Intracellular location regulates calcium-calmodulin-dependent activation of organelle-restricted eNOS

Davin Jagnandan,1 William C. Sessa,2 and David Fulton1

1Vascular Biology Center and Department of Pharmacology, Medical College of Georgia, Augusta, Georgia; and 2Department of Pharmacology and Molecular Cardiobiology Division, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, Connecticut

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Jagnandan, Davin, William C. Sessa, and David Fulton. Intracellular location regulates calcium-calmodulin-dependent activation of organelle-restricted eNOS. Am J Physiol Cell Physiol 289: C1024–C1033, 2005. First published May 25, 2005; doi:10.1152/ajpcell.00162.2005.—Mislocalization of endothelial nitric oxide (NO) synthase (eNOS) in response to oxidized low-density lipoprotein, cholesterol depletion, elevated blood pressure, and bound eNOS interacting protein/NOS traffic inducer is associated with reduced NO release via unknown mechanisms. The proper targeting of eNOS to the plasma membrane or intracellular organelles is an important regulatory step controlling enzyme activity. Previous studies have shown that plasma membrane eNOS is constitutively phosphorylated on serine 1179 and highly active. In contrast, the activity of eNOS targeted to intracellular organelles is more complex. The cis-Golgi eNOS is fully activated by Akt-dependent phosphorylation. However, eNOS targeted to the trans-Golgi is decidedly less active in response to all modes of activation, including mutation to the phosphomimetic aspartic acid. In this study, we establish that when expressed within other intracellular organelles, such as the mitochondria and nucleus, the activity of eNOS is also greatly reduced. To address the mechanisms underlying the impaired catalytic activity of eNOS within these locations, we generated subcellular-targeted constructs that express a calcium-independent NOS isoform, iNOS. With the use of organelle specific (plasma membrane, cis- vs. trans-Golgi, plasma membrane, and Golgi, nucleus, and mitochondria) targeting motifs fused to the wild-type iNOS, we measured NO release from intact cells. With the exception of the Golgi lumen, our results showed no impairment in the ability of targeted iNOS to synthesize NO. Confirmation of correct targeting was obtained through confocal microscopy using identical constructs fused to the green fluorescent protein. We conclude that the reduced activation of eNOS within discrete cytoplasmic regions of the Golgi, the mitochondria and the nucleus is primarily due to insufficient access to calcium-calmodulin.

ENDOTHELIAL NITRIC OXIDE (NO) synthase (eNOS) is one of three distinct enzymes that catalyze the synthesis of NO (28). Genetic deletion of eNOS confirmed many previously established cardiovascular roles for endothelium-derived NO, including the regulation of vascular tone, permeability, inflammation, cellular proliferation, angiogenesis, and vascular remodeling. eNOS-derived NO is vital for cardiovascular homeostasis and the reduced synthesis or bioavailability of NO which is invariably linked to compromised blood vessel function, is a hallmark of cardiovascular disease (2). The amount of NO produced by eNOS is tightly controlled by the interplay of several distinct posttranslational modifications, including phosphorylation, protein-protein interactions, and subcellular localization (6, 11). These controls have evolved to facilitate the delivery of sufficient quantities of NO at the right place and the right time to initiate the appropriate signaling response.

In endothelial cells, both in culture and in situ within isolated blood vessels, eNOS resides in two distinct subcellular locations, the perinuclear Golgi complex and the plasma membrane. Recently, we reported (8) that subcellular targeting of eNOS to either the cytoplasmic face of the Golgi or to the plasma membrane strongly influenced the ability of the enzyme to produce NO in response to both the calcium-mobilizing agent, thapsigargin, and to Akt-dependent phosphorylation. Plasma membrane-targeted eNOS was constitutively active and highly responsive to transmembrane calcium fluxes, whereas cis-Golgi-targeted eNOS was less responsive to calcium and fully activated by the protein kinase Akt. In contrast, eNOS targeted to the trans-Golgi or to the cytoplasm exhibited both reduced calcium and Akt-dependent activation. Furthermore, the translocation of eNOS from the plasma membrane to internal endomembranes, induced via binding the proteins eNOS interacting protein/NOS traffic inducer (NOSTRIN/ NOSIP) or through exposure to oxidized low-density lipoprotein, is also associated with reduced capacity to make NO (4, 30, 42). The mechanisms underlying the reduced activity of eNOS within these intracellular locations remain to be identified.

The site-specific phosphorylation of eNOS modulates enzyme activity in response to a variety of stimuli (6). The extent of phosphorylation is strongly influenced by subcellular localization as shown by the intense serine 1179 (S1179) phosphorylation of plasma membrane-targeted eNOS and the reduced phosphorylation of cytoplasmic G2A eNOS (8, 15). To exclude the possibility that Akt and/or other kinases are spatially restricted from activating eNOS in different compartments, we mutated S1179 to the phosphomimetic aspartic acid (S1179D) (8). However, the cytosolic and trans-Golgi targeted eNOS S1179D were not fully activated compared with the native enzyme. These data suggest that mechanisms other than phosphorylation constrain the activity of eNOS in various subcellular compartments.

The activation of endothelial cells by “classic” transmembrane receptor ligands such as bradykinin and acetylcholine and by shear stress is accompanied by the elevation of intracellular calcium and subsequent liberation of NO (27). The
initial depletion of calcium from intracellular stores triggers the influx of extracellular calcium through a variety of different plasma membrane calcium channels (32). The location of signaling molecules within the cell and their proximity to the influx of calcium plays a major role in the efficiency of coupling enzyme activity to local calcium concentrations. In this regard, membrane associated eNOS is more sensitive to transmembrane calcium fluxes than a cytosolic eNOS mutant (21). However, because of the presence of eNOS in both locations it is not known whether the influx of calcium primarily activates the plasma membrane eNOS or eNOS bound to intracellular membranes such as the Golgi. The presence of eNOS or eNOS-like activity has also been reported in the mitochondria and the nucleus (1, 5, 13) and the contribution of calcium to the activity of eNOS in these discreet intracellular environments, i.e., cis- vs. trans-Golgi, mitochondria, and nuclei remains unknown.

Therefore, the goal of the present study is to determine the calcium dependency of eNOS in these organelles and at the Golgi and plasma membrane by using targeted fusion proteins of eNOS and iNOS. eNOS and iNOS are structurally similar enzymes and have virtually identical co-factor and substrate affinities with one major exception, calcium-calmodulin. These properties will enable us to investigate both the calcium and substrate/co-factor dependency of NOS activity within discrete subcellular locations.

EXPERIMENTAL PROCEDURES

Generation of eNOS Targeting Proteins

The wild type (WT), 17 amino acid transmembrane sequence derived from syntaxin-3 (S17), 87 amino acid targeting sequence derived from Golgin-97 (GRIP), 81 amino acid transmembrane sequence of β-1,4-galactosyltransferase (GAL), and 15 amino acid membrane targeting sequence from K-ras (CAAX) eNOS constructs were generated as previously described (8).

Nuclear-targeted eNOS. Nuclear-targeted eNOS (NLS) was achieved by fusion of a tripartite nuclear localization sequence (PKKKRKVD) derived from SV40 to the untargeted G2A-eNOS by using the following primers: sense, 5′-GGG AAC TTG-3′, and antisense, 5′-GCG GCC GCC CGC ACC AGG ACG GTG GCC GTG TCT GGG CCG CGG A-3′.

Mitochondrial targeted eNOS. Mitochondrial (Mito) eNOS was constructed by fusion of the targeting sequence derived from human cytochrome-c oxidase subunit VIII (MSVLTPLLRGLTGSAR-RLPVFRA KIHS1) to G2A-eNOS by using the following primers: 5′-ATT CTA CAT GAT CAC CAT GCT TAC ATT CTC TTC TTT TTT GGA TCT ACC TTC TTC TTT TTT GGA TCG TGG GGG CCG GGG GTG TCT GGG CCG CGG A-3′.

Plasma membrane-targeted eNOS. eNOS was targeted to the plasma membrane via fusion to the amino terminal neuromodulin targeting sequence (accession no. NM_002045) using the following primers: sense 5′-AGC TTG CCA TCA TCG TGC CCT GCT ACC TAC GC CAC GCC G-3′, antisense 5′-GCG AAG AAT CAT TCG TGG TCG GCC GCA TCG TAA-3′.

Golgi lumen targeted eNOS. Targeting the lumen of the Golgi apparatus was achieved via fusion of a Golgi-targeting sequence from the glycosyltransferase FKRP (accession no. NM_024305) using the following primers: sense 5′-ATG CGG CTC ACC CGC TGC GCT GCC CTC GTG GCC GCC ACC ACC ACC ATC C-3′, and antisense 5′-GCG GCC GCC CGC ACC AGG ACG GTG ACA A-3′.

Generation of iNOS Targeting Proteins

Golgi-targeted iNOS constructs. The trans-Golgi GRIP domain, and cis-Golgi S17 were derived from the eNOS constructs described above and fused to the full-length mouse iNOS cDNA (accession no. M87039) via 3′ XhoI and XbaI sites.

Golgi and plasma membrane-targeted iNOS. The first 75 amino acids of iNOS, required for targeting of eNOS to Golgi and plasma membrane [MGNLKSVGEPGPGCGGLGLGLGCGQGP-SPAPESPRAAPATPAPHDPNSPSTLRPPEPGPKFPRVKN (24)] were fused to the NH2 terminus of iNOS using the following primers: sense, 5′-AAAT CTG ACC ATG GG GGC AAC TTG AAG AGT G-3′; and antisense, 5′-GCG GCC GCC CGC TTC TTT AGC CGA GGG AAC TTG-3′.

Plasma membrane-targeted iNOS constructs. The CAAX motif was derived from eNOS-CAAX as described above and fused to the COOH terminus of iNOS via Xhol-XbaI restriction sites. Neomodulin (MLCCMRRTKQVEKNDNDQKI)-tagged iNOS was generated using the eNOS targeting sequence described above.

Nuclear and mitochondria-targeted iNOS constructs. Nuclear, mitochondria, and intra-Golgi lumen targeting sequences were obtained from the eNOS constructs described above and ligated to the Xhol-XbaI sites and HindIII-NotI sites, respectively, of iNOS.

Green fluorescent protein-tagged iNOS. Enhanced green fluorescent protein (EGFP) was amplified by PCR from the plasmid pEGFP-N1 (BD Bioscience Clontech) using the following primers and fused to the NH2- and COOH-terminus of iNOS via HindIII-NotI and Xhol-XbaI sites, respectively. Sense, 5′-AAG CTT GCC ACC ACC ATG GCC AAA GGC G-3′; antisense, 5′-GCG GCC GCC CGC TTC TAC AGC TCG TCC ATG CC-3′; sense, 5′-CCT GAG CAT GGT GAG CAA GGG CGA GGA GC-3′; and antisense, 5′-TCT AGA TTA CTA GTA CAG CTC GTC TCC ATG CC-3′.

Nuclear- and Golgi-targeted red fluorescent protein. Monomeric red fluorescent protein (accession no. AF506027) was a generous gift from Roger Tsien (University of California, San Diego). Nuclear-targeted RFP was achieved via fusion to the nuclear targeting sequence described above, using the following primers: sense, 5′-AAG CTT GCC ACC ACC ATG GCC AAC AGC AAC GGC G-3′; and antisense, 5′-GCG GCC GCC CGC TTC TAC AGC TCG TCC ATG CC-3′.

Golgi targeting of RFP was achieved via fusion with β-galactosyltransferase as described previously (8) using the following primers: sense, 5′-GCG GCC GCC GCC GCC ACC ATG GCC TCC ATG GCC AAC AGC AAC GGC G-3′; and antisense, 5′-TGT AGA TTA CTA GTA CAG CTC GTC TCC ATG CC-3′.

Cell Culture Conditions and Transfection

COS-7 cells were grown in Dulbecco’s modified Eagle’s medium containing penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% (vol/vol) fetal calf serum (complete Dulbecco’s modified Eagle’s medium). For transfection, COS-7 cells were seeded at a density of 3 × 10^6 cells/60-mm dish and transfected the next day with the cDNAs according to the manufacturer’s instructions (Lipofectamine 2000, Invitrogen).

Western Blot Analysis

Cells were washed twice with phosphate-buffered saline (PBS), lysed on ice in 50 mM Tris·HCl, pH 7.5, 1% Nonidet P-40 (vol/vol), 10 mM NaF, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and lysates were transferred to an Eppendorf tube and rotated for 45 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, size fractionated by SDS-PAGE, and were Western blotted as described previously (9).
Reagents. All buffer reagents and chemicals were acquired from Sigma-Aldrich.

Immunofluorescence

COS-7 cells were transfected as described and plated onto sterile coverslips. Cells were then fixed in acetone/methanol 1:1 for 3 min at −20°C and rinsed twice with PBS plus 0.1% bovine serum albumin (PBS/BSA) for 5 min at room temperature. The cells were incubated with 5% goat serum in PBS/BSA for 30 min at room temperature, followed by incubation for 2-h with primary antibody either (polyclonal or monoclonal) at room temperature. Anti-rabbit Texas Red-labeled (diluted 1:100) or anti-mouse FITC-labeled (1:100) secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) was incubated for 1 h at room temperature. The slides were mounted with Slowfade (Molecular Probes; Eugene, OR), and the cells were observed with an inverted Zeiss microscope fitted with a Bio-Rad MRC 600 confocal imaging system. Primary antibodies for eNOS were obtained from BD Transduction Labs (eNOS MAb) and GM130 from G. Warren (Yale University). Mitotracker and 4,6-diamidino-2-phenylindole (DAPI) were obtained from Invitrogen.

Live Cell Imaging

COS-7 cells were transfected with cDNAs encoding fusion proteins of EGFP and monomeric RFP as described above. From 24 to 48 h later, cells were replated onto glass-bottomed culture dishes (MatTek). All imaging was performed with the use of the confocal microscopy (LSM 510 Meta 3.2, Zeiss). Magnification power was set at ×40 with oil.

NO Release

Media (100 μl) containing nitrite and nitrate (primarily NO3−) was ethanol precipitated to remove proteins and refluxed in sodium iodide/glacial acetic acid to convert NO3− to NO. NO was measured via specific chemiluminescence after reaction with ozone (Sievers). Net NO3− from cells transfected with eNOS cDNAs was calculated after subtracting NO3− levels from mock transfected cells (10). NO release from cells transfected with iNOS constructs was determined in a 120-min period immediately after the addition of fresh media (DMEM) containing ~400 μM L-arginine.

Statistical Analysis

NO release data are expressed as means ± SE. All analyses were performed using InStat software (GraphPad) and were made using a two-tailed Student’s t-test or ANOVA with a post hoc test where appropriate. Differences were considered as significant at P < 0.05.

RESULTS

Targeting of eNOS Constructs

The restricted expression of eNOS in various intracellular locations was obtained by inframe fusion of the cytosolic G2A mutant of eNOS to the targeting sequences of select organelle resident proteins. The strategy, as illustrated in Fig. 1 (also see Table 1), shows cis- (S17) and trans- (GRIP) Golgi, plasma membrane (CAAX, NEURO), nuclear (NLS), and mitochondria (Mito)-targeted eNOS. To demonstrate that these constructs do indeed target their intended domains, we used confocal microscopy to record their intracellular location. As shown in Fig. 2A, WT-eNOS is found at both the perinuclear/Golgi complex and the plasma membrane. eNOS targeted to the cis-Golgi with S17 is exclusively perinuclear (Fig. 2B), whereas trans-Golgi expression of eNOS co-localizes with the Golgi marker GM130 (Fig. 2E). In Fig. 2, C and D, targeting of eNOS to the plasma membrane is clearly evident by the presence of eNOS in the outer membrane. eNOS targeted to the nucleus (Fig. 2F) is present exclusively within the nucleus, as determined by comparison with DAPI staining (Fig. 2F, right). Restricted expression of eNOS within the mitochondria is shown by overlap with the mitochondria stain with Mitotracker (Fig. 2G, left and right).

Reduced Activity of eNOS Targeted to Mitochondria, Nucleus, and trans-Golgi

To assess the functional significance of eNOS subcellular localization, COS-7 cells were transfected with the targeted eNOS constructs and NO release was determined via NO-specific chemiluminescence with the use of a NO analyzer (model 280, Sievers) (Fig. 3). In Fig. 3A, basal NO was measured in unstimulated COS-7 cells. Plasma membrane-targeted eNOS is constitutively active and releases large amounts of NO from unstimulated cells (Fig. 3A, right). The cis-Golgi-targeted S17 eNOS shows comparable activity to the WT enzyme, whereas the trans-Golgi-targeted (GRIP), mitochondrial (Mito), and nuclear (NLS) enzymes produce very little basal NO. As shown in the Western blot in Fig. 3A, bottom, equal amounts of eNOS enzyme were present in the lysates of cells transfected with the fusion proteins. To ensure that the capacity of the organelle-restricted enzymes to produce NO is not compromised by the targeting sequence, NO assay activity assays were performed in cell free extracts. In detergent-soluble lysates, the enzymatic activity, as determined by arginine to citrulline conversion, of NLS and Mito-eNOS were not significantly different from that of WT eNOS (81 ± 7, 79 ± 10, vs. 73 ± 5 pmol·min−1·mg protein−1, respectively). In Fig. 3B, stimulated NO release was determined from transfected COS-7 cells using the calcium mobilizing agent thapsigargin. Stimulated NO release was similar from WT and cis-Golgi targeted S17 eNOS and enhanced in plasma membrane targeted eNOS. In contrast, the trans-Golgi, mitochondria, and nucleus-targeted eNOS had greatly attenuated calcium-dependent NO synthesis. To further address the ability of localized calcium to activate targeted eNOS, we stimulated transfected COS cells with a G protein coupled receptor agonist, ATP and the calcium ionophore, ionomycin. As shown in Fig. 3, C and D, both ATP and ionomycin stimulated significantly less NO release from the NLS, Mito, and GRIP eNOS constructs. However, in response to ionomycin there was less difference between the activities of the WT and the Mito and GRIP eNOS (44% and 65% of WT for ATP and 70% and 80% of WT activity in response to ionomycin, respectively).

Calcium Dependence of NOS isoforms in Transfected COS-7 Cells

In the next set of experiments, our goal was to determine the calcium dependence of eNOS and iNOS in transfected COS-7 cells. As shown in Fig. 4, the calcium chelator EGTA, effectively eliminated NO release from eNOS, but not the calcium-independent iNOS. In contrast, the calcium ionophore, ionomycin, stimulated a large increase in NO release from eNOS transfected cells, but did not influence NO release from iNOS. Thus the activity of iNOS is completely independent from calcium with regard to both positive and negative regulation.
Subcellular targeting does not influence the ability of iNOS to produce NO. We next determined the ability of subcellular-targeted iNOS constructs to produce NO from different areas of the cell. COS-7 cells were transfected with iNOS fusion proteins and 48 h after transfection, the media was changed to replenish arginine and NO release was determined over a period of 2 h. Immediately after NO release, the cells were lysed and the abundance of iNOS protein, determined via Western blot analysis (Fig. 5, bottom). As shown in Fig. 5A, targeting iNOS to the Golgi and plasma membrane with the eNOS targeting sequence does not alter the ability of iNOS to make NO. Similarly, when iNOS is targeted to the trans- or cis-Golgi, it produces as much NO as the WT enzyme (Fig. 5, B and C). Furthermore, directed expression of iNOS in the mitochondria (Fig. 5E), nucleus (Fig. 5D), or plasma membrane (Fig. 5F), areas of low and high eNOS activity, respectively, did not influence the activity of iNOS.

Reduced NO release from the Golgi lumen. Previously, by using the targeting sequence derived from β-1,4-galactosyltransferase (GAL), we showed that eNOS targeted to the lumen of the Golgi had very low-to-nonexistent activity (8). In the present study, by using a distinct targeting sequence derived from the glycosyltransferase FKRP, the restricted expression of eNOS within the Golgi lumen negates its ability to produce NO in response to thapsigargin (data not shown). To determine whether the Golgi lumen would influence the activity of iNOS, we fused the enzyme to the two distinct Golgi targeting sequences derived from GAL and FKRP. The intralumen location of iNOS within the Golgi apparatus reduces the activity of the enzyme by 50% (data not shown).

Table 1. Sequences of select organelle resident proteins

<table>
<thead>
<tr>
<th>Targeting Sequence</th>
<th>Destination</th>
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<tbody>
<tr>
<td>WT</td>
<td>Golgi and plasma membrane</td>
</tr>
<tr>
<td>GRIP</td>
<td>Trans-Golgi</td>
</tr>
<tr>
<td>S17</td>
<td>Cis-Golgi</td>
</tr>
<tr>
<td>Mito</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear-targeted eNOS</td>
</tr>
<tr>
<td>Neuro</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>CAAX</td>
<td>Plasma membrane</td>
</tr>
</tbody>
</table>

WT, wild type; GRIP, 87 amino acid targeting sequence derived from Golgin-97; S17, 17 amino acid transmembrane sequence derived from syntaxin-3; and CAAX, 15 amino acid membrane targeting sequence from K-ras.
calmodulin. Thus the calcium-independent activity of iNOS in transfected COS-7 cells presents a novel way of isolating this variable to determine whether the reduced activity of eNOS in discrete intracellular locations is due to reduced access to cofactors or substrate or calcium. iNOS was targeted to the cis and trans Golgi, mitochondria, nucleus, both Golgi and plasma membrane and the plasma membrane as outlined by the strategy in Fig. 1 and Table 1. As shown in Fig. 6A, WT murine iNOS is predominantly cytosolic, distributed throughout the cytosol, and excluded from the nucleus of transfected COS-7 cells. Attaching the first 75 amino acids of eNOS, which are responsible for both its membrane association and subcellular targeting (24), to iNOS resulted in the enzyme being transported to both the perinuclear Golgi and plasma membrane (Fig. 6B) as per eNOS. Targeting iNOS to the cis- and trans-Golgi with the S17 and GRIP motifs is confirmed by the predominant perinuclear expression and the absence of plasma membrane staining (Fig. 6, C and D, respectively). iNOS targeted to the mitochondria exhibits a classic mitochondrial staining pattern (Fig. 6E) and colocalizes with mitochondria targeted RFP and Mitotracker (data not shown). The nuclear targeting of iNOS is clearly shown in Fig. 6F and is distinct from the perinuclear, Golgi targeted RFP. The plasma membrane targeting of iNOS via fusion to the CAAX or NEURO motif is shown in Fig. 6, G and H. The iNOS constructs were fused in frame with either COOH or NH2 terminal GFP and cotransfected with either nuclear or Golgi-targeted RFP to aid in the identification of subcellular localization. The fusion of GFP to either the NH2 or COOH terminus of iNOS did not reduce the ability of the enzyme to produce NO (1135.7375 ± 82.5613 pmol/ml for WT-iNOS, 1228.6102 ± 83.5684 pmol/ml for GFP-iNOS, and 1417.0998 ± 227.3476 pmol/ml for iNOS-GFP, n = 4).

**DISCUSSION**

Within cells, the spatial separation of proteins provides an additional level of control to ensure the fidelity of signaling from the extracellular to the intracellular environment. Previously, we have shown that eNOS is subject to such spatial regulation in that targeting of eNOS to the cytoplasm, Golgi, or plasma membrane regulates both its activity and mechanism of activation within cells. Specifically, the plasma membrane-targeted eNOS is highly phosphorylated on serine 1179 (S1179) and is constitutively active. In contrast, the cis-Golgi restricted eNOS is less responsive to transmembrane calcium fluxes, but is fully activated by Akt-dependent phosphorylation of S1179 (8). In addition to these locations, eNOS has also been reported to translocate to the nucleus and to target to and regulate mitochondrial function (1, 5, 13). The goal of the current manuscript was to identify the functional consequences of a mitochondrial and nuclear location and to identify the mechanisms underlying the activity of eNOS at various locations within the cell. We found that the cytosol, trans-Golgi, mitochondria, and nucleus are inefficient microenvironments.
for NO production. To identify the underlying mechanisms, we targeted the calcium-calmodulin-independent iNOS to the same intracellular locations. This strategy isolates a single variable, as the affinities for L-arginine, and co-factors such as BH4 or NADPH, are not different between eNOS and iNOS. iNOS targeted to discrete intracellular regions of the cell behaves similarly to cytosolic iNOS and is distinctly different from the activity of eNOS within the same regions. These results suggest that the reduced activity of eNOS, both cytosolic and targeted to the trans-Golgi, mitochondria, and nucleus is not due to limited substrate or other co-factors, but rather due to insufficient calcium-calmodulin.

Fig. 3. Basal and calcium-activated NO release is reduced in trans-Golgi, mitochondria and nuclear targeted eNOS. WT-, S17-, GRIP-, Mito-, NLS-, Neuro-, and CAAX-targeted eNOS fusion proteins were transfected into COS cells and basal (A) and calcium-dependent (B–D) NO release was determined via NO-specific chemiluminescence. A: NO release was determined over a 24-h period and cell lysates were immunoblotted for total eNOS (bottom). B: cells were stimulated with thapsigargin (100 nM) for 20 min. C and D: COS cells were transfected with WT, GRIP, Mito, NLS, and CAAX-targeted eNOS fusion proteins. In C, cells were stimulated with ATP (5 μM) for 20 min, and in D, cells were stimulated with ionomycin (1 μM) for 20 min. Data are means ± SE (n = 5). *P < 0.05 vs. the WT enzyme.
Calcium-calmodulin is an essential requirement for NO production from all NOS isoforms. The inducible iNOS isoform tightly binds calmodulin in an almost irreversible manner. In combination with the absence of calmodulin-sensitive auto-inhibitory loops, the activity of iNOS within cells is essentially independent of calcium, a finding confirmed in transfected COS-7 cells. In contrast, eNOS and nNOS are calcium-dependent isoforms. Both genes have evolved a targeting strategy to efficiently couple enzyme activity to discrete changes in the intracellular calcium environment. eNOS is anchored to intra-

**Table 2. NOS affinities**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$Ca^{2+}$, nM</th>
<th>Arginine, μM</th>
<th>NADPH, μM</th>
<th>BH4, μM</th>
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<tr>
<td>nNOS</td>
<td>233 (36)</td>
<td>2–3 (12, 29)</td>
<td>0.2–5 (17, 31)</td>
<td>0.08 (25)</td>
</tr>
<tr>
<td>iNOS</td>
<td>0 (36)</td>
<td>2–16 (18)</td>
<td>0.3–4 (17, 40)</td>
<td>0.1–1.3 (7, 14)</td>
</tr>
<tr>
<td>eNOS</td>
<td>300 (26)</td>
<td>2–5 (22, 26)</td>
<td>0.7–10 (23, 26, 34)</td>
<td>0.2–0.3 (23)</td>
</tr>
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</table>

nNOS, neuronal nitric oxide synthase; iNOS, inducible NOS; eNOS, endothelial NOS. Numbers in parentheses are references in the text.
cellular membranes via a combination of myristoylation, palmitoylation, and possibly polybasic domains (13, 16). The targeting of eNOS to these intracellular domains facilitates the efficient coupling of enzyme activity to intracellular second messengers as evidenced by the reduced NO release seen from cells expressing mistargeted acylation-deficient mutants (23, 37). Similarly, nNOS is concentrated at synaptic junctions and motor endplates via the interaction of its NH2-terminal PDZ domain to adapter proteins such as PSD93/95, CAPON, and \( \gamma \)-syntrophin (20, 41). The central dogma of eNOS targeting is that eNOS must target plasma membrane organelles such as caveolae or lipid rafts to be fully active. Consistent within this theme, plasma membrane-targeted eNOS is highly responsive to transmembrane calcium fluxes (8). The clustering of proteins that facilitate calcium entry, such as voltage independent-calcium channels and the Na/Ca exchanger within these organelles (38) and the detection of higher concentrations of calcium at the plasma membrane (19, 21, 33) strengthen this concept. When stimulated with ionomycin, which produces larger calcium transients than thapsigargin or the G protein-coupled receptor agonist, ATP, the difference between the WT enzyme and the targeted eNOS constructs was reduced, suggesting that calcium is the key variable influencing eNOS activity. In addition, the data showing that the activity of iNOS is invariant when targeted to the plasma membrane and peripheral aspects of the Golgi is also consistent with this concept and argues against the intracellular limitation of substrate or cofactors.

The reduced activity of eNOS within the nucleus suggests that a physiological role of eNOS within this location is unlikely, except perhaps as a mechanism to silence eNOS activity. Similarly, the inner leaflet of the mitochondria is a less efficient site of NO production and the possibility remains that mitochondrial production of NO is more effective on the peripheral membranes of this organelle (13). Despite their close proximity, it is not clear why substantial differences in eNOS activity occur between the trans- and cis-Golgi-targeted eNOS constructs. The local concentration of calcium within the microenvironment of the perinuclear/Golgi is not known; however, the ability of iNOS to produce equivalent amounts of NO at both locations suggests that there are significant differences.
In transfected COS cells, iNOS-GFP displays a predominantly cytosolic distribution, which is in agreement with the localization of iNOS reported in many other cell types (3, 35). iNOS immunoreactivity has also been reported in perinuclear organelles, peroxisomes, and the plasma membrane (3, 39). The significance of iNOS in these locations is poorly understood and it is not clear whether intracellular location facilitates NO synthesis or whether it has a functional or protective role. Evidence for the latter comes from studies demonstrating the colocalization of iNOS and catalase in peroxisomes, where the presence of antioxidant enzymes has been proposed to reduce iNOS-derived oxidative stress (3) and from our current findings, which to a large extent, demonstrates that iNOS functions independently of its intracellular location. The invariant activity of iNOS also suggests that the concentrations of NOS substrates and cofactors are sufficient throughout the cell to support NO activity. However, within the lumen of the Golgi both eNOS and iNOS activities were significantly reduced suggesting that one and more factors essential for NOS activity is deficient.

In conclusion, we have shown that targeted expression of eNOS within the cell dictates the calcium-dependent activation of the enzyme. Restricted expression of eNOS at the plasma membrane produces an enzyme that is highly active in response to the elevation of intracellular calcium and subsequent transmembrane calcium fluxes. The activity of the cis-Golgi eNOS was comparable to the WT enzyme, whereas eNOS targeted to the same locations, produced equivalent levels of NO as the WT enzyme. Therefore, the proximity of eNOS to local pools of NO, WT enzyme. Thus the proximity of eNOS to local pools of NO synthesis or whether it has a functional or protective role. Evidence for the latter comes from studies demonstrating the colocalization of iNOS and catalase in peroxisomes, where the presence of antioxidant enzymes has been proposed to reduce iNOS-derived oxidative stress (3) and from our current findings, which to a large extent, demonstrates that iNOS functions independently of its intracellular location. The invariant activity of iNOS also suggests that the concentrations of NOS substrates and cofactors are sufficient throughout the cell to support NO activity. However, within the lumen of the Golgi both eNOS and iNOS activities were significantly reduced suggesting that one and more factors essential for NOS activity is deficient.

In conclusion, we have shown that targeted expression of eNOS within the cell dictates the calcium-dependent activation of the enzyme. Restricted expression of eNOS at the plasma membrane produces an enzyme that is highly active in response to the elevation of intracellular calcium and subsequent transmembrane calcium fluxes. The activity of the cis-Golgi eNOS was comparable to the WT enzyme, whereas eNOS expressed in the trans-Golgi, mitochondria, and nucleus displayed significantly reduced capacity to produce NO. The reduced NO release seen with the trans-Golgi, mitochondrial, and nuclear-targeted eNOS cannot be due to insufficient cofactors or substrate as the calcium-independent iNOS, targeted to the same locations, produced equivalent levels of NO as the WT enzyme. Thus the proximity of eNOS to local pools of intracellular calcium is the major factor governing the synthesis of NO.

REFERENCES


