with and incubated in Hanks' balanced salt solution supplemented with L-arginine (100 μM) and $CaCl_2$ (1.3 mM) in the presence or absence of ionomycin (3 μM) for 30 min at 37 °C and the supernatant was collected for analysis of NO_x. Samples containing NO_x were analyzed by NO-specific chemiluminescence (Sievers, Boulder, CO) as previously described (Sessa et al., 1995). Cells were lysed with 1 N NaOH for total protein determination using the Lowry assay or lysed in Laemmli's SDS sample buffer for Western blotting to determine eNOS expression. Stimulated NO_x release per milligram of cellular protein was calculated after subtracting unstimulated basal release from the control wells.

Purification of WT and C15S eNOS. Five hundred milliliters of a suspension culture of WT or C15S stably transfected HEK 293 cells was harvested and lysed in 50 mL of lysis buffer (homogenization buffer containing 10% glycerol, 1% Triton X-100, 100 μM L-arginine, and 5 μM BH_4). After removal of insoluble materials by centrifugation, lysate was incubated in batch with ADP−Sepharose resin (1 mL) for 1 h and loaded into a chromatography column. The column was washed sequentially with lysis buffer (10 column volumes), lysis buffer containing 0.5 M NaCl (10 column volumes), and then lysis buffer without EDTA/EGTA (buffer A, 10 column volumes). Proteins were eluted with buffer A containing 5 mM 2′-AMP and 2 mM $CaCl_2$. The eluate was further purified in batch by incubation with calmodulin−Sepharose resin (1 mL) for 1 h. After the mixture was packed into a column, the column was washed with buffer A containing 2 mM $CaCl_2$(10 column volumes) and then with buffer A containing 2 μM $CaCl_2$ (10 column volumes). eNOS was eluted with the lysis buffer containing 1 mM EGTA. All above procedures were carried out at 4 °C, and the purity of samples was determined using silver staining after SDS−PAGE.

Determination of Enzyme Kinetic Properties. NOS activity was measured by the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline as described (Bredt & Snyder, 1991). Fifty nanograms of protein of WT and C15S eNOS was used for all kinetic measurements. For K_m and V_{max} measurements, 1−100 μM L-arginine (including [^{3}H]-L-arginine) was used in reaction mixtures in the presence of 100 nM calmodulin/2.5 mM $CaCl_2$/30 μM BH_4/1 mM NADPH/10 mM Tris-HCl, pH 7.4. For measurements of calcium affinity, 0.01−100 μM free Ca^{2+} (calculated in the presence of 1 mM EGTA) was used in addition to 100 nM calmodulin/10 μM L-arginine/30 μM BH_4/1 mM NADPH/10 mM Tris-HCl, pH 7.4. For measurements of calmodulin affinity, 0.5−100 nM calmodulin was used in the presence of 10 μM L-arginine/2.5 mM $CaCl_2$/30 μM BH_4/1 mM NADPH/10 mM Tris-HCl, pH 7.4. For measurements of BH_4 affinity, 10 nM to 10 μM BH_4 was added into the reaction containing 100 nM calmodulin/2.5 mM $CaCl_2$/10 μM L-arginine/1 mM NADPH/

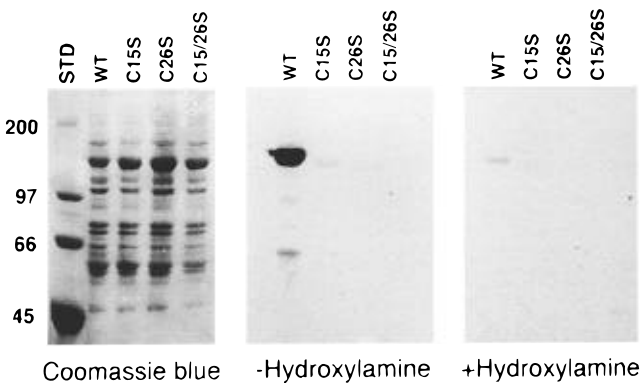


FIGURE 1: Mutation of cysteines-15 or/and -26 abolishes palmitoylation of eNOS. HEK 293 cells stably transfected with WT or mutant eNOS were labeled with [^{3}H]palmitic acid. After ADP−Sepharose affinity purification and SDS−PAGE, proteins were then visualized by Coomassie Blue staining (a, left, STD = molecular weight standards). Duplicate gels were treated with 1 M Tris-HCl, pH 10 (control, b, center) or 1 M hydroxylamine (NH_2OH), pH 10 (c, right) for 18 h followed by fluorographic analysis.

10 mM Tris-HCl, pH 7.4. For measurements of NADPH affinity, 0.01−100 μM NADPH was assayed in the presence of 100 nM calmodulin/2.5 mM $CaCl_2$/30 μM BH_4/10 μM L-arginine/10 mM Tris-HCl, pH 7.4. For NOS specific activity assay of total cell lysate, 50 μg of total proteins was used in addition to 100 nM calmodulin/2.5 mM $CaCl_2$/30 μM BH_4/10 μM L-arginine/1 mM NADPH/10 mM Tris-HCl, pH 7.4. All of the reactions were carried out at 37 °C for 5 min.

RESULTS

Mutation of Cys-15 and -26 Abolishes Palmitoylation of eNOS in Stably Transfected HEK Cells. WT, C15S, C26S, or C15/26S eNOS stably transfected HEK 293 cells were labeled with [^{3}H]palmitic acid, and labeled eNOS was partially purified by affinity chromatography, separated by SDS−PAGE, and detected by fluorography. As shown in Figure 1, mutation of cysteine-15 and/or −26 abolished the incorporation of [^{3}H]palmitic acid into eNOS. The labeling in WT eNOS was hydroxylamine-sensitive (Figure 1c), demonstrating that the palmitoylation of eNOS is in a thioester linkage. The hydroxylamine-resistant residual labeling in Figure 1c may be due to the conversion of palmitate into myristate in the cells. Identical results for palmitate labeling, hydroxylamine sensitivity, and, in addition, fatty acid analysis were obtained in transiently transfected COS cells (Liu et al., 1995).

Palmitoylation-Deficient Mutants Associate with Membrane via Hydrophobic Interaction. Previously, we demonstrated that mutation of eNOS palmitoylation sites does not significantly alter its overall membrane association (Liu et al., 1995). To examine if the mutants bind to membrane due to hydrophobic, ionic, or protein−protein interactions, membranes prepared from stably transfected HEK 293 cells were treated with 1 M NaCl or pH 11 $NaHCO_3$ buffer. As shown in Figure 2, palmitoylation-deficient mutants were still associated with membrane as tightly as WT eNOS after either treatment, implying that the mutants were associated with membranes through strong hydrophobic interactions not dependent on palmitate incorporation.

Next, we examined Triton X-114 temperature-induced phase separation to examine the hydrophobic character of

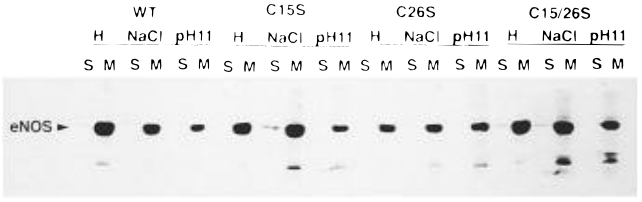


FIGURE 2: WT and palmitoylation-deficient mutants of eNOS are tightly associated with membranes after treatment with high salt or pH. Membranes prepared from HEK 293 cells stably transfected with WT or mutant eNOS were homogenized and treated with homogenization buffer (H, control), 1 M NaCl (NaCl), or sodium bicarbonate buffer (pH 11), and eNOS in supernatant (S) and membrane (M) fractions was determined by Western blotting. Similar results were obtained in an additional experiment.

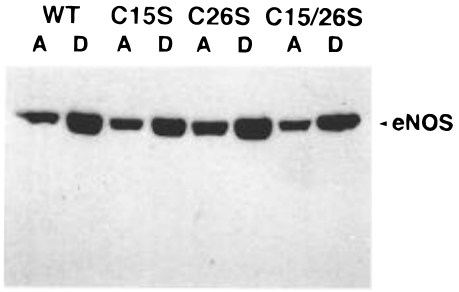


FIGURE 3: WT and palmitoylation-deficient mutants of eNOS partition similarly between Triton X-114 aqueous and detergent phases. Stably transfected HEK cells were solubilized with 1% Triton X-114 at 4 °C, and lysates were subjected to phase separation (aqueous, A, and detergent, D) at 30 °C. eNOS in each phase was partially purified with ADP−Sepharose, and samples were analyzed by Western blotting. Similar results were obtained in an additional experiment.

WT and palmitoylation-deficient mutant eNOS. Partitioning into the aqueous or detergents phases of Triton X-114 is a standard technique to discern the relative hydrophobicity of membrane proteins. If cysteine palmitoylation contributed significantly to the overall membrane association of eNOS, then the palmitoylation-deficient mutants of eNOS should partition more into the aqueous phase compared to the WT protein. Transfected HEK 293 cells were lysed with Triton X-114 at 4 °C and aqueous and detergent phases separated at 30 °C. As seen in Figure 3, WT and eNOS mutants partitioned similarly between the aqueous and detergent phases, with more eNOS in the detergent phase (77%, 72%, 71%, and 77% of WT, C15S, C26S, and C15/26S in the detergent phase, respectively, determined by laser densitometry). Thus, mutation of the palmitoylation sites of eNOS does not influence partitioning into the Triton X-114 detergent phase.

Cells Expressing Palmitoylation-Deficient Mutants Release Less NO upon Stimulation. To determine if cellular compartmentalization of eNOS influences NO release, we initially characterized NOS specific activity and immunoreactive protein in total cell lysates prepared from stably transfected HEK 293 cells. NOS specific activities (determined under V_{max} conditions with optimal amounts cofactors and substrate) were 33 ± 5, 41 ± 7, 35 ± 4, and 37 ± 5 pmol of citruline min^{-1} (mg of protein)$^{-1}$ for WT, C15S, C26S, and C15/26S eNOS, respectively (n = 2 experiments in duplicate). Also, the cells expressed roughly equal amounts of immunoreactive protein (see inset, Figure 4). Next, we challenged the cells with the calcium ionophore, ionomycin, and measured NO_x release. Agonist-dependent increases in cytoplasmic calcium

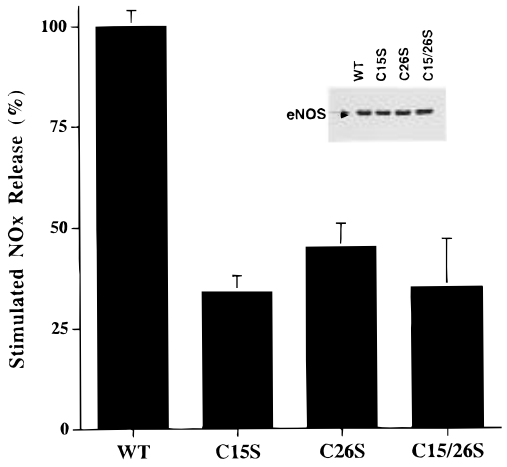


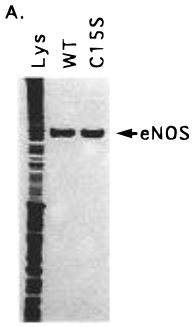
FIGURE 4: Palmitoylation-deficient mutants of eNOS release less NO upon ionomycin stimulation. Stably transfected HEK cells were stimulated with ionomycin (3 μM) as described, and NO_x release was measured by NO-specific chemiluminescence. Stimulated NO_x release from WT eNOS transfected HEK cells (typically, 310 ± 15 pmol/mg of protein) was taken as 100%. Bars represent means; capped vertical lines represent SE (n = four experiments in duplicate). As seen in the inset, stable cell lines expressed roughly equal amounts of eNOS protein in total cell lysates (60 μg of protein loaded in each lane) as determined by Western blotting.

facilitate calcium−calmodulin-dependent activation of eNOS and cause the subsequent release of NO (Pollock et al., 1991; Sessa et al., 1992). Ionomycin (3 μM) induced the release of NO_x from both WT and palmitoylation-deficient mutant eNOS transfected HEK cells. However, cells expressing palmitoylation-deficient mutants released less NO_x (60% less) than did cells expressing WT eNOS (Figure 4), suggesting that palmitoylation was necessary for optimal stimulated NO release. Partial inhibition of stimulated NO_x release (40−50%) was also seen in NIH 3T3 cells stably transfected with palmitoylation-deficient mutant forms of eNOS compared to cells stably expressing WT enzyme (data not shown). As shown previously, palmitoylation deficient eNOS does not localize to caveolae in transfected cells (García-Cardeña et al., 1996; Shaul et al., 1996). These data show that in spite of equal catalytic quantities of eNOS, the mislocalization of the palmitoylation mutants influences the optimal release of NO from intact cells.

The Palmitoylation-Deficient Mutant Has Kinetic Properties Identical to Those of WT eNOS. Although the stable cell lines expressed similar amounts of eNOS protein and activity, less NO release from palmitoylation-deficient mutants could be ascribed to their different kinetic properties compared to WT eNOS. To address this possibility, we purified a palmitoylation-deficient mutant (C15S) and WT eNOS to homogeneity using two steps of affinity chromatography (Figure 5A). As seen in the table in Figure 5B, WT and C15S eNOS behaved virtually identically in terms of their specific activities and affinities for substrate and cofactors, indicating that mutation of the palmitoylation site does not significantly affect kinetic parameters of eNOS. Therefore, palmitoylation does not affect NOS activities *in vitro.*

DISCUSSION

The present study demonstrates that palmitoylation of eNOS does not significantly affect its overall membrane affinity or catalytic activity. More importantly in intact cells,



B.

		WT	C15S
Km (L-Arg) (μM)		5.0	5.4
Vmax (nmol/min/mg)		585	614
Km (NADPH) (μM)		0.65	0.73
EC_{50}	Ca^{2+} (μM)	0.11	0.09
	CaM (nM)	9.0	8.6
	BH_4 (μM)	0.22	0.30

FIGURE 5: Palmitoylation-deficient mutant eNOS (C15S) is catalytically and kinetically similar to WT eNOS. Panel A represents a silver-stained gel of eNOS in total lysate (Lys) or after two affinity purification steps of WT or mutant. In panel B is a summary of kinetic data performed with WT or C15S eNOS.

expression of palmitoylation-deficient mutant forms of eNOS reduces the total amount of NO released relative to fully acylated eNOS. These data show for the first time that protein cysteine palmitoylation, a posttranslational modification of eNOS necessary for localization into caveolae (García-Cardeña et al., 1996; Shaul et al., 1996), has a functional consequence in intact cells and suggests that caveolae localization optimizes the ability of eNOS to produce NO.

Cysteine palmitoylation has been demonstrated to be necessary for overall membrane association of certain proteins (such as G protein α subunit Gs; Wedegaertner & Bourne, 1994) but not all proteins (Hancock et al., 1990; Koegl et al., 1994). Mutation of the palmitoylation sites of eNOS does not significantly alter its overall membrane association when expressed transiently (Liu et al., 1995) or stably (this paper) in HEK 293 cells. Similar results have been reported for palmitoylation-deficient mutants of GAD 45 and the G protein α subunit, Gqα (Shi et al., 1994; Hepler et al., 1996). For example, mutation of the palmitoylation sites of GAD 45 or Gqα inhibits palmitoylation but does not influence membrane association or partitioning into the Triton X-114 detergent phase.

Previously, we have shown that N-myristoylation is necessary but not sufficient for membrane association of eNOS due to the presence of myristoylated eNOS in both cytosolic and membrane fractions. In addition, the association of *in vitro* translated eNOS with membranes and endogenous eNOS in EC membranes is resistant to high salt and pH (Pollock et al., 1991; Busconi & Michel, 1994). Therefore, there must be other hydrophobic determinant(s) in eNOS, besides N-myristoylation and palmitoylation, important for its membrane association. Recently, it was shown that the basic amphipathic, α-helical calmodulin (CAM) binding domain of eNOS was involved in the binding of eNOS to acidic phospholipid vesicles and that CAM binding would inhibit WT or fatty acylation-defective (G2A, which is neither N-myristoylated nor palmitoylated) eNOS−phospholipid interactions, suggesting that CAM could regulate eNOS membrane association (Venema et al., 1995). In the present experiments, we did not examine if CAM could displace WT or palmitoylation-deficient mutant forms of eNOS; however, *in vivo* expression of fatty acylation-defective G2A eNOS in the cytosol of transiently transfected COS cells or of stably transfected HEK cells argues against CAM binding as a major determinant of membrane associa-

tion (Sessa et al., 1993; Busconi & Michel, 1993). However, it does not rule out the possibility that the eNOS CAM domain may cooperate with amino-terminal *N*-myristate to facilitate membrane association.

Although palmitoylation-deficient mutant forms of eNOS have similar hydrophobic and kinetic properties compared to WT eNOS when expressed in HEK293 cells, these mutants release significantly less NO than WT eNOS did. Because NO can be released, albeit at lower levels, from cells expressing either the membrane-associated, palmitoylation mutants (this paper) or cytosolic, acylation-defective G2A mutant eNOS (Sessa et al., 1995) argues against membrane association being sufficient for maximal NO release. However, compartmentalization of eNOS into specific intracellular membrane domains (Golgi and caveolae) appears necessary for optimal, stimulated NO release. Previously, it was shown that acylation-defective eNOS is cytosolic and does not target into the Golgi complex of cells and more recently that palmitoylation-deficient mutants of eNOS do not target to plasmalemma caveolae (García-Cardeña et al., 1996; Shaul et al., 1996). Thus, it is likely that targeting onto the cytoplasmic face of the Golgi via N-myristoylation and then into caveolae via cysteine palmitoylation is an important event necessary for placing eNOS into a milieu rich in NOS substrates (L-arginine, NADPH, oxygen), cofactors (Ca^{2+}, tetrahydrobiopterin, and flavins), and NOS regulatory proteins (CAM). The enrichment of CAM in purified caveolae has been recently described (Shaul et al., 1996). Alternatively, other proteins with regulatory functions localized in caveolae such as nonreceptor tyrosine kinases (Shenoy-Scaria et al., 1994; Robbins et al., 1995; Song et al., 1996) may influence eNOS activation. The concept of protein−protein interactions regulating the localization of NOS isoforms has recently been described for neuronal NOS (nNOS, NOS 1). The postsynaptic localization of nNOS in neurons occurs via an interaction with postsynaptic density protein 95 (Brenman et al., 1996), which may be a scaffolding molecule necessary to assemble a signaling complex in specific domains of the plasma membrane. By analogy to nNOS, palmitoylation of eNOS is likely to play a role in its targeting near to other signaling proteins or substrates to form a highly efficient signal transduction cascade in discrete regions of endothelial cells.

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