

## Acylation Targets Endothelial Nitric-oxide Synthase to Plasmalemmal Caveolae\*

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Endothelial nitric-oxide synthase (eNOS) generates the key signaling molecule nitric oxide in response to intraluminal hormonal and mechanical stimuli. We designed studies to determine whether eNOS is localized to plasmalemmal microdomains implicated in signal transduction called caveolae. Using immunoblot analysis, eNOS protein was detected in caveolar membrane fractions isolated from endothelial cell plasma membranes by a newly developed detergent-free method; eNOS protein was not found in noncaveolar plasma membrane. Similarly, NOS enzymatic activity was 9.4-fold enriched in caveolar membrane versus whole plasma membrane, whereas it was undetectable in noncaveolar plasma membrane. 51–86% of total NOS activity in postnuclear supernatant was recovered in plasma membrane, and 57–100% of activity in plasma membrane was recovered in caveolae. Immunoelectron microscopy showed that eNOS heavily decorated endothelial caveolae, whereas coated pits and smooth plasma membrane were devoid of gold particles. Furthermore, eNOS was targeted to caveolae in COS-7 cells transfected with wild-type eNOS cDNA. Studies with eNOS mutants revealed that both myristoylation and palmitoylation are required to target the enzyme to caveolae and that each acylation process enhances targeting by 10-fold. Thus, acylation targets eNOS to plasmalemmal caveolae. Localization to this microdomain is likely to optimize eNOS activation and the extracellular release of nitric oxide.

The endothelial isoform of nitric-oxide synthase (eNOS)<sup>1</sup> is one of three isoenzymes that converts L-arginine to L-citrulline plus the key signaling molecule nitric oxide (NO). eNOS is acutely activated by increases in endothelial intracellular calcium induced by the stimulation of diverse G-protein-coupled cell surface receptors and by physical stimuli such as hemodynamic shear stress and varying oxygenation. Endothelium-derived NO regulates blood pressure, platelet aggregation, and vascular smooth muscle mitogenesis. Diminished NO production has been implicated in the pathogenesis of a variety of

vascular disorders including atherosclerosis and pulmonary hypertension (1–4).

eNOS is a unique isoform of the enzyme in that it is primarily localized to the particulate subcellular fraction (1–4). Studies evaluating NOS enzymatic activity have demonstrated that functional eNOS is primarily associated with the plasma membrane (5). Since eNOS activity is acutely regulated by intraluminal factors and the NO produced is a labile, cytotoxic messenger molecule (1–4), the intracellular site of NO synthesis is likely to have a major influence on its biological activity in the vascular wall. We therefore sought to determine the specific subcellular location to which eNOS is targeted and designed studies to delineate whether the enzyme is localized to plasmalemmal caveolae. Caveolae are plasmalemmal microdomains that have been implicated in the transcytosis of macromolecules, the uptake of small molecules by potocytosis, and the compartmentalization of signaling molecules (6–9). The latter function is particularly relevant to the regulation of eNOS activity, as numerous signaling molecules known to modulate the enzyme are concentrated in caveolae. These signaling molecules include G-protein-coupled receptors such as the muscarinic acetylcholine receptor, a plasma membrane Ca<sup>2+</sup> pump, an IP<sub>3</sub>-sensitive Ca<sup>2+</sup> channel, and protein kinase C (6, 7, 10). In this report we use three independent methods to demonstrate that eNOS is localized to endothelial plasmalemmal caveolae. In addition, we demonstrate that the enzyme is targeted to caveolae in COS-7 cells transfected with wild-type eNOS cDNA. Targeting does not occur with myristoylation- or palmitoylation-deficient eNOS mutants, indicating that acylation is necessary for caveolar localization of the enzyme. The posttranslational targeting to caveolae is likely to optimize both eNOS activation and the extracellular release of NO.

### MATERIALS AND METHODS

**Cell Culture**—Pulmonary artery endothelial cells (PAEC) were obtained from third-generation intrapulmonary arteries of fetal lambs at 125–135 days gestation (term = 144 ± 4 days) by collagenase digestion and were propagated as described previously (11). Animal care and euthanasia procedures were approved by the Institutional Review Board for Animal Research. PAEC were studied at passage 4–6. COS-7 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (Life Technologies Inc.) supplemented with 10% heat-inactivated fetal bovine serum plus 200 units/ml penicillin and 200 µg/ml streptomycin.

**Isolation of Caveolae**—Caveolae were isolated from highly purified plasma membrane fractions prepared from PAEC or COS-7 cells using a newly developed detergent-free method that takes advantage of the unique buoyant density of caveolar membrane (12). This approach was chosen because the detergent solubility of certain caveolar components such as the β subunit of heterotrimeric G-proteins is well established (13). To optimize the recovery of active enzyme, 2 mM β-mercaptoethanol was added to all solutions, and the protease inhibitors pepstatin (50 µg/ml), leupeptin (50 µg/ml), N<sup>ε</sup>-p-tosyl-L-lysine chloromethyl ketone (50 µg/ml), and trypsin soybean inhibitor (50 µg/ml) were added during

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<sup>1</sup> The abbreviations used are: eNOS, endothelial isoform of nitric-oxide synthase; PAEC, pulmonary artery endothelial cells.

cell disruption and membrane sonication.

**Immunoblot Analyses**—Immunoblot analyses were performed as described previously (14, 15). The membranes were probed with either an antiserum generated to the unique mid-molecule peptide PYNS-SPRPEQHKSYK of eNOS (16, 17), a monoclonal antibody to caveolin (mAb 2234, kindly provided by Dr. John Glenney, Glentech, Inc., Lexington, KY), which is a 22-kDa integral membrane protein and an accepted marker for caveolae (6, 7, 15), or an antiserum to the eNOS cofactor calmodulin (Transduction Laboratories, Lexington, KY).

**Nitric-oxide Synthase Activity**—NOS activity was determined in the subcellular fractions of PAEC or COS-7 cells by measuring the conversion of [<sup>3</sup>H]arginine to [<sup>3</sup>H]citrulline (14). Preliminary experiments revealed that the gradient media does not interfere with NOS activity in cell lysates. NOS activity was linear with time for up to 2 h, and in all subcellular fractions activity was inhibited >95% by 4.0 mM nitro-L-arginine methyl ester. In all studies, NOS activity was evaluated in aliquots of non-caveolar fractions with at least 3-fold greater protein content than caveolar samples. Results were statistically analyzed by analysis of variance with Newman-Keuls *post hoc* testing.

**Immunoelectron Microscopy**—Immunogold localization of eNOS and caveolin was carried out using whole mount plasma membrane preparations as described previously (18). PAEC membranes attached to grids were incubated sequentially for 30 min each with the indicated primary antibodies (anti-eNOS IgG diluted 1:100 or mAb 2234 diluted 1:100), followed by 50 μg/ml of goat anti-mouse IgG, and then a 1:30 dilution of gold-conjugated rabbit anti-goat IgG. Phosphate-buffered saline containing 0.15% bovine serum albumin was used to dilute all of the antibodies. After each antibody incubation, the grids were washed 3 times for 30 min each in phosphate-buffered saline containing 0.15% bovine serum albumin. The membranes were then fixed with 2.5% glutaraldehyde in phosphate-buffered saline for 10 min followed by 1% osmium tetroxide in phosphate-buffered saline for 10 min. Each sample was stained sequentially for 10 min with 1% tannic acid and 1% uranyl acetate. The grids were examined and photographed with an electron microscope (100 CX; JEOL USA Inc., Peabody, MA). Primary human fibroblasts that possess caveolae but do not express eNOS served as control cells (10).

**Cell Transfection**—COS-7 cells were transiently transfected with eNOS cDNAs using DEAE-dextran as reported previously (19). Experiments were performed with either wild-type eNOS cDNA (19), a myristoylation-deficient mutant of eNOS (17), or a palmitoylation-deficient mutant of eNOS subcloned into the eukaryotic expression vector pBK-CMV (Stratagene, La Jolla, CA) (20). Transfection efficiency was assessed at 48 h by evaluating NADPH-diaphorase staining (19). The proportion of transfected cells ranged from 10 to 30%. NOS activity was determined at 72 h in postnuclear supernatant (37–51 μg of protein/sample), cytosol (29–119 μg), plasma membrane (12–22 μg), noncaveolar plasma membrane (5–12 μg), and caveolar membrane (1–2 μg). NOS activity was undetectable in nontransfected COS-7 cells and cells sham-transfected with vector alone.

## RESULTS AND DISCUSSION

We first examined eNOS protein abundance in subcellular fractions from PAEC. Immunoblot analysis showed that caveolin was found exclusively in the caveolar membrane fraction (Fig. 1). eNOS protein was also detected solely in caveolar membranes. We could not detect eNOS in noncaveolar plasma membrane even when greater than 10-fold excess of protein was analyzed (data not shown). Triton X-100-based methods of caveolar purification did not allow the co-isolation of eNOS (data not shown). Calmodulin (17 kDa) was also found in the caveolar fraction, and it was not detected in noncaveolar plasma membrane, suggesting that this critical cofactor is spatially associated with eNOS. We have previously shown that the purified caveolae membranes are not contaminated with coated pits, focal adhesion sites, or a variety of calcium-binding proteins (12).

We then determined if the localization of eNOS protein to caveolae correlates with NOS enzymatic activity. NOS activity was 7-fold greater in the plasma membrane fraction than in cytosol (Fig. 2). Within the plasma membrane, NOS activity was undetectable in the noncaveolar fraction, whereas it was 9.4-fold enriched in caveolar membranes compared with whole plasma membrane. In three independent experiments, 51–86%

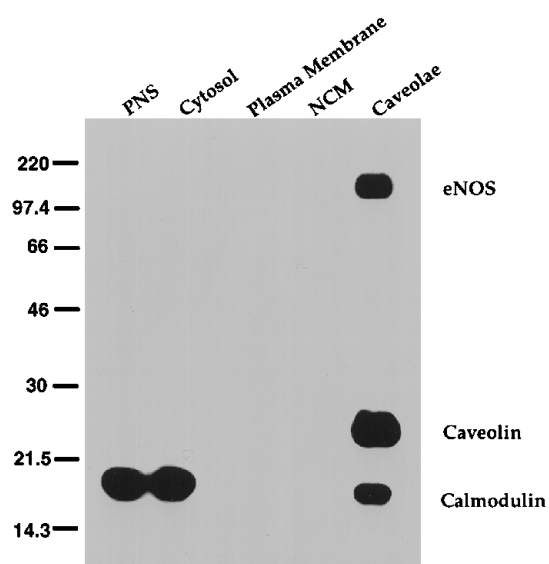


FIG. 1. Immunoblot analysis for eNOS, caveolin and calmodulin in subcellular fractions from PAEC. 5-μg samples of post-nuclear supernatant (PNS), cytosol, plasma membrane, noncaveolar membrane (NCM), and caveolar membrane were evaluated. Signal for eNOS was evident at 135 kDa in caveolae only. Signal for eNOS was absent when preimmune serum was substituted for antiserum to eNOS and when the purified antigenic eNOS peptide was added to the immune serum (100 μg/ml) before incubation to adsorb the eNOS antibody (data not shown). Caveolin protein was detectable at 22 kDa in caveolar membrane only. The blot was also probed for calmodulin (17 kDa), a cofactor required for eNOS activity. Comparable findings were obtained in three independent experiments.

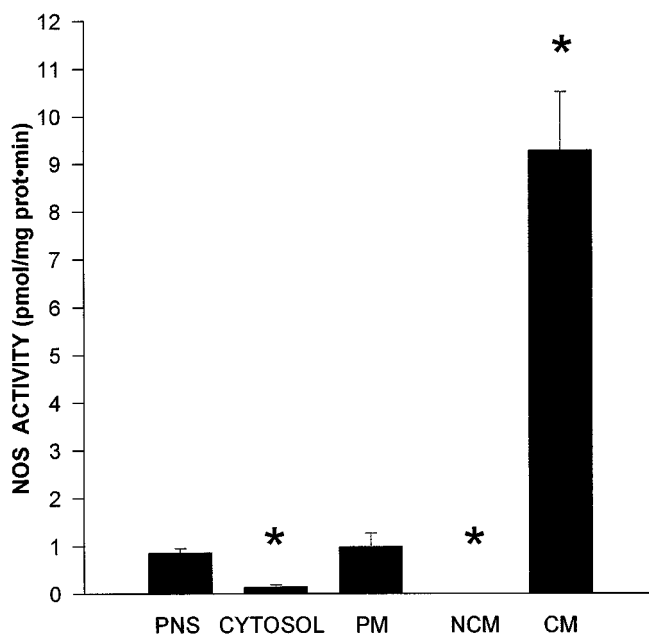
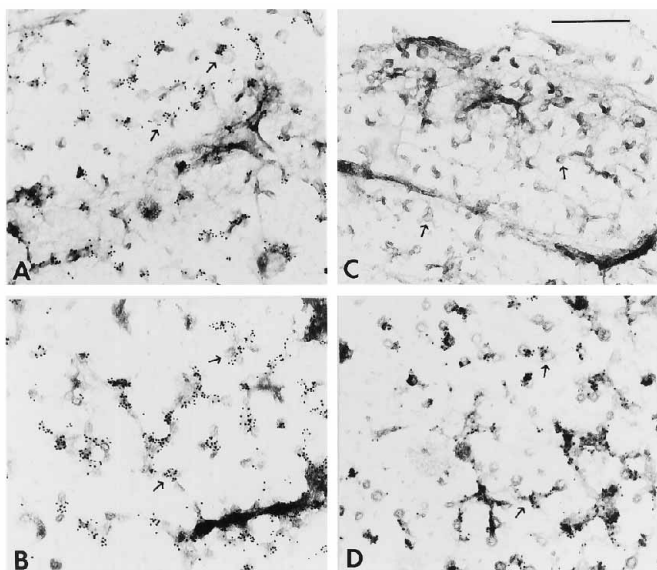


FIG. 2. NOS enzymatic activity in subcellular fractions from PAEC. Samples of postnuclear supernatant (PNS, 11 μg of protein/sample), cytosol (23 μg), plasma membrane (PM, 5 μg), noncaveolar membrane (NCM, 2.7 μg), and caveolar membrane (CM, 0.7 μg) were evaluated. NOS activity was determined by measurement of L-[<sup>3</sup>H]arginine conversion to L-[<sup>3</sup>H]citrulline. Enzymatic activity was undetectable in NCM. Comparable findings were obtained in three independent experiments. Mean ± S.E., *n* = 4–6, \**p* < 0.05 versus plasma membrane.

of the total activity in postnuclear supernatant was recovered in the plasma membrane fraction, and 11–29% was recovered in cytosol, consistent with previous studies indicating localization of eNOS enzymatic activity primarily to the plasma membrane (5). More importantly, 57–100% of the total activity in



**FIG. 3. Localization of caveolin and eNOS in plasma membranes by immunoelectron microscopy.** Immunogold labeling was performed using mAb anti-caveolin IgG in fibroblasts (A) and PAEC (B), and using anti-eNOS antiserum in fibroblasts (C) and PAEC (D). Caveolae (arrows) and coated pits are evident in both cell types. Bar = 0.45  $\mu\text{m}$ .

plasma membrane was recovered in the caveolar fraction, indicating that plasmalemmal NOS is primarily localized to caveolae. These observations suggest that studies that have relied solely on immunohistochemical techniques to localize eNOS to the Golgi apparatus may have been confounded by the labeling of Golgi vesicles destined for the plasma membrane (21–23). With the subfractionation method employed in the present experiments, the Golgi marker enzyme galactosyl transferase is absent in the purified plasma membrane preparation, which yields the caveolar fraction (12).

We have shown by biochemical subfractionation and by enzymatic assays that the primary location of eNOS in the plasma membrane is in caveolae. To confirm these findings in an entirely independent manner, eNOS was localized by immunoelectron microscopy. In fibroblast and endothelial membranes processed for the localization of caveolin, gold particles were found over small invaginations on the membrane surface that had the characteristic appearance of caveolae (Fig. 3, A and B, respectively, arrows). In contrast, coated pits and smooth membrane areas were not decorated with gold. When identically processed membranes from fibroblasts were labeled with anti-eNOS antiserum, gold particles were not found over caveolar structures, coated pits, or smooth membrane (Fig. 3C). However, when endothelial membranes were labeled with anti-eNOS antiserum, caveolar structures were specifically decorated with gold but the coated pits and smooth membrane were not (Fig. 3D). These data demonstrate that the detection of eNOS in isolated endothelial caveolar fractions is not due to a nonspecific association of the enzyme with caveolae membranes during the purification process.

We next sought to delineate the mechanism(s) underlying the localization of eNOS to caveolae. First, we determined if the targeting of eNOS to caveolae is unique to endothelial cells by transiently transfecting wild-type eNOS cDNA into COS-7 cells that possess caveolae but do not express NOS constitutively. 72 h following transfection, there was 6–16-fold greater NOS activity in plasma membrane than cytosol (Table I). This finding mimicks the 7-fold difference between the same subcellular fractions in endothelial cells (Fig. 2). Within the plasma mem-

brane, NOS activity in caveolae far exceeded activity in non-caveolar plasma membranes (27–46-fold difference), also simulating the findings in endothelial cells. The relative overexpression of eNOS in the transfected cells may account for the modest enzymatic activity detected in noncaveolar plasma membranes. Caveolar NOS activity was enriched 7–12-fold relative to whole plasma membrane (Table I), which also mimicks the observations in endothelium (9-fold enrichment, Fig. 2). The comparable findings in the endothelial cells and the transfected epithelial cell line suggest that eNOS and caveolae membranes collectively possess all of the properties necessary for posttranslational targeting of the protein to caveolae.

We then determined the characteristics of the eNOS protein that program the targeting of the enzyme to caveolae. A variety of signaling molecules including G-protein  $\alpha$  subunits, and nonreceptor Src-like kinases have N-terminal consensus sequences for dual acylation that appear to regulate their localization to caveolae (6, 7). Dual acylation involves *N*-myristoylation and palmitoylation. We have previously identified an N-terminal myristoylation consensus sequence in eNOS (19). A glycine residue is located in position 2, similar to the N terminus of caveolar G-protein subunits and Src-like kinases (6, 7, 19). Furthermore, we have previously demonstrated that wild-type eNOS is myristoylated and also palmitoylated and that the mutagenesis of the myristoylation site blocks both types of acylation. These changes also attenuate the association of the protein with the particulate fraction (17, 24). To investigate the role of acylation in the targeting of eNOS to caveolae, cDNA encoding a myristoylation-deficient mutant of eNOS ( $\text{myr}^-$ ) was transiently transfected into COS-7 cells (17). NOS activity in the postnuclear supernatant was generally comparable in cells expressing the  $\text{myr}^-$  mutant or wild-type eNOS (Table I). However, in  $\text{myr}^-$  mutant cells, the ratio of NOS activity in plasma membrane *versus* cytosol was less than one, contrasting with the 6–16-fold greater activity in plasma membrane *versus* cytosol in wild-type eNOS cells. A ratio of one or less indicates a lack of enrichment, while a ratio greater than one indicates enrichment. In the  $\text{myr}^-$  mutant cells, the ratio of NOS activity in caveolar *versus* noncaveolar plasma membranes was also less than one; this contrasts markedly with the observed 27–46-fold greater NOS activity in caveolar *versus* noncaveolar plasma membranes in wild-type eNOS cells. These findings indicate that acylation targets eNOS to caveolae. However, because the  $\text{myr}^-$  mutation of eNOS blocks both myristoylation and palmitoylation (17, 24), it is not possible to determine the contribution of myristoylation alone. The requirement for prior myristoylation has also been observed for the palmitoylation of caveolar nonreceptor Src-like kinases (25).

Experiments were then performed to elucidate the specific role of palmitoylation in targeting the enzyme to caveolae. We have recently shown that the cysteine residues at positions 15 and 26 are the palmitoylation sites for eNOS (20). The substitution of serines for cysteines 15 and 26 ( $\text{palm}^-$  mutant) completely prevents the palmitoylation of the protein, while myristoylation is unaffected (20). Following the transient transfection of  $\text{palm}^-$  mutant eNOS cDNA into COS-7 cells, enzymatic activity in the postnuclear supernatant was generally similar to that in wild-type eNOS cells (Table I). However, the relative abundance of NOS activity in plasma membrane *versus* cytosol was diminished in  $\text{palm}^-$  mutant cells compared with wild-type cells (1.4–2.4-fold greater activity in plasma membrane than cytosol, *versus* 6–16-fold difference, respectively). Importantly, this diminution in relative plasma membrane NOS abundance was not as great as in  $\text{myr}^-$  mutant cells (0.3–0.9-fold difference). In addition, in  $\text{palm}^-$  mutant

TABLE I

Nitric-oxide synthase activity in subcellular fractions of COS-7 cells transfected with wild-type, *myr*<sup>-</sup> mutant, or *palm*<sup>-</sup> mutant eNOS

Activity (pmol of citrulline formed/mg of protein/min) was measured in postnuclear supernatant (PNS), cytosol (CYTO), plasma membrane (PM), noncaveolar membrane (NCM), and caveolar membrane (CM). Results from three independent transfection experiments (A, B, and C) are shown for each construct. The ratios of NOS activity in different fractions are shown in parentheses. Mean  $\pm$  S.E.,  $n = 4-6$ .

	PNS	CYTO	PM	(PM/CYTO)	NCM	CM	(CM/NCM)	(CM/PM)
Wild-type								
A.	2.2 $\pm$ 0.1	0.11 $\pm$ 0.01 <sup>a</sup>	1.7 $\pm$ 0.2	(15.5)	0.41 $\pm$ 0.04 <sup>a</sup>	15.4 $\pm$ 2.4 <sup>a</sup>	(37.6)	(9.1)
B.	8.8 $\pm$ 0.2 <sup>a</sup>	12.9 $\pm$ 0.2 <sup>a</sup>	16.7 $\pm$ 0.1	(5.8)	4.5 $\pm$ 0.3 <sup>a</sup>	205.2 $\pm$ 3.4 <sup>a</sup>	(45.6)	(12.3)
C.	31.7 $\pm$ 0.3 <sup>a</sup>	4.3 $\pm$ 0.1 <sup>a</sup>	63.2 $\pm$ 2.0	(14.7)	15.3 $\pm$ 0.5 <sup>a</sup>	417.8 $\pm$ 10.7 <sup>a</sup>	(27.3)	(6.6)
Myr <sup>-</sup> mutant								
A.	16.2 $\pm$ 0.7 <sup>a</sup>	9.0 $\pm$ 0.3 <sup>a</sup>	4.3 $\pm$ 0.2	(0.48)	3.3 $\pm$ 0.3	1.7 $\pm$ 0.2 <sup>a</sup>	(0.52)	(0.40)
B.	18.6 $\pm$ 0.3 <sup>a</sup>	11.3 $\pm$ 0.2 <sup>a</sup>	3.1 $\pm$ 0.2	(0.27)	1.9 $\pm$ 0.04 <sup>a</sup>	1.2 $\pm$ 0.2 <sup>a</sup>	(0.63)	(0.39)
C.	35.8 $\pm$ 1.6 <sup>a</sup>	11.3 $\pm$ 0.9	10.5 $\pm$ 0.9	(0.93)	5.1 $\pm$ 0.9 <sup>a</sup>	2.7 $\pm$ 0.4 <sup>a</sup>	(0.26)	(0.26)
Palm <sup>-</sup> mutant								
A.	17.1 $\pm$ 0.7 <sup>a</sup>	7.1 $\pm$ 0.1	9.6 $\pm$ 0.2	(1.4)	3.8 $\pm$ 0.2 <sup>a</sup>	10.6 $\pm$ 0.3	(2.8)	(1.10)
B.	7.0 $\pm$ 0.1 <sup>a</sup>	2.0 $\pm$ 0.03	3.0 $\pm$ 0.6	(1.5)	0.6 $\pm$ 0.04 <sup>a</sup>	3.8 $\pm$ 0.6	(6.3)	(1.27)
C.	53.8 $\pm$ 1.2 <sup>a</sup>	15.2 $\pm$ 0.2 <sup>a</sup>	36.2 $\pm$ 0.9	(2.4)	13.2 $\pm$ 0.6 <sup>a</sup>	38.1 $\pm$ 1.0	(2.9)	(1.05)

<sup>a</sup>  $p < 0.05$  versus plasma membrane.

(myristoylation-competent) cells, there was a decrease in relative NOS activity in caveolar versus noncaveolar plasma membrane (3–6-fold difference) compared with wild-type cells (27–46-fold difference), but the decrease was less than that found in the *myr*<sup>-</sup> mutant, palmitoylation- and myristoylation-deficient cells (0.3–0.6-fold difference). As such, there is approximately a 10-fold enhancement in the targeting of eNOS to caveolae due to myristoylation alone, and the targeting is augmented an additional 10-fold by palmitoylation. These results indicate that both acylation processes are necessary for optimal targeting of eNOS to the caveolae. The findings also support the overall hypothesis that dual acylation targets proteins to caveolae (13).

The present observations are consistent with the recent findings that the loss of palmitoylation alone decreases the percentage of membrane-bound eNOS from approximately 80% (wild-type) to approximately 40%, whereas the additional loss of myristoylation essentially eliminates association with the membrane fraction (20). The myristoyl group might anchor eNOS to caveolar membranes via hydrophobic interactions with membrane lipids. However, thermodynamic studies have demonstrated that the free energy of membrane binding for myristoylated proteins is low and the interactions may be readily reversible (26). Palmitoylation of eNOS may provide additional hydrophobic interactions that stabilize the weak caveolar membrane association conferred by myristoylation alone. Other potential protein-membrane interactions cannot be excluded since caveolar membranes from cells transfected with acylation-deficient eNOS, although not enriched compared with whole plasma membrane, were not completely devoid of NOS enzymatic activity. Venema *et al.* (27) have recently proposed that the calmodulin binding domain of eNOS may play a role in membrane binding by providing electrostatic interactions with negatively charged phospholipids (27). A similar role has been proposed for the calmodulin binding domain of the MARCKS protein, another myristoylated protein exhibiting regulated binding to cell membranes (28). However, the present findings of a lack of NOS enrichment in caveolae from cells transfected with myristoylation- and palmitoylation-deficient eNOS indicate that dual acylation is required for targeting eNOS to caveolae.

The caveolar localization of eNOS has important potential ramifications on endothelial cell NO production and secretion. Since signaling molecules including G-protein coupled receptors, modulators of calcium flux, and protein kinase C are also concentrated in caveolae (6, 7, 10), the caveolar localization of eNOS may be necessary for acute regulation of the enzyme by intraluminal hormones. In addition, since caveolae interact

with elements of the cytoskeleton through actin and microtubules, changes in the organization of the cytoskeleton due to stretching or compression of the endothelial cell may deform the caveolae, thereby opening caveolar membrane channels and carriers (6, 7, 12, 15). This mechanism may be critically important in the acute, calcium-mediated activation of eNOS by physical stimuli such as shear stress. Furthermore, it has recently been noted in studies of submucosal blood vessels from guinea pig ileum that eNOS-labeled vesicles are found on both the luminal and abluminal side of endothelial cells (21). This suggests that caveolae may release NO to both the vascular lumen and the underlying smooth muscle. Moreover, since membrane cholesterol is essential for normal caveolar function (29), pathologic alterations in cholesterol balance may adversely influence endothelial cell NO production through effects on caveolae, thereby contributing to atherosclerotic processes. Thus, the caveolar localization of eNOS has major implications on vascular health and disease.

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