Trafficking of Endothelial Nitric-oxide Synthase in Living Cells

QUANTITATIVE EVIDENCE SUPPORTING THE ROLE OF PALMITOYLATION AS A KINETIC TRAPPING MECHANISM LIMITING MEMBRANE DIFFUSION

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To examine endothelial nitric-oxide synthase (eNOS) trafficking in living endothelial cells, the eNOS-deficient endothelial cell line ECV304 was stably transfected with an eNOS-green fluorescent protein (GFP) fusion construct and characterized by functional, biochemical, and microscopic analysis. eNOS-GFP was co-localized with Golgi and plasma membrane markers and produced NO in response to agonist challenge. Localization in the plasma membrane was dependent on the palmitoylation state, since the palmitoylation mutant of eNOS (C15S/C26S eNOS-GFP) was excluded from the plasma membrane and was concentrated in a diffuse perinuclear pattern. Fluorescence recovery after photobleaching (FRAP) revealed eNOS-GFP in the perinuclear region moving 3 times faster than the plasmalemmal pool, suggesting that protein-lipid or protein-protein interactions are different in these two cellular domains. FRAP of the palmitoylation mutant was two times faster than that of wild-type eNOS-GFP, indicating that palmitoylation was influencing the rate of trafficking. Interestingly, FRAP of C15S/C26S eNOS-GFP but not wild-type eNOS-GFP fit a model of protein diffusion in a lipid bilayer. These data suggest that the regulation of eNOS trafficking within the plasma membrane and Golgi are probably different mechanisms and not due to simple diffusion of the protein in a lipid bilayer.

The appropriate targeting of lipid-modified signaling proteins into discrete membrane domains is important for the fidelity of signal transduction events. For example, dual, acylation reactions via co-translational N-myristoylation and post-translational cysteine palmitoylation of proteins such as G proteins, Src family members, and endothelial nitric-oxide synthase (eNOS) are required for correct cellular trafficking and for coupling intracellular signaling to extracellular stimuli in intact cells (1–3). In general, mutation of the N-myristoylation sites of dually acylated molecules prevents subsequent cysteine palmitoylation and typically results in mislocalization of the protein into cytosolic compartments, thus providing compelling evidence that lipid modifications support the directional membrane trafficking of otherwise soluble proteins.

eNOS is a peripheral membrane protein that targets to specific intracellular domains, including Golgi and cholesterol and sphingolipid-rich microdomains of the plasma membrane, caveolae. Inhibition of dual acylation prevents Golgi targeting (4), and mutation of the palmitoylation sites prevents caveolae targeting (5, 6). In both of the above situations, the mislocalization of eNOS has functional consequences; the stimulated production of nitric oxide (NO) in intact cells is attenuated, although the purified mutant enzymes are catalytically identical to wild-type (WT) eNOS (3, 4, 7, 8). These data suggest that dual fatty acylation is critical for eNOS localization into specific regions of the cell and for optimal NO production. The amount of eNOS detected in Golgi versus plasma membrane varies in different endothelial cell populations as determined by in vitro and in vivo techniques, suggesting a dynamic, complex trafficking pattern of this protein. Interestingly, the first 35 amino acids, inclusive of the N-myristoylation and palmitoylation sites has been shown to direct the green fluorescent protein (GFP) into the Golgi region of living NIH 3T3 cells, whereas N-myristoylation per se cannot, suggesting that N-myristoylation and cysteine palmitoylation can participate in the Golgi targeting and stable membrane association of this protein.

To gain an appreciation for the dynamic nature of eNOS trafficking in its native environment, we have generated endothelial cells that stably express WT eNOS and the palmitoylation mutant eNOS (C15S/C26S eNOS) coupled to GFP. Here we show that the pattern of eNOS-GFP trafficking into the apical plasma membrane depends on palmitoylation. In addition, fluorescence recovery after photobleaching (FRAP) demonstrates that the rate of eNOS-GFP movement differs in Golgi versus plasma membrane and that palmitoylation slows eNOS movement in the cell. These data provide the first direct measurement of the movement of a dually acylated peripheral membrane protein in the Golgi and plasma membrane and support the idea that palmitoylation permits the kinetic trapping of acylated proteins in discrete microenvironments of the cell and caveolae.
facilitates the movement of proteins between Golgi and plasma membrane.

**MATERIALS AND METHODS**

**Generation of eNOS-GFP Fusions**—WT eNOS-GFP and the palmitoylation-deficient mutant generated by mutation of the two cysteine palmitoylation sites, cysteine 15 and 26, to serines (C15S/C26S eNOS-GFP) were prepared as described previously (9). In brief, polymerase chain reaction was used to introduce an NheI site into the 3′-end of the eNOS coding sequence and into the 5′-end of a GFP clone that was optimal for expression (10). These sequences were joined via frame in the NheI site and inserted into the mammalian expression vector pcDNA3. The C15S/C26S mutant of eNOS in pcDNA3 was subcloned as a HindIII/SrfI fragment into the above vector.

**Cell Culture Conditions and Transfection**—The immortalized human umbilical vein endothelial cell (HUVEC) line, ECV304 (15) and primary cultures of bovine aortic endothelial cells were grown in Dulbecco’s modified Eagle’s medium containing penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% (v/v) fetal calf serum (complete Dulbecco’s modified Eagle’s medium). For transfection, ECV304 cells were seeded at a density of 1 × 10⁵ cells/100-mm dish and transfected the next day with the cDNAs for WT eNOS-GFP or C15S/C26S eNOS-GFP according to the standard calcium phosphate precipitation method. Transfected ECV304 cells were selected for growth in the presence of G418 (800 μg/ml). After 3 weeks, G418-resistant colonies were selected and maintained in the complete Dulbecco’s modified Eagle’s medium containing G418 (200 μg/ml). Several clones of WT and mutant transfected cells were isolated and characterized.

**Immunoprecipitation and Western Blotting**—Cells were washed twice with Tris-buffered saline, lysed on ice in a modified immune precipitation assay buffer (100 mM Tris HCl, pH 7.4, 1% Nonidet P-40 (v/v), 10 mM NaF, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin), and lysates were transferred to an Eppendorf tube and rotated for 30 min at 4 °C. Lysates were Dounce-homogenized (50 strokes), insoluble material was removed by centrifugation at 12,000 × g for 10 min at 4 °C, Fasansorin complexes were removed by ultracentrifugation, and eNOS was immunoprecipitated in the absence of eNOS monoclonal antibody (Zymed Laboratories Inc., clone 9D10) and Western blotted as described previously.

**Fluorometric Assessment of eNOS-GFP**—Nontransfected and transfected cells were trypsinized, washed and resuspended in complete cell culture medium; counted; washed twice with Tris-buffered saline and then resuspended in complete Dulbecco’s modified Eagle’s medium containing penicillin (150 μg/ml NaCl), and lysed at equal density, i.e. 10⁵ cells/ml. Immune precipitation assay buffer (150 mM NaCl, pH 7.4, 1% Nonidet P-40 (v/v), 10 mM NaF, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Lysates were transferred to an Eppendorf tube and rotated for 30 min at 4 °C. Lysates were Dounce-homogenized (50 strokes), insoluble material was removed by centrifugation at 12,000 × g for 10 min at 4 °C, supernatants were transferred to 96-well plates (Dynatech), and the amount of eNOS-GFP in transfected cells was calculated based on the standard curve of purified GFP diluted in lysates of the exactly same number of nontransfected cells to exclude both the background and the possible quenching of fluorescence by some cell lysate components. Preliminary data demonstrated that eNOS-GFP extracted from cells was intact and GFP was not proteolytically cleaved (based on sequential Western blots with GFP and eNOS antisera); therefore, measurements of GFP actually reflect intact eNOS-GFP (data not shown).

**Release of Biologically Active NO from ECV Cells Stably Transfected with eNOS-GFP**—ECV304 cells unlike the parental cells (HUVEC, Ref. 11) do not express the soluble isofrom of guanylate cyclase as assessed by the lack of cGMP accumulation in response to 100 μM sodium nitroprusside (data not shown). Thus, in order to investigate if ECV304 or ECV304 stably transfected with eNOS-GFP produces biologically active NO, a rat vascular smooth muscle cell-endothelium co-culture system was used (12), and cGMP accumulation in vascular smooth muscle cells determined by radiocimetry or measured as described above. cGMP values, protein content in each well was measured by the Bradford method following solubilization of the protein with hot 1 N NaOH. Statistical comparisons between groups were performed using Student’s t test. Differences among means were considered significant at p < 0.05.

**Immunofluorescence Microscopy**—Transfected ECV304 cells were plated onto Permanox eight-chamber slides. For colocalization with the resident Golgi protein mannosidase II (Man II), cells were fixed in acetone for 5 min at −20 °C and then rinsed twice with Dulbecco’s phosphate-buffered saline plus 0.1% bovine serum albumin (w/v, Dulbecco’s phosphate-buffered saline/bovine serum albumin) for 5 min at room temperature. The cells were then incubated sequentially with 5% (v/v) G418 in Dulbecco’s phosphate-buffered saline/bovine serum albumin for 30 min at room temperature, followed by a 3-h incubation with Man II polyclonal antibody (kindly provided by Dr. Kelley Moreman; 1:500) and a 45-min incubation with Texas Red-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:500. Slides were mounted with Slowfade (Molecular Probes, Eugene, OR), and cells were observed with an inverted Zeiss microscope fitted with a Bio-Rad MRC 600 confocal imaging system. For colocalization of eNOS-GFP with caveolin-1 (Cav-1), cells were fixed for 15 min with freshly prepared paraformaldehyde (3%, w/v) at room temperature, washed two times for 5 min, and then incubated sequentially with 0.1% Triton X-100 (v/v) in Dulbecco’s phosphate-buffered saline for 10 min, Dulbecco’s phosphate-buffered saline plus 5% goat serum for 30 min, and thereafter with Cav-1 polyclonal antibody (Transduction Laboratories; 1:300) for 2 h. All of the next steps were the same as described above for colocalization with Man II. The specificity of the Man II and Cav-1 antibodies has been demonstrated previously (4, 5).

**Isolation of Caveolin-enriched Membrane Fractions**—Low-density cholesterol-stabilized membrane fractions were isolated as described (13). Briefly, confluent WT or C15S/C26S eNOS-GFP-transfected ECV304 cells cultured in 100-mm dishes were washed twice with DBPS, scraped into 2 ml of 500 mM sodium carbonate (pH 11), Dounce-homogenized, and sonicated (three 20-s bursts at 30% of maximal power). The homogenate was then adjusted to 42.5% of sucrose by the addition of 2 ml of 85% sucrose prepared in MBS (25 mM Mes, pH 6.5, 0.15 mM NaCl) and placed at the bottom of an ultracentrifuge tube. A 5–30% discontinuous sucrose gradient was formed above (3 ml of 5% sucrose, 5 ml of 30% sucrose; both in MBS containing 250 mM sodium carbonate) and centrifuged at 35,000 rpm for 18 h in an SW40 rotor (Beckman Instruments, Palo Alto, CA). A light-scattered band confined to the 5–30% sucrose interface was observed that contained caveolin but excluded most other proteins. Gradient fractions (1 ml) were collected from the top of the tube to yield a total of 12 fractions, and 30 μl of each fraction was separated by SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) and transferred to nitrocellulose. After transfer, nitrocellulose membranes were stained with Ponceau S to visualize protein bands and subjected to immunoblotting as described previously. Blots were probed with eNOS monoclonal antibody, rabbit anti-Cav-1 polyclonal antibody (1:500; Transduction Laboratories), and anti-C-ROP polyclonal antibody (1:100; Affinity BioReagents) followed by horseradish peroxidase-conjugated, goat anti-rabbit secondary antibody and ECL to detect the immunoreactive proteins. The percentage of total membrane protein in different gradient fractions was determined by laser densitometry.

**Electron Microscopy of ECV304 Cells**—ECV304 cells were grown on coverslips. After washing in PBS at 37 °C, cells were fixed with 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), postfixed (0.1% osO₄, 0.1% K₄Fe(CN)₆, in 0.1 M cacodylate, 1 h), stained with uranyl acetate, and embedded in Epon (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections were mounted on 200-mesh grids and stained with 2% aqueous uranyl acetate followed by lead citrate. Micrographs were taken at 40,000× on a Philips CM10 microscope at 80 kV.

**Quantitation and FRAP of eNOS-GFP in Living Cells**—A confocal microscope was used to image the eNOS-GFP in living cells. Cells were seeded on gelatin-coated (2%, w/v) 35-mm plates (MatTek, Ashland, MA). To assess the amount of eNOS-GFP in different subcellular compartments, Z-series (0.2-μm steps, approximately 20–30 sections) were collected from live cells, and the percentage of eNOS-GFP in each step was measured using IPLAB software. To study the dynamics of eNOS in living cells, we used the fluorescence recovery after photobleaching (FRAP) method of Axelrod et al. (21). Focusing 100% of krypton/argon laser power (blue light of 488-nm length) on small areas of either plasma membrane or perinuclear region, membrane-associated eNOS was photobleached irreversibly for 20 s. 3% of laser power was then used to image the GFP at 15, 30, or 60-s intervals after photobleaching. The first image after photobleaching was recorded after 20–30 s, then 15 s before adjusting the optics for subsequent image emissions. The images were analyzed using IPLAB software. The mean fluorescence signal intensity before photobleaching and in the subsequent time points after photobleaching was measured. If there was any fluorescence signal drop during the recording, FRAP values were normalized to an adjacent cell. We also assessed the mobile fraction of eNOS (in WT or C15S/C26S...
eNOS-GFP cells) within the perinuclear region after partial bleaching. For this purpose, the regions of interest were marked within the bleached and unbleached part of the perinuclear region, and the fluorescence intensity in those regions was measured before bleaching and after recovery. The percentage of mobile eNOS (percentage of mobile fraction) was calculated by dividing the ratio of fluorescence intensity within the photobleached region to the respective one out of the photo-bleached region after recovery by the respective ratio before photo-bleaching as described previously (14).

RESULTS

eNOS-GFP Fusion Protein Is Functionally Expressed in Stably Transfected ECV304 Cells—To assess the trafficking of eNOS in endothelial cells, the immortalized endothelial cell line ECV304 was stably transfected with WT eNOS-GFP expression plasmid. ECV304 cells retain the typical cobblestone morphology appearance of native endothelial cells and retain expression of several but not all cell markers (15). As seen in Fig. 1A, eNOS-GFP (162 kDa) was amply expressed in transfected ECV304 cells, whereas the nontransfected ECV304 cells did not express a detectable amount of the endogenous protein (135 kDa). To assess the expression more quantitatively, we used a fluorometric assay to measure the amount of GFP per cell, reflecting the amount of eNOS-GFP fusion protein. Fig. 1B shows a typical standard curve with purified GFP diluted in lysates of 10^7 nontransfected ECV304 cells in radioimmune precipitation assay buffer (1 ml). The amount of eNOS-GFP fusion protein from the same number of transfected cells (per ml of lysate) was 744 \pm 18.6 ng (n = 3 separate experiments from serial passaged cells) as calculated from the standard curve; thus, the calculated average number of eNOS-GFP molecules per cell was 2.74 \times 10^5.

To address the question of whether eNOS-GFP is catalytically active once expressed in ECV304 cells, we employed a sensitive bioassay to quantify the level of cGMP accumulation in detector vascular smooth muscle cells. As seen in Fig. 1C, the calcium ionophore ionomycin significantly increased cGMP accumulation, an effect that was attenuated by coincubation of the cultures with the NOS inhibitor, nitro-L-arginine methyl ester (n = 4). Ionomycin did not increase cGMP accumulation in cultured vascular smooth muscle cells coincubated with nontransfected ECV304 cells (10.1 \pm 0.33 and 4.4 \pm 3.0 pmoles of cGMP/mg of protein for control and ionomycin-treated cells, respectively, n = 4). In addition, two receptor-dependent agonists, ADP and vascular endothelial growth factor (VEGF), also stimulated cGMP accumulation. Thus, eNOS-GFP couples NO production to both receptor-dependent and-independent forms of stimulation as seen in native endothelial cells.

eNOS-GFP Colocalizes with Golgi and Caveolae Markers—To determine if eNOS-GFP in ECV304 cells colocalized with the resident protein of cis-medial region of the Golgi apparatus, Man II immunofluorescence was performed. The majority of perinuclear eNOS-GFP (left) in ECV304 cells is colocalized with Man II (middle) as described previously (Fig. 2A; the arrows denote areas of colocalization in the right panel). To delineate if eNOS-GFP colocalized with Cav-1, eNOS-GFP cells were immunolabeled with a Cav-1 antibody and observed as above. eNOS-GFP and caveolin-1 appeared to concentrate into the plasma membrane (Fig. 2B, left and middle), and eNOS-GFP partially colocalized with Cav-1 (right; the arrows denote regions of colocalization, and the arrowheads demonstrate regions with little colocalization between eNOS-GFP and caveolin-1).

Since there is evidence that some immortalized or oncogenically transformed cells do not contain caveolae, we performed transmission electron microscopy on confluent ECV304 cells. In contrast to several primary endothelial cells in culture that lose their caveole, ECV304 cells indeed contain membrane-attached, subplasmalemmal, and racemose flask-shaped organelles of approximately 50-nm diameter, defined as caveole (Fig. 2C).

The Palmitoylation Mutant of eNOS-GFP Does Not Target into the Plasma Membrane—In order to determine the role of palmitoylation of eNOS in its subcellular distribution in living cells, ECV304 cells were stably transfected with the palmitoylation-deficient mutant C155S/C265S eNOS-GFP. As seen in Fig. 3A, at all time points after plating, C155S/C265S eNOS-GFP cells had a similar perinuclear appearance to that of cells expressing WT eNOS-GFP. However, the palmitoylation mutant, although tightly membrane-associated biochemically (3), was more diffusely distributed in this cellular domain and occupied a larger area of the cell compared with cells expressing WT eNOS-GFP. More importantly, as seen in postconfluent cells (5 days), WT eNOS-GFP was particularly strong in cell junctions in contrast to C155S/C265S eNOS-GFP, which did not yield any enhancement of the fluorescence intensity in cell peripheral borders and was confined to a diffuse perinuclear pattern. Vertical sections through the monolayer (delineated by the horizontal bar in the bottom panel of Fig. 3A) to examine the plasma membrane distribution demonstrated that WT eNOS-GFP was localized specifically to both apical and basolateral membranes with the greatest concentration of the protein at intercellular junctions (Fig. 3B), whereas the palmitoylation mutant was excluded from the junctions.
We also determined the percentage of WT and C15S/C26S eNOS-GFP in different subcellular compartments by quantitative horizontal Z-series analysis. WT eNOS-GFP was mostly abundant in plasma membrane (53.6 ± 6.2%), followed by Golgi (34.8 ± 4.6%) and cytosol (11.6 ± 3.3%, n = 6). Thus, based on our fluorimetric assessments of WT eNOS-GFP in cell lysates, the average number of eNOS-GFP molecules was 1.47, 0.93, and 0.32 × 10^6 for plasma membrane, Golgi, and cytosol, respectively. On the other hand, C15S/C26S eNOS-GFP was not present in the plasma membrane, and the majority of it was found in the perinuclear region (65.8 ± 10.5%), followed by the remaining part of cytosol (34.2 ± 9%, n = 7).

Next, we examined the subcellular localization of WT and mutant eNOS using sodium carbonate disruption followed by discontinuous sucrose gradients. This methodology allows for the separation of proteins that localize to cholesterol and glycolipid-rich domains (interface of 5–30% sucrose) from other membrane proteins (bottom of gradient) (Fig. 4). Cav-1-enriched fractions (fractions 4 and 5) contained 97% of the total eNOS in the cell, we photobleached a region of the perinuclear compartments in confluent cells were photo-bleached, and the FRAP was determined. The recovery was reasonably fit by a single exponential function, \[ F(t) = F_i \exp(-t/\tau) \], where \( F_i \) represents measured fluorescence, \( F_0 \) represents the initial fluorescence, \( t \) represents time (s), and \( \tau \) is the time constant. Fig. 5A shows the fluorescence recovery in plasma membrane of eNOS-GFP. The average time constant of this process was 716 ± 209 s (n = 4 cells from four experiments).

Next, a part of perinuclear region was photobleached to determine the kinetics of FRAP within the Golgi region (Fig. 5B). The speed of recovery was about 3 times faster in the Golgi (222 ± 50 s; n = 5) compared with that of plasma membrane, suggesting that protein-protein or lipid-protein interactions with eNOS were different in these two membrane compartments. To assess the role of palmitoylation in the movement of eNOS in the cell, we photobleached a region of the perinuclear C15S/C26S eNOS-GFP in ECV304 cells. As seen in Fig. 5C, the recovery of the palmitoylation mutant was faster than that of WT eNOS, with a time constant of 103 ± 16 s (n = 5). These data suggest that palmitoylation, in addition to contributing to the targeting of eNOS to the plasma membrane, slows the movement of eNOS in the perinuclear region. Moreover, the percentage of mobile fraction of the palmitoylation mutant was closely than that of Cav-1 (only 12.5% in fractions 4 and 5). Fig. 4B represents densitometric evaluation of these data.

**Different Kinetics of WT eNOS-GFP and C15S/C26S eNOS-GFP Movement in Endothelial Cells**—To investigate the dynamics of WT eNOS-GFP movement, plasma membrane and perinuclear compartments in confluent cells were photobleached, and the FRAP was determined. The recovery was reasonably fit by a single exponential function, \[ F(t) = F_i \exp(-t/\tau) \], where \( F_i \) represents measured fluorescence, \( F_0 \) represents the initial fluorescence, \( t \) represents time (s), and \( \tau \) is the time constant. Fig. 5A shows the fluorescence recovery in plasma membrane of eNOS-GFP. The average time constant of this process was 716 ± 209 s (n = 4 cells from four experiments).
greater than that of the wild type enzyme (93 ± 4.5 and 71 ± 13% mobile protein for C15S/C26S eNOS-GFP compared with WT, respectively, n = 5). These data suggest a more homogeneous distribution and mixing of the bleached and unbleached pools of the mutant compared with the WT protein.

The Movement of Palmitoylation Mutant but Not of WT eNOS-GFP Can Be Described by a Random Process Resembling Lateral Diffusion—To evaluate the FRAP data further, we inverted the data values of the images and subtracted the prebleach image from the images acquired during recovery. This procedure generated an image of the bleaching pattern (Fig. 6A). Analysis of the initial bleach pattern did not fit to a Gaussian function for WT eNOS-GFP, and the distribution of the bleaching patterns did not change over time in a manner consistent with a random process of diffusion of a protein in a lipid bilayer. In striking contrast to the WT protein, the initial bleach \( F_o \) of the palmitoylation mutant was readily fit by a simple Gaussian function described by the equation \( F_o = C* \exp(-x^2/\sigma_x^2), \) and the subsequent images of the spreading bleach pattern were fit with the equation \( F_n = C* (\sigma_x^2/\sigma_n^2) \exp(-(x - m)^2/\sigma_n^2) \) (16), where \( C \) represents the peak bleach value, \( x \) represents a data point, and \( m \) represents the mean of the curve. The mean of the Gaussian fits for the palmitoylation mutant remained constant over time (Fig. 6B), and the change in the S.D. squared plotted against time (Fig. 6C) was well fit by a single line with a slope directly related (\( \Delta y/\Delta x = 4D \)) to a diffusion coefficient \( (D) \) calculated to be 0.051 ± 0.013 \( \mu \)m\(^2\)/s (n = 8). The \( D \) for C15S/C26S eNOS-GFP was 3 orders of magnitude smaller than that for GFP alone in solution (17) or inside the cell (16, 18). The measured \( D \) for C15S/C26S eNOS-GFP was well within the range of those reported for other proteins. For example, the \( D \) for C15S/C26S eNOS-GFP was about 6 times smaller than that for the resident protein of the Golgi, Man II-GFP (14), or 10 times smaller than elastase-GFP in the endoplasmic reticulum (19). However, the \( D \) for C15S/C26S eNOS-GFP was 5 times larger than that for ADP-ribosylation factor 1-GFP in the Golgi (20) and 10 times larger than for the acetylcholine receptor in plasma membrane (21).
tors, P2U-purinoceptors (23), and thromboxane A2 receptors levels similar to that seen in native HUVEC (25). Moreover, the

The calculated coefficient is proportional to the slope of the linear fit ($\Delta y/\Delta x = 4^*D$).

DISCUSSION

To study the dynamics of eNOS movement in intact endothelial cells, stable cell lines were generated expressing wild-type and palmitoylation mutant forms of eNOS-GFP. As seen in the present study, eNOS-GFP is functionally capable of producing NO and targets into the Golgi region and plasma membrane. Utilizing FRAP, we demonstrate that the kinetics of eNOS-GFP movement are different in the Golgi versus plasma membrane and are dramatically different from that of a myristoylated, nonpalmitoylated form of the enzyme. These data point toward different mechanisms for regulating the movement of the same protein at different places in the cell and support the concept that palmitoylation reduces the mobility of peripheral membrane proteins.

Since eNOS is active as a dimer and nonacylated forms can heterodimerize with wild-type protein, we used the endothelial cell line, ECV304, which does not express eNOS, as a host to study eNOS-GFP targeting. ECV304 cells originate from HUVEC by spontaneous transformation (15) and are polarized (22), and they retain typical cobblestone endothelial cell morphology and the expression of endothelial marker proteins such as angiotensin-converting enzyme (15), $\text{H}_1$ histamine receptors, $\text{P}_{2\text{U}}$-purinoceptors (23), and thromboxane $\text{A}_2$ receptors (24). Stable transfection of these cells with eNOS-GFP resulted in the establishment of cell lines that express eNOS protein levels similar to that seen in native HUVEC (25). Moreover, the fusion protein was biologically responsive, since ionomycin, ADP, and VEGF induced the release of NO release as measured by cGMP accumulation. Our previous studies have shown that the GFP tag did not affect the catalytic function of eNOS, since specific activities of eNOS and eNOS-GFP measured in lysates from transfected NIH 3T3 cells were identical. Also, the subcellular localization of GFP-tagged eNOS was virtually the same as nontagged eNOS in these cells (9).

In cultured endothelial cells and in the endothelium of intact blood vessels, eNOS has been localized in Golgi and plasma membrane by immunofluorescence, immunoperoxidase, and electron microscopy (4, 5, 26, 27). Based on subcellular fractionation data, using discontinuous sucrose gradients or caveolae isolation (employing distinct methodologies), eNOS has been found in varying amounts in Golgi- and plasmalemma-enriched fractions, suggesting a cycle between this two membrane compartments (5, 6, 28). In the present study, eNOS-GFP in the perinuclear region colocalizes with Man II, as previously reported (4, 9). In postconfluent cells, some but not all eNOS in the plasma membrane colocalizes with Cav-1, suggesting that eNOS can exist in other domains of the plasma membrane. A recent study in intact coronary blood vessels reveals the complexity of defining the subcellular distribution of eNOS. In all endothelia examined, including endocardial, coronary arterial, venous, capillary, and thoracic aorta, eNOS was found in the perinuclear region colocalizing with the 58-kDa Golgi protein. eNOS in the plasma membrane was only visualized in endocardial and venous endothelium, suggesting that the relative distribution between the Golgi and plasma membrane is related to the pattern of endothelial cell heterogeneity and function (29).

To gain an understanding of the mechanisms contributing to the heterogeneity of eNOS localization, the use of eNOS-GFP to image its trafficking in living cells is advantageous, since the method does not rely on antibody specificity, cell fixation, permeabilization, or other harsh biochemical procedures (i.e. sonication). The present results show that eNOS-GFP localization differs dramatically depending on the state of cellular confluency. In subconfluent cells, there are intense signals for eNOS-GFP in the perinuclear region. In confluent cells, the polarized distribution of eNOS into Golgi-like and plasmalemmal compartments, as reported in fixed cells, became more apparent. This suggests that the process of contact inhibition either triggers the condensation of eNOS into these discrete domains or that the tight apposition of cells during confluency condenses what appears to be “patchy” patterns of eNOS seen in subconfluent cells. At present, we cannot rule out either possibility.

Recent evidence suggests that protein palmitoylation can serve as a mechanism to target peripheral membrane proteins into glycolipid, cholesterol-rich domains of the Golgi and plasma membrane, and caveolae. This is based on experiments demonstrating that palmitoylation mutants of proteins that are still N-myristoylated such as eNOS (5, 6) and Src family members (30, 31) do not target into Triton X-100-insoluble microdomains or the plasmalemmal domains. More recently, there is evidence that palmitoylation is an important constituent of a Golgi targeting signal for several different classes of proteins such as eNOS, GAP-43, rhodopsin, and the neuronal growth cone protein SCG10 (9, 32–34). For eNOS, both N-myristoylation and cysteine palmitoylation are necessary for the ability of agonists to activate eNOS and produce the biologically active gas, NO. As seen in the present study, the myristoylation-replete palmitoylation mutant of eNOS, C15S/C26S eNOS-GFP (9), diffusely distributes into the perinuclear region of stably transfected ECV304 cells and does not target into the plasma membrane of postconfluent cells as does WT.
eNOS-GFP. These results confirm and extend experiments in NIH 3T3 cells transiently transfected with wild type and palmitoylation mutants of eNOS (3, 5). Interestingly, vertical sectioning of cells reveals WT eNOS-GFP concentrated in cellular junctions and lightly distributed on both apical and basolateral surfaces of the cell, whereas the palmitoylation mutant of eNOS is excluded from junctions and appears diffusely distributed in the perinuclear region and in subplasmalemmal aspects on both sides of the cell. Also, the confocal horizontal Z-series evaluation of the relative amount of eNOS-GFP in different subcellular pools reveals that approximately half of the total WT eNOS protein is in the plasma membrane. These results were further complemented by biochemical studies showing that eNOS-GFP is found primarily in two domains, one enriched in Cav-1 (approximately 40% of the total eNOS) and the other enriched in β-COP, as described previously by us and others (5, 28). However, the palmitoylation mutant of eNOS was not found in the plasma membrane and was more uniformly distributed, strongly suggesting its exclusion from caveolae-like, Cav-1-enriched domains. Collectively, these data demonstrate that the palmitoylation of eNOS is required for stable association with the plasma membrane, in particular at intercellular junctions of confluent cells.

To better understand the interaction of eNOS with intracellular compartments, we photobleached small portions of eNOS-GFP fusion proteins and monitored the recovery of the fluorescence over time. Fluorescence photobleaching allows for the irreversible bleaching of protein pools in a defined region of the cell. We assumed that any increase in fluorescence intensity after photobleaching was a function of the movement of eNOS-GFP from other nonbleached regions of the cell and not due to de novo protein synthesis for two reasons: 1) the biosynthetic half-life of eNOS is 18 h (3); and 2) preliminary experiments revealed that complete photobleaching of eNOS-GFP in the entire cell did not result in the recovery of the fluorescence during the time frame of the experiment (data not shown).

All FRAP experiments produced recovery processes that appeared relatively uniform and could be readily fit by a simple exponential function. Interestingly, the FRAP of perinuclear fluorescence was 3 times faster than the FRAP for eNOS in the plasma membrane, suggesting that eNOS interacts differently with the Golgi apparatus compared with the plasma membrane. Since eNOS requires both N-myristoylation and cysteine palmitoylation to target to Golgi (9) and plasma membrane (5, 6), the differences in FRAP are probably due to regulated protein-protein and/or lipid-protein interactions that may occur in the Golgi and plasma membrane. To our knowledge, this is the first observation of different kinetics of movement of eNOS or any other peripheral membrane protein in different subcellular compartments.

While there are simple explanations that may explain the above differences of eNOS trafficking in Golgi and plasma membrane, virtually nothing is known about the molecular mechanisms of anterograde movement of eNOS or any other acylated protein to or between caveolae or lipid rafts, which may be critical for interpretation of the kinetics of eNOS movement. It seems likely that Cav-1 may be partially involved in this process, since it was shown to interact with both acylated and nonacylated forms of eNOS (35, 36) and to recirculate between Golgi complex and caveolae (37, 38). Also, the involvement of other chaperone proteins, such as Hsp90 (39), Hsp65 (40), cytoskeleton, or elements of the classic secretory machinery remains to be tested. It is possible that part of eNOS-GFP, which contributes to FRAP in the plasma membrane comes from lateral diffusion or from the trans-Golgi network-derived cytoplasmic vesicles (5, 41). An alternative explanation is that the lipid composition of the Golgi seems to be less cholesterol-rich than that of plasmalemma (42), which could contribute to the faster movement of eNOS-GFP in the Golgi compared with the plasma membrane.

To better understand the role of palmitoylation of eNOS in directing interactions with the Golgi and plasma membrane, we compared the FRAP of wild-type eNOS in the Golgi region with that of the palmitoylation mutant localized in the perinuclear region. The FRAP data for both proteins were fit by a single exponential function; however, FRAP for the palmitoylation mutant in the perinuclear region was 2 times faster than that for the wild-type protein. There were also differences in the mobile fractions of the two proteins, with the palmitoylation mutant being more mobile than wild type. Interestingly, the mixing of bleached with unbleached C15S/C26S eNOS-GFP appeared uniformly within the entire perinuclear region, whereas the wild-type protein was not as uniformly distributed throughout the Golgi and did not mix as well. Because FRAP data produced by both proteins can be readily fit by a single exponential function, a priori, one might assume that the differences in FRAP were due to the speed of diffusion of the proteins in membranes. Upon inspection of the fine structure of the bleaching patterns, the data for C15S/C26S eNOS-GFP could be fit by a Gaussian function; i.e. the distribution of protein occurred in a manner consistent with a random process of diffusion of a protein in a lipid bilayer. However, the WT eNOS-GFP in Golgi and plasma membrane was not distributed normally, and the data cannot be explained by diffusion of a protein in a lipid bilayer. These data suggest that direct calculation of diffusion coefficients from FRAP data on membrane proteins may be misleading, since FRAP represents many cellular processes (i.e. protein-protein interactions, protein-lipid interactions, and organelle movement) in addition to diffusion of protein in a lipid bilayer. With this in mind, our data highlight the importance of palmitoylation in slowing the intramembrane movement of eNOS in particular and probably of other dually acylated proteins, perhaps by stabilizing the interaction of proteins with resident lipids and/or other proteins in order to limit their random diffusion and facilitate directional trafficking.

In summary, by fusing eNOS to GFP and introducing it into endothelial cells, we have demonstrated that eNOS movement is dynamic and requires palmitoylation. Moreover, the use of GFP has permitted us to show that palmitoylation of eNOS renders the protein less mobile in lipid bilayers, thus supporting the idea that this post-translational modification is a "kinetic trapping mechanism" for peripheral membrane proteins. The ability to use GFP to make quantitative measurements of eNOS trafficking should make it possible to generate rigorous models of peripheral membrane protein trafficking and to understand the regulation of eNOS activity by protein-protein interactions in Golgi and plasmalemmal microdomains.

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