Biosynthesis and Palmitoylation of Endothelial Nitric Oxide Synthase: Mutagenesis of Palmitoylation Sites, Cysteines-15 and/or -26, Argues against Depalmitoylation-Induced Translocation of the Enzyme†

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ABSTRACT: The presence of myristoylated endothelial nitric oxide synthase (eNOS) in cytosolic fractions of bovine aortic endothelial cells (BAEC) suggests that N-myristoylation of eNOS is not sufficient for membrane localization. Therefore, we examined if posttranslational palmitoylation was another molecular signal for the membrane attachment of eNOS. Metabolic labeling showed incorporation of [^{3}H]palmitic acid into the membrane-bound, but not the cytosolic, form of eNOS. Fatty acid analysis demonstrated the labeled fatty acid incorporated into eNOS was palmitate, linked via a hydroxylamine-labile thioester bond. Biosynthesis and turnover studies show that the turnover of palmitate is much faster than the protein itself. However, the rates of palmitoylation and depalmitoylation were not affected by bradykinin or ionomycin treatment of BAEC. To examine the contribution of palmitoylation to the membrane association of eNOS, we mutated cysteine-15 and -26. Both mutations markedly diminished palmitoylation of eNOS, but did not significantly alter its membrane association. Additionally, [^{3}H]palmitic acid was not incorporated into nonmyristoylated mutant eNOS (G2A eNOS), suggesting that myristoylation is necessary for subsequent palmitoylation of the enzyme. Taken together, our data imply that palmitoylation does not play a major role in membrane association of eNOS and other amino acid sequences, in conjunction with N-myristoylation, are necessary and sufficient for membrane association of the enzyme.

The nitric oxide synthase (NOS)[1] family of proteins is a novel class of mammalian enzymes that convert L-arginine to nitric oxide (NO) and L-citrulline. Three isoforms of NOS have been elucidated to date based on their purification, cDNA cloning, and genomic localization: neuronal (nNOS), cytokine-inducible (iNOS), and endothelial (eNOS). These proteins share 50% amino acid identity, and are separate gene products (Sessa, 1994).

Although all the NOS isoforms can exist as soluble or membrane-associated proteins (Nathan & Xie, 1994), most insights into the biochemical mechanism for membrane association have been derived from studies on eNOS. eNOS is an N-myristoylated protein (Liu & Sessa, 1994). Myristic acid, a 14-carbon saturated fatty acid, is attached to eNOS cotranslationally through an amide linkage to glycine-2. Mutation of glycine-2 to alanine (G2A eNOS) inhibits the incorporation of [^{3}H]myristic acid into eNOS and converts the enzyme from a membrane to a cytosolic activity (Sessa et al., 1993a; Busconi & Michel, 1993), implying that N-myristoylation is necessary for its membrane association. However, it is unlikely that myristoylation, *per se*, is sufficient for membrane localization. The Gibbs free energy for binding of a myristoylated peptide to a phospholipid vesicle is not sufficient to anchor a myristoylated protein to membrane stably (Peitzsch & McLaughlin, 1993). Indeed, several N-myristoylated proteins have been found in cytosolic fractions (Gordon et al., 1991). High-salt treatment or membrane denaturation failed to release eNOS from membrane fractions (Pollock et al., 1991; Busconi & Michel, 1994), suggesting that association of eNOS with membranes is mainly due to strong hydrophobic interactions rather than to weak ionic interactions. Therefore, additional factors must be involved in membrane association of eNOS.

Another common fatty acid modification of proteins involved in signaling pathways is palmitoylation. The 16-carbon saturated fatty acid is attached posttranslationally to proteins. This modification increases protein hydrophobicity, thus facilitating interactions with lipid bilayers (Peitzsch & McLaughlin, 1993; James & Olson, 1990a). Palmitoylation is a reversible process, unlike myristoylation, which is irreversible (Gordon et al., 1991). Palmitoylation and depalmitoylation of proteins can be regulated by extracellular signals providing a mechanism for the dynamic regulation of protein localization (James & Olson, 1990b; Degtyarevet al., 1993; Mumby et al., 1994; Wedegaertner & Bourne, 1994). Several myristoylated proteins including G proteins and members of the Src family are also palmitoylated (Resh, 1994), and myristoylation is necessary for subsequent palmitoylation (Galbiati et al., 1994; Grassie et al., 1994). While this paper was in preparation, it was demonstrated that eNOS is palmitoylated and that bradykinin could cause depalmitoylation of the enzyme suggesting that the cycle of

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[1] Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; BAEC, bovine aortic endothelial cells; HEK, human embryonic kidney.

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palmitoylation/depalmitoylation could regulate the amount of eNOS found in membranes and cytosol (Robinson et al., 1995). Therefore, the main purpose of the present study was to further charaterize the palmitoylation of eNOS, to identify the potential palmitoylation sites, and to directly determine if palmitoylation could contribute to its membrane association.

MATERIALS AND METHODS

Generation of Cell Lines Stably Transfected with Wild-Type or G2A eNOS. Wild-type and G2A mutant (in which glycine-2 has been mutated to alanine) eNOS cDNAs were constructed (Sessa et al., 1993a) and subcloned into the mammalian expression vector pcDNA3 (containing the neomycin-resistant gene). Human embryonic kidney cells (HEK 293, ATCC) were transfected with wild-type or G2A eNOS cDNAs in pcDNA3 (15 μg of plasmid DNA per 1 $\times$ 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). In preliminary experiments, HEK 293 cells did not contain the mRNA for eNOS nor express NOS activity (data not shown). After 14 days, G418-resistant colonies were isolated with cloning cylinders, trypsinized, and proliferated in the complete DMEM containing maintenance concentrations of G418 (250 μg/mL). Six stable cloned lines of each wild-type and G2A were characterized, and lines WT.HEK5 and G2A.HEK6 were used for this study.

Determination of Fatty Acyl Modification of eNOS. Wild-type eNOS-transfected HEK 293 cells or BAEC were labeled with [^{3}H]palmitic acid for 4 h. The labeled eNOS was purified by immunoprecipitation, followed by electrophoresis as described below. 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 described above.

To identify the fatty acid incorporated into eNOS, eNOS was metabolically labeled with [^{3}H]palmitic acid, immunoprecipitated, separated, and excised from SDS−PAGE. The ^{3}H-labeled eNOS, [^{3}H]myristic acid, or [^{3}H]palmitic acid was hydrolyzed with 800 μL of 2 N HCl/83% methanol *in vacuo* at 110 °C for 18 h. The hydrolysates were extracted several times with chloroform/methanol (Bligh & Dyer, 1959), and organic extracts were evaporated to dryness under a stream of nitrogen and redissolved in 10 μL of methanol. Fatty acids were analyzed by thin-layer chromatography on Whatman KC-18 reversed phase plates, with acetonitrile/acetic acid (1:1) as the mobile phase (Randerath, 1963). After chromatography, plates were air-dried, treated with En^{3}Hance spray (New England Nuclear), and exposed to Hyperfilm-MP for a month.

Metabolic Labeling of Cells. Bovine aortic endothelial cells (BAEC, passages 3−5) were maintained in the complete DMEM as previously described (Liu & Sessa, 1994). Stably transfected HEK 293 cells, transiently transfected COS cells (60−70 h post-transfection), or BAEC (in 100 mm dishes) 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]myristic acid or [^{3}H]palmitic acid (45−50 Ci/mmol, New England Nuclear) for 4 h in the same medium. The ^{3}H-fatty acids were dried under N_2 and redissolved in DMSO so that the final concentration of DMSO in the labeling medium was 0.1%.

In pulse−chase experiments, BAEC were labeled with 300 μCi/mL ^{3}H-fatty acids or 150 μCi/mL [^{35}S]methionine for 1 h in serum-free medium. The cells were quickly rinsed once and chased for various time intervals with complete DMEM containing 0.1 mM of an appropriate cold fatty acid or 10 $\times$ concentration of methionine. In some experiments, BAEC were labeled with [^{3}H]palmitic acid in serum free media, in the absence or presence of bradykinin (10 μM) or ionomycin (10 μM). To examine if the turnover of palmitic acid was modulated by agonists, BAEC were labeled with [^{3}H]palmitic acid for 1 h and then chased at various time points with the complete DMEM, in the absence or in the presence of bradykinin (10 μM) or ionomycin (10 μM). At the end of each incubation, cells were lysed with 1 mL of modified RIPA buffer (homogenization buffer containing 1% Nonidet P-40/0.15 M NaCl/0.5% sodium deoxycholate/0.05% SDS) for immunoprecipitation.

Immunoprecipitation. Cells or the above samples were solubilized by incubation in 1 mL of the modified RIPA buffer for 30 min at 4 °C. Lysates were centrifuged at 10000*g* for 5 min to remove insoluble material, and 2 μg of the eNOS monoclonal antibody H32 (ascites fluid, IgG_2a isotype; kindly provided by Dr. Jennifer Pollock, Abbott Laboratories) was added and incubated for 2 h at 4 °C. Fifteen microliters of protein A−Sepharose (Pharmacia) suspension (1:1 in RIPA buffer) was added and incubated for 1 h at 4 °C. The beads were pelleted, washed 3 times with 1 mL of RIPA buffer, and then boiled in SDS−PAGE sample buffer for 5 min. Preliminary experiments using [^{35}S]-methionine-labeled BAEC cells demonstrated that H32 quantitatively and specifically precipitated eNOS in the above conditions (data not shown).

Intracellular Calcium Measurements. Ca^{2+} measurements were performed on an ACAS 570 Interactive Laser Cytometer using a 488 argon source (Meridian, Inc.), and $[Ca^{2+}]_i$ was monitored by using the fluorescent Ca^{2+} probe Fluo-3. This fluorescein-based dye gains fluorescence intensity proportionally with increasing $[Ca^{2+}]_i$. BAEC were plated onto 35 mm culture dishes and grown overnight in BAEC medium. The next day cells were loaded with Fluo-3 (final concentration 2 μM) for 60 min using pluronic acid at 22 °C, washed twice, and placed into Krebs'−Ringers−HEPES buffer (composed of 125 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM HEPES, and 6 mM glucose with 5% FBS, pH 7.4). Tissue culture dishes were placed onto the stage of the ACAS 570 and cells visualized with an integrated, inverted microscope to select appropriate fields. Repeated laser excitiation of a field (generally containing 8−10 cells) was performed with continuous recording of fluorescence data. Bradykinin (10 μM) or ionomycin (1 μM) was added, and the scanning was continued for an additional 10 min. Fluorescence intensity curves for individual fields were generated with the Kinetic software data program available with the ACAS 570.

Measurement of Nitrite (NO_2^-) Release from BAEC. For measurement of NO_2^- (the primary breakdown product of NO in aqueous media) release from BAEC, cells were grown in 35 mm dishes. The medium was changed to Hanks' balanced salt solution supplemented with L-arginine (100 μM) and $CaCl_2$ (1.3 mM), and cells were equilibrated for 30 min at 37 °C. To stimulate NO release, bradykinin (10 μM) or ionomycin (1 μM) was added for 30 min, and the supernatant was collected for analysis of NO_2^- by chemiluminescence. Samples (100 μL) containing NO_2^- were refluxed in 0.1 M H_2SO_4 with potassium iodide (to stoichiometrically convert NO_2^- to NO), and NO was quantitated by an NO chemiluminescence detector (Sievers Instruments) after reaction with ozone. Sodium nitrite served as a standard for quantification purposes. BAEC cell numbers were determined and data presented as picomoles of NO_2^- per 10^6 cells per 30 min.

Fluorography and Western Blotting. Samples were separated by 5−15% gradient or 7.5% SDS-PAGE gels (Laemmli, 1970). For fluorographic analysis, the gels were treated with En^{3}Hance (New England Nuclear) as directed by the manufacturer and exposed to Hyperfilm-MP (Amersham). For Western blotting, proteins were electroblotted onto nitrocellulose. Blots were probed with the eNOS monoclonal antibody H32 and detected with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody as previously described (Pollock et al., 1993; Sessa et al., 1993b).

Site-Directed Mutagenesis of Cysteines-15 and -26 in eNOS and Transient Transfection of COS Cells. Oligonucleotide-mediated mutagenesis was performed with the Altered Sites mutagenesis kit (Promega) essentially as described by the manufacturer. In brief, the full-length eNOS cDNA in Bluescript plasmid (Sessa et al., 1992) was digested with *Hin*dIII/*Xba*I and subcloned into the corresponding sites of the mutagenesis vector pALT. Single-stranded DNA was made and annealed with mutagenic primers, and the complementary strand was synthesized. The mutagenic primer sequences that converted cysteine codon (tgc) to serine (tcc) were designed, introducing an *Avr*II restriction site to aid selection of mutant cDNAs. Mutagenic primers (antisense) were 5′-CCCCAGCCCTAGGCCGGAGGGGGGCCC-3′ (C15S) and 5′-TGCTTGCCGGATAGCCCTAGGCCCAG-CC-3′ (C26S). Mutants were isolated, and mutations were confirmed by DNA sequencing. C15S and C26S mutants and wild-type eNOS cDNAs were subcloned (*Hin*dIII/*Xba*I) into the mammalian expression vector pcDNA3. COS-7 cells were transiently transfected with mutant and wild-type eNOS cDNAs (15 μg of plasmid DNA per 1×10^6 cells in 100 mm dishes) using the DEAE-dextran procedure (Ausubel, 1990).

Cell Fractionation and NOS Activity Assay. Cells were rinsed with ice-cold phosphate-buffered saline, scraped into the same buffer, and centrifuged at 600*g* for 5 min. Cell pellets were resuspended in 1 mL of homogenization buffer (50 mM Tris-HCl/0.1 mM EDTA/0.1 mM EGTA/2 μM leupeptin/1 μM pepstatin A/1 μM aprotinin/1 mM phenylmethanesulfonyl fluoride/10 mM NaF/1 mM Na_3VO_4, pH 7.5) and homogenized with a Dounce homogenizer at 4 °C. Cell homogenates were centrifuged at 100000*g* for 90 min at 4 °C. NOS activities of samples were assayed by determining the conversion of [^{3}H]-L-arginine into [^{3}H]-L-citruline as described (Sessa et al., 1992). Briefly, membrane or cytosolic proteins (50−100 μg) were incubated (total volume 0.2 mL) in homogenization buffer containing 1 mM NADPH, 3 μM tetrahydrobiopterin, 100 nM calmodulin, 2.5 mM $CaCl_2$, and 10 μM L-arginine and [^{3}H]-L-arginine (0.2 μCi, 55 Ci/mmol) for 45 min at 37 °C. After the incubation period, the reaction was quenched by the addition of 1 mL of 20 mM HEPES stop buffer, pH 5.5 (containing 2 mM EDTA and 2 mM EGTA). The reaction mix was then passed over a 1 mL column containing Dowex AG 50WX-8 (Na^+ form) resin (preequilibrated in stop buffer); washed with 1 mL of water, and collected directly into a 20 mL liquid scintillation vial containing scintillation cocktail for counting.

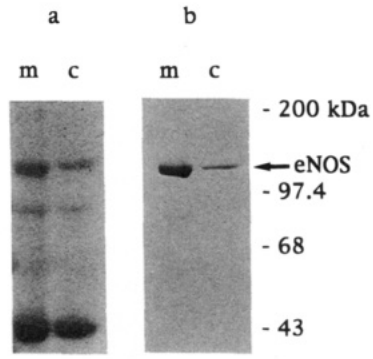

FIGURE 1: Both membrane and cytosolic eNOS are myristoylated. BAEC were labeled with [^{3}H]myristic acid. After subcellular fractionation into membrane (m) and cytosolic (c) fractions, eNOS was immunoprecipitated, electrophoresed, and visualized by Coomassie Blue staining (a) and then by fluorography after a 1 month exposure (b). eNOS migrates as a 135 kDa protein on SDS−PAGE, and the lower band in (a) is the immunoglobulin heavy chain. Similar labeling results were observed in five other experiments.

RESULTS

A Cytosolic Pool of eNOS Is Myristoylated. Previous studies examining the subcellular distribution of eNOS have shown that 5−10% of eNOS protein or activity was detectable in cytosolic fractions with the remainder of the protein/activity in particulate fractions. N-Myristoylation of eNOS is necessary for its membrane localization. However, it is not known if myristoylation is also sufficient for its particulate localization. To examine if the cytoplasmic form is myristoylated, BAEC were labeled with [^{3}H]myristic acid and fractionated into cytosol and membranes, and eNOS was immunoprecipitated. Immunoprecipitated eNOS was subjected to SDS−PAGE and radiolabeled protein visualized by fluorography. Figure 1a shows that approximatley 90% of eNOS is associated with membranes (based on densitometry of the negative of the Coomassie-stained gel), and Figure 1b shows that the cytosolic form of eNOS is also myristoylated and accounts for about 5% of total myristoylated eNOS (5 ± 2%, mean ± standard deviation, $n = 6$ experiments, determined by densitometry).

eNOS Is Palmitoylated via a Thioester Linkage. To examine if eNOS is palmitoylated, BAEC were labeled with [^{3}H]palmitic acid for 4 h and then fractionated into cytosol and membranes. eNOS was immunoprecipitated and subjected to SDS−PAGE, and labeled protein was visualized by fluorography. As seen in Figure 2a, [^{3}H]palmitic acid was incorporated into eNOS, and the label was found in the membrane fraction. Comparison of eNOS by Western blotting (Figure 2b), however, revealed that a small amount (8%, determined by densitometry) of eNOS was present in

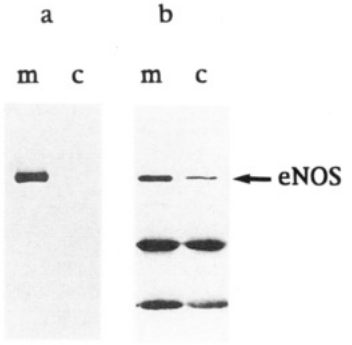

FIGURE 2: Membrane-bound eNOS is labeled with [^{3}H]palmitic acid. BAEC were labeled with [^{3}H]palmitic acid in the presence of cereulenin. After subcellular fractionation into membrane (m) and cytosolic (c) fractions, eNOS was immunoprecipitated and subjected to SDS−PAGE and fluorographic analysis after a 1 month exposure (a) or Western blot analysis (b). Proportional aliquots of membrane and cytosolic proteins were loaded on both gels. The two large bands below eNOS in (b) are the immunoglobulin heavy and light chains, respectively. Similar results were observed in two additional experiments.

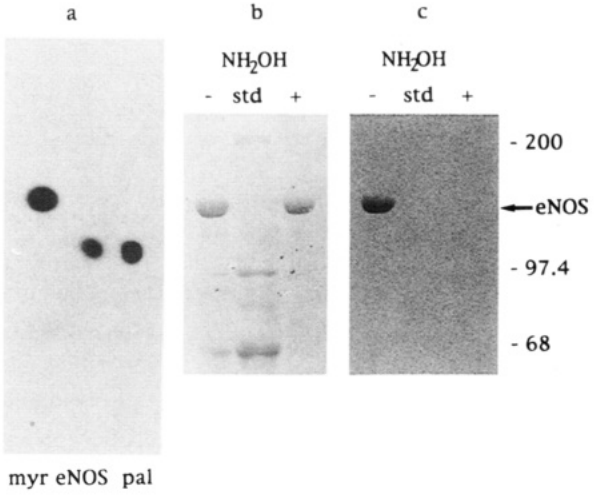

FIGURE 3: eNOS is palmitoylated via a thioester linkage. In (a), labeled eNOS, [^{3}H]palmitic acid, and [^{3}H]myristic acid were acid-methanolysed, extracted, and identified by reversed-phase thin-layer chromatography. In (b) and (c), [^{3}H]palmitic acid-labeled eNOS was immunoprecipitated and separated by SDS−PAGE. Duplicate gels were treated with 1 M hydroxylamine (NH_2OH), pH 10, or as a control, 1 M Tris-HCl, pH 10, for 18 h. Proteins in the gels were then visualized by Coomassie Blue staining (b, std = molecular weight standards) followed by fluorographic analysis (c).

the cytosolic fraction, suggesting that there is a cytosolic myristoylated, nonpalmitoylated pool of eNOS in the cells and that the palmitoylated form is exclusively associated with the membrane fraction.

Since cells can convert palmitic acid into other metabolites prior to its attachment to proteins (Olson et al., 1985; McIlhinney et al., 1985; Kamps et al., 1985), it was necessary to determine if the ^{3}H-label in eNOS was still palmitate. Labeled eNOS from BAEC was immunoprecipitated, separated by SDS−PAGE, and excised from the gel. The protein-bound fatty acid was released by acid methanolysis, extracted, and identified by reversed-phase thin-layer chromatography. As shown in Figure 3a, the ^{3}H-labeled material was recovered as a single species that comigrated with the methyl ester of authentic [^{3}H]palmitic acid, but not the methyl ester of [^{3}H]myristic acid. These results demonstrate that eNOS is palmitoylated.

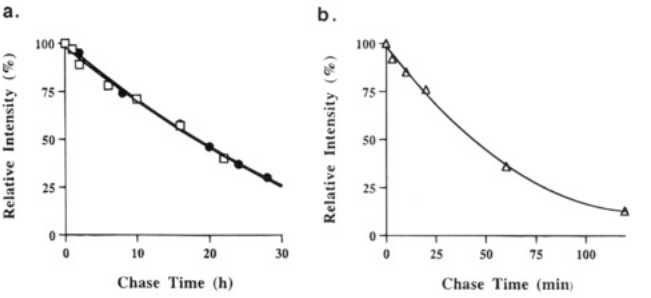

FIGURE 4: Turnover of eNOS and of its lipid modifications. BAEC were labeled with 150 μCi/mL [^{35}S]methionine (open squares in panel a), 300 μCi/mL [^{3}H]myristic acid (solid circles in panel a), or [^{3}H]palmitic acid (b) for 1 h. The cells were quickly rinsed once and chased for various time intervals with the complete DMEM containing 0.1 mM of the appropriate cold fatty acid or 10 × concentration of methionine. At the end of each incubation, the cells were lysed for immunoprecipitation and subjected to SDS−PAGE. After visualization of the protein by Coomassie Blue staining, labeled eNOS was quantitated by densitometry of fluorograms with the highest intensity taken as 100%.

Although most palmitoylated proteins incorporate palmitic acid via a hydroxylamine-labile thioester linkage to cysteine residues (Towler et al., 1988), palmitic acid has been reported to be incorporated via a hydroxylamine-resistant amide bond to lysine residues (Hackett et al., 1994; Stanleyet al., 1994). To determine if the label was attached to eNOS via a thioester or amide bond, duplicate samples of labeled eNOS were isolated from wild-type eNOS-transfected HEK 293 cells, electrophoresed, and treated with hydroxylamine or Tris buffer. Figure 3b,c demonstrates that linkage of radiolabel to eNOS was sensitive to alkaline hydroxylamine treatment, suggesting that the palmitic acid was linked to eNOS via a thioester bond.

Turnover of Palmitate of eNOS Is Much Faster than the Enzyme Itself. To examine the turnover of eNOS and of its lipid modification, BAEC were pulse-labeled with an appropriate ^{3}H-fatty acid or [^{35}S]methionine and then chased for various time intervals. At the end of each chase, the cells were lysed, and eNOS was immunoprecipitated and subjected to SDS−PAGE. After visualization of the protein by Coomassie Blue staining, labeled eNOS was subjected to fluorographic and densitometric analysees. As shown in Figure 4, turnovers of [^{35}S]methionine-labeled eNOS and [^{3}H]myristate-labeled eNOS are 19 ± 1 h (n = 4) and 18 ± 1 h (n = 3), respectively, whereas the turnover of palmitate of eNOS is 45 ± 5 min (n = 3). The rapid turnover of palmitate in eNOS may be due to the reversible nature of palmitoylation, and the identical half-lives of [^{35}S]methionine and [^{3}H]myristate labeled are consistent with the idea that N-myristoylation is an irreversible process.

Neither Palmitoylation nor Depalmitoylation of eNOS Is Modulated by Agonists. To address the possibility that agonists modulate the palmitoylation or depalmitoylation of eNOS, BAEC were labeled with [^{3}H]palmitic acid in the absence or presence of bradykinin for the indicated time periods and then immunoprecipitated. After visualization of the protein by Coomassie Blue staining, labeled eNOS was subjected to fluorographic and densitometric analyses. As shown in Figure 5a, the rate and extent of eNOS palmitoylation were not affected by bradykinin treatment. When BAEC were pulse-labeled with [^{3}H]palmitic acid and then chased in the absence or presence of bradykinin for the indicated time periods, the rate of depalmitoylation of eNOS

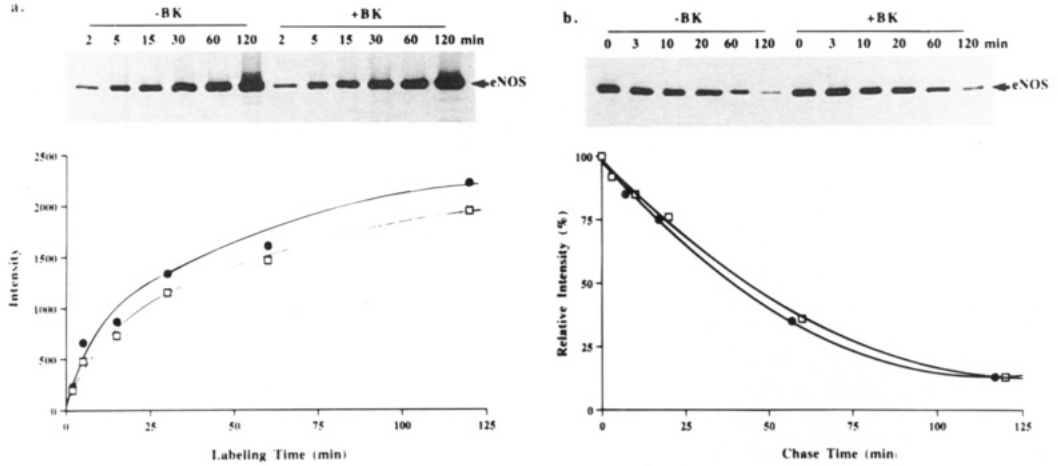

FIGURE 5: Palmitoylation/depalmitoylation of eNOS is not affected by bradykinin (BK). BAEC were labeled with 300 μCi/mL [^{3}H]-palmitic acid for various time periods in the presence (solid circles) or absence (open squares) of 10 μM bradykinin (a) or chased for various time intervals with the complete DMEM containing 0.1 mM cold palmitic acid in the presence (solid circles) or absence (open squares) of 10 μM bradykinin after being labeled with 300 μCi/mL [^{3}H]palmitic acid for 1 h (b). At the end of each incubation, the cells were lysed for immunoprecipitation and subjected to SDS−PAGE. A similar amount of the protein was visualized by Coomassie Blue staining. The labeled eNOS was determined by densitometry of fluorograms (top inserts). Similar results were observed in two additional experiments.

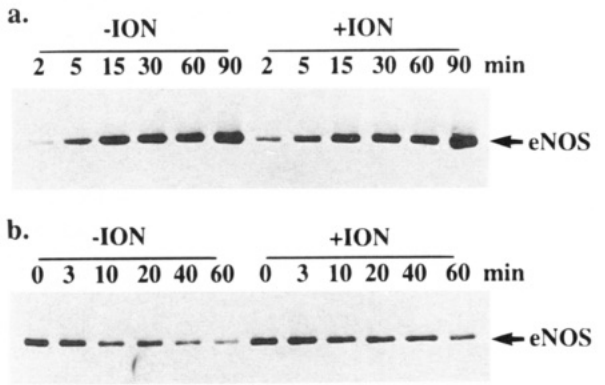

FIGURE 6: Palmitoylation/depalmitoylation of eNOS is not affected by ionomycin (ION). BAEC were labeled with 300 μCi/mL [^{3}H]-palmitic acid for various time periods in the presence or absence of 10 μM ionomycin (a) or chased for various time intervals with the complete DMEM containing 0.1 mM cold palmitic acid in the presence or absence of 10 μM ionomycin after being labeled with 300 μCi/mL [^{3}H]palmitic acid for 1 h (b). At the end of each incubation, the cells were lysed for immunoprecipitation and subjected to SDS−PAGE. A similar amount of the protein was visualized by Coomassie Blue staining. The labeled eNOS was analyzed by fluorography and densitometry. Identical results were observed in an additional experiment.

was not affected by bradykinin (Figure 5b). Similar results were obtained when ionomycin was used as an agonist (Figure 6). However, both bradykinin (10 μM) and ionomycin (1 μM) increased cytoplasmic calcium levels measured by Fluo-3 fluorescence. Relative peak fluorescence units for control BAEC were 163 and 130, and for stimulated BAEC are 307 and 247, representing a 1.88- and 1.90-fold increase in relative peak fluorescence for bradykinin- and ionomycin-treated BAEC, respectively. Additionally, both bradykinin and ionomycin caused the release of nitric oxide (measured as the breakdown product NO_2^- by chemiluminescence). NO_2^- levels were 20.9 ± 1.0, 32 ± 1.6, and 48.3 ± 2.7 pmol $(10^6$ cells$)^{-1}$ (30 min)$^{-1}$ for control, bradykinin- (10 μM), and ionomycin (1 μM)-treated BAEC, respectively (mean ± standard error, $n = 3$ experiments). Under these conditions, we did not observe significant stimulus-dependent translocation of eNOS (stimulated with bradykinin or ionomycin for 5 and 20 min) from membranes to cytosol assessed by Western blot analysis of crude membranes and cytosol ($n = 3$) or semipurified (ADP−Sepharose) membranes and cytosol ($n = 5$). Nor did we observe significant translocation with [^{35}S]methionine ($n = 3$) or [^{3}H]myristic acid labeling ($n = 2$) of BAEC protein followed by immunoprecipitation of membrane and cytosolic eNOS. There was some variability in the total amount of eNOS in cytosol (from 4−10%); however, this variability did not consistently change with agonist stimulation. Similar results were also found in another laboratory (Dr. D. G. Harrison, Emory University, personal communication). However, bradykinin did increase ^{32}P incorporation (approximately 2-fold) into immunoprecipitated eNOS as previously described (Michel et al., 1993).

Site-Directed Mutagenesis of C15 and C26 Inhibits Palmitoylation but Not Membrane Association of eNOS. To identify the potential palmitoylation site(s) of eNOS, immunopurified, [^{3}H]palmitic acid labeled and [^{3}H]myristic acid labeled eNOS were partially digested with endoproteinase Glu-C (V8 protease). V8 protease mapping demonstrated similar labeled mapping patterns between [^{3}H]palmitic acid labeled and [^{3}H]myristic acid labeled eNOS, implying that the palmitoylation site(s) must be close to the unique, N-terminal myristoylation site of eNOS. Therefore, we mutated the first two cysteines in the amino terminus into serines (named C15S and C26S, respectively). COS cells were then transiently transfected with the cDNAs, and labeled with [^{3}H]palmitic acid for 4 h or approximately four half-lives of palmitate on eNOS. Under these conditions, more than 94% of eNOS should be labeled with palmitate. Mutation of either cysteine-15 or cysteine-26 markedly diminished the incorporation (by 95%) of [^{3}H]palmitic acid into eNOS compared to [^{3}H]palmitic acid labeling of wild-type eNOS (Figure 7). Moreover, the double mutation of cysteine-15 and cysteine-26 into serine also reduced the incoporation of palmitate into the double mutant by 95% (data not shown). The residual labeling in the three mutants was hydroxylamine-resistant (data not shown), suggesting that the residue labeling on the mutants may arise from conversion of palmitate into myristate. Surprisingly, the

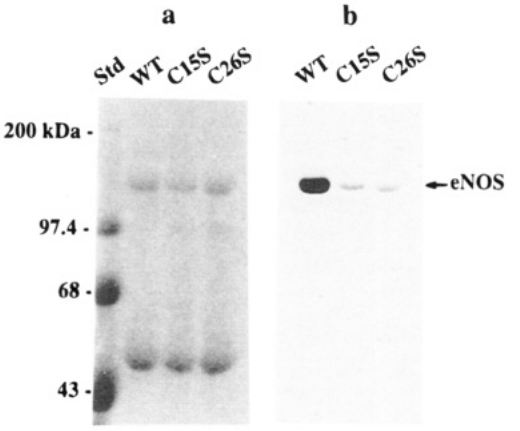

FIGURE 7: Mutation of Cys-15 or Cys-26 markedly inhibits palmitoylation of eNOS. Wild-type (WT), C15S, or C26S eNOS-transfected COS cells were labeled with [^{3}H]palmitic acid for 4 h. eNOS was immunoprecipitated, separated by SDS−PAGE, and stained with Coomassie Blue (a, std = molecular weight standards), and labeled protein was detected by fluorography after a 3-week exposure (b). Similar results were obtained in three separate experiments.

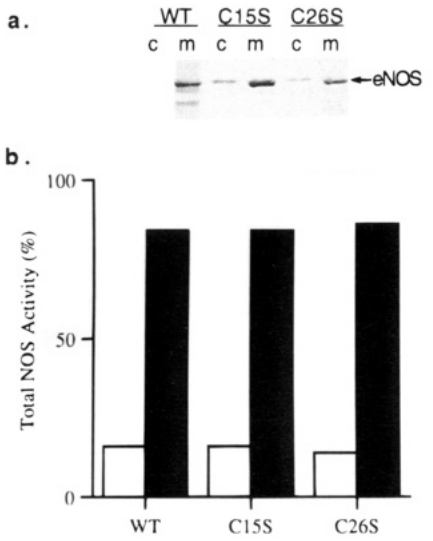

FIGURE 8: Nonpalmitoylated eNOS mutants remain membrane-bound. Wild-type (WT), C15S, or C26S eNOS-transfected COS cells were fractionated into cytosol (c or open bars) and membranes (m or solid bars). In panel a, eNOS was immunoprecipitated and subjected to Western blot analysis, and in panel b, NOS activity was assayed by determining the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citruline. The total NOS specific activities were 18, 15, and 17 pmol of citruline min^{-1} (mg of protein)$^{-1}$ for WT, C15S, and C26S eNOS , respectively. Similar results were obtained in two addtional experiments.

majority (approximately 80−90%) of C15S or C26S proteins and their NOS activities were in high-speed membrane fractions of transiently transfected COS cells. Similar fractionation of protein and activity is seen with wild-type eNOS transiently expressed in COS cells (Figure 8).

N-Myristoylation of eNOS Is Required for Its Palmitoylation. To examine the effects of eNOS N-myristoylation on its palmitoylation state, wild-type or nonmyristoylated mutant G2A eNOS-transfected HEK cells were labeled with [^{3}H]palmitic acid for 4 h. Cell homogenates were fractionated into cytosol and membranes, labeled eNOS was immunoprecipitated and separated by SDS−PAGE, and the labeled protein was detected by fluorography. As shown in Figure 9, wild-type eNOS expressed in HEK cells incorporated the radiolabel only into particulate eNOS, which is consistent with the results obtained from BAEC (Figure 2). Interestingly, the myristoylation-defective mutant, G2A eNOS, was not labeled with [^{3}H]palmitic acid, demonstrating that myristoylation is necessary for palmitoylation of eNOS.

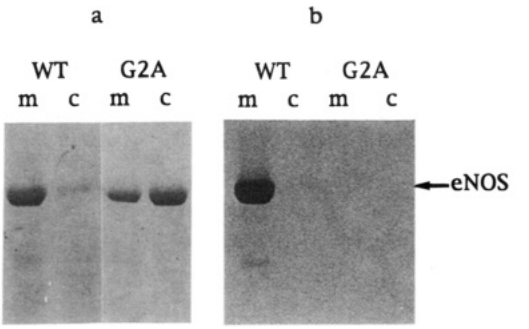

FIGURE 9: N-Myristoylation of eNOS is required for its palmitoylation. Wild-type or G2A eNOS-transfected HEK cells were labeled with [^{3}H]palmitic acid for 4 h. After cell fractionation into membranes (m) and cytosol (c), labeled eNOS was immunoprecipitated, separated by SDS−PAGE, and stained with Coomassie Blue (a), and labeled protein was detected by fluorography after a 1 month exposure (b). Similar results were obtained in two separate experiments.

DISCUSSION

The central findings of this study are that palmitoylation is not regulated by agonists that cause NO release nor is it required for membrane association of eNOS. These conclusions are based on the observations that bradykinin and ionomycin, agonists that mobilize intracellular calcium and release nitric oxide from endothelial cells, do not significantly influence the rate and extent of eNOS palmitoylation or depalmitoylation. More importantly, identification of the major palmitoylation sites, cysteines-15 and/or -26, and mutation of these sites, markedly inhibits the incorporation of [^{3}H]palmitic acid (>95%), but does not influence the amount of eNOS protein or activity partitioned into high-speed membrane fractions. Collectively, our data argue against the importance of palmitoylation for the membrane association of eNOS and are opposed to the hypothesis that depalmitoylation, *per se*, is necessary for translocation of eNOS from membranes to cytosol (Robinson et al., 1995).

The molecular mechanisms responsible for the membrane association of eNOS are not known. A majority of eNOS (>90%), isolated from freshly harvested or serially passaged endothelial cells, or mammalian cells expressing the eNOS cDNA, fractionates into high-speed membrane pellets (Sessa, 1994). eNOS is extracted from membranes by various detergents, but not by boiling or treatment of membranes with high concentrations of salt, implying that hydrophobic interactions account for membrane association of the enzyme (Pollock et al., 1991; Busconi & Michel, 1994). Clearly, N-myristoylation is necessary for the membrane association of eNOS (Sessa et al., 1993a; Busconi & Michel, 1993), but the presence of myristoylated enzyme in cytosolic fractions (Figure 1) indicates that N-myristoylation is not sufficient for membrane association of the protein.

Palmitoylation of several proteins has been shown to contribute to their membrane association (Resh, 1994). The present study and a recent report (Robinson et al., 1995) demonstrate that eNOS is palmitoylated via a hydroxylamine-sensitive, thioester linkage. While palmitoylation may

function to enhance the hydrophobicity of integral and peripheral membrane proteins (Mumby et al., 1994; Wedegaertner et al., 1993), the faster turnover of palmitate (45 min) compared to the eNOS protein backbone and myristate (approximately 20 h, Figure 4) suggested that the cycle of palmitoylation and depalmitoylation could be regulated. Palmitoylation of nonmyristoylated Gs protein α subunit is enhanced by β-adrenergic receptor activation (James & Olson, 1990b; Degtyarev et al., 1993; Mumby et al., 1994; Wedegaertner & Bourne, 1994). The mechanism for the enhanced palmitoylation is via the rapid, stimulus-dependent depalmitoylation of the G protein, thus generating a pool of nonpalmitoylated protein for subsequent repalmitoylation (Wedegaertner & Bourne, 1994), an effect that may account for the rapid, agonist-induced translocation of the protein from membranes to cytosol (Levis & Bourne, 1992; Ransnas et al., 1989). In the present study, two compounds that increase cytosolic calcium, evoke the release of nitric oxide, and cause phosphorylation of eNOS, bradykinin (a receptor-dependent agonist) and ionomycin (a receptor-independent agonist), did not cause significant translocation of eNOS from membranes to cytosol or influence the rate or amount of eNOS palmitoylation or depalmitoylation. During the course of preparing this paper, Robinson et al. (1995) reported that depalmitoylation of eNOS is stimulated by bradykinin and thus proposed that depalmitoylation, but not phosphorylation, as was originally hypothesized (Michel et al., 1993), accounts for the purported translocation of eNOS. However, the rate and extent of palmitoylation (synthesis) in the presence of bradykinin and identification and mutation of the palmitoylation sites were not reported. The reasons for the difference between our results and those of the above workers are not clear. In our experiments, BAEC were early passage cultures, labeled in serum-free media and stimulated in the presence of serum (for depalmitoylation studies), whereas Robinson et al. performed their experiments in later passage cultures using dialyzed, serum-replete media. Also, we included cerulenin, a specific inhibitor of fatty acid synthetase, to increase the specific activity of the [^{3}H]palmitate pool. However, these experimental differences probably do not account for the discrepant results because the turnover of palmitate in our study (in the presence of cerulenin) is identical to that previously reported (Robinson et al., 1995) and bradykinin or ionomycin did not modulate palmitoylation or depalmitoylation of eNOS in BAEC labeled in dialyzed serum-replete, cerulenin-free media (unpublished observation).

To directly test if palmitoylation is necessary for the membrane association of eNOS and if depalmitoylation allows for the reported translocation of eNOS from membrane to cytosol, we rationalized that identification and mutation of the palmitoylation site(s) should render the enzyme more soluble. Site-directed mutagenesis of either cysteine-15 or cysteine-26 dramatically diminished the incorporation of [^{3}H]palmitic acid (by >95%) but did not influence the proportion of membrane-associated enzyme or activity, suggesting that palmitoylation, *per se*, does not play a major role in membrane association of eNOS and that depalmitoylation probably could not solely account for the reported translocation of eNOS (Michel et al., 1993). Since palmitoylation of eNOS is not required for its membrane association, the origin of the cytosolic, myristoylated, nonpalmitoylated form is presently not known.

To our surprise, mutation of either cysteine-15 or cysteine-26 substantially reduced the incorporation of [^{3}H]palmitic acid into eNOS, suggesting that palmitoylation of either cysteine is highly dependent on the presence of the other. This effect is probably not due to misfolding of the protein or inhibition of dimer formation because the mutants were similarly active in direct measurement of NOS activity and have similar apparent K_m's for L-arginine compared to wild-type enzyme. The interdependency of cysteines-15 and -26 may be due to the amino acids immediately flanking these sites. Adjacent to the two palmitoylation sites are three proline residues (at positions 14, 31, and 34) separated by a hydrophobic-rich stretch of five glycine/leucine repeats. Thus, mutation of Cys-15 or -26 to a polar serine residue may interfere with recognition of the neighboring cysteine by a membrane-associated palmitoyltransferase. Similar interdependency of cysteine palmitoylation sites has been described for bovine opsin and neuromodulin (Karnik et al., 1993; Liu et al., 1993). An alternative explanation, although less likely, is that mutation of either Cys-15 or Cys-26 could inhibit the palmitoylation of another N-terminal cysteine residue, thereby inhibiting the incorporation of [^{3}H]palmitic acid.

Although palmitoylation of several proteins accounts for their membrane association, several exceptions have been reported. For example, glutamic acid decarboxylase (GAD_{65}) is synthesized as a soluble protein and becomes anchored to membranes by posttranslational modification(s). Mutation of its palmitoylation sites results in a loss of palmitoylation; however, nonpalmitoylated GAD_{65} remains membrane-bound (Shi et al., 1994). Also, mutation of the palmitoylation sites of vesicular stomatitis virus G protein, transferrin receptor, and the heavy chain of the human class I histocompatibility antigen inhibits palmitoylation, but does not influence stable membrane association (Rose et al., 1984; Jing & Trowbridge, 1987; Kaufman et al., 1984). Our present data with eNOS support the growing body of evidence that palmitoylation is not necessary or sufficient for the membrane association of all proteins. If palmitoylation is not required for membrane association of these proteins, then what is its role? Recent data suggest that palmitoylation is important for protein−protein or protein−lipid interactions. For instance, palmitoylation of Src family members enhances their interactions with glycosylphosphatidylinositol-linked proteins (Shenoy-Scaria et al., 1993). In the transferrin receptor, lack of palmitoylation increases its rate of endocytosis (Alvarez et al., 1990). Since palmitoylation of eNOS is not required for membrane binding, it may increase its relative hydrophobicity, thus influencing its orientation on biological membranes. Such orientation may facilitate protein−protein and protein−lipid interactions and be important for the function of the enzyme in endothelial cells. The precise biological significance of eNOS palmitoylation is currently being investigated.

Like most G protein α subunits, mutation of the eNOS N-myristoylation site prevents not only myristoylation but also palmitoylation. The amino-terminal consensus sequence, Met-Gly-Cys-X-X-Ser/Cys (shared by both myristoylated and palmitoylated α subunits of G proteins as well as some of the Src family of proteins), is postulated as a motif for both myristoylation and palmitoylation (Mumby et al., 1994; Resh, 1994). For these proteins, the palmitoylation site, cysteine-3, is adjacent to the myristoylation site, glycine-2. However, eNOS does not have such a motif, and

the cysteine closest to the N-myristoylation site, glycine-2, is located at position 15. Palmitoylation of Ras proteins has been reported to be dependent upon their isoprenylation (McIlhinney, 1990). Hence, either myristoylation (of G proteins, Src, or eNOS) or isoprenylation (of Ras) most likely allows the proteins to weakly associate with membranes, permitting access to a membrane-bound palmitoyltransferase (Gutierrez & Magee, 1991). Alternatively, it is possible that N-myristoylation is needed for the proper conformation of the substrate for subsequent palmitoylation. Since a cytosolic fraction of myristoylated eNOS is not palmitoylated and G2A eNOS is neither myristoylated nor palmitoylated, myristoylation in conjunction with other unidentified factors may contribute to membrane association of eNOS.

In summary, eNOS is reversibly palmitoylated in a thioester linkage at cysteines-15 and/or -26 and is a new member of a class of proteins dually acylated by both myristic acid and palmitic acid. Since mutation of cysteines-15 and/or -26 inhibits the incorporation of [^{3}H]palmitic acid, but not membrane association, the role of palmitoylation in the localization or activation of eNOS remains to be elucidated.

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