 Functional Relevance of Golgi- and Plasma Membrane–Localized Endothelial NO Synthase in Reconstituted Endothelial Cells

Qian Zhang, Jarrod E. Church, Davin Jagnandan, John D. Catravas, William C. Sessa, David Fulton

Objective—We have previously shown in COS-7 cells that targeting of endothelial NO synthase (eNOS) to the Golgi or plasma membrane (PM) regulates the mechanism and degree of eNOS activation. However, little is known about the functional significance of eNOS targeting in endothelial cells (ECs). The goal of the current study was to isolate these 2 pools of enzyme in ECs and determine their functional significance in response to agonist stimulation and manipulation of membrane cholesterol levels.

Methods and Results—Using an RNA interference strategy, we generated stable populations of ECs that had >90% inhibition of eNOS expression and lacked the ability to produce NO. Reconstitution of these eNOS “knockdown” ECs with Golgi- and PM-targeted eNOS restored the ability of ECs to produce NO. Calcium-dependent agonists were the more efficient stimulus for the PM-restricted eNOS in ECs. In contrast, Golgi eNOS was less responsive to both calcium- and Akt-dependent agonists. eNOS restricted to the PM was more sensitive to manipulation of membrane cholesterol levels and was significantly attenuated by modified low-density lipoprotein.

Conclusions—Within ECs, the PM is the most efficient location to produce NO but is more vulnerable to cholesterol levels and modified low-density lipoprotein. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: eNOS ■ cholesterol ■ Golgi ■ plasma membrane ■ RNAi.

Endothelium-derived NO is an important regulator of cardiovascular homeostasis. The amount of NO produced by the endothelium is tightly controlled by transcriptional and post-translational control of endothelial NO synthase (eNOS). Although numerous studies have shown that eNOS has a protective role in vascular remodeling and atherosclerosis, others have shown that dysregulation of eNOS can exacerbate atherosclerotic lesions.

The activity of eNOS is regulated by numerous post-translational modifications, including protein–protein interactions, phosphorylation, and subcellular location. The phosphorylation of eNOS, and in particular the phosphorylation of S1179 by the protein kinase Akt, increases enzyme activity by enhancing reductase activity and calcium sensitivity. When restricted to the PM, eNOS is constitutively phosphorylated and highly active. In contrast, Golgi eNOS is hypophosphorylated and produces less NO in response to calcium-dependent agonists. Coexpression of active Akt increases the phosphorylation and activity of the Golgi eNOS but does not influence the activity of the constitutively phosphorylated PM eNOS. These results suggest that in ECs, eNOS at the PM would be more responsive to agonists that elicit large calcium transients and that agonists selectively activating the kinase Akt could preferentially activate the Golgi pool of eNOS. However, the functional significance of eNOS targeting in ECs is not known.

The modulation of membrane cholesterol and exposure of ECs to oxidized low-density lipoprotein (LDL) have been shown to reduce eNOS activity by displacing eNOS from the PM to intracellular sites. Therefore, our goals for the current study were to determine the functional significance of eNOS targeting in ECs and to establish whether manipulation...
of membrane cholesterol or exposure to oxidized LDL differentially influences eNOS activity in the Golgi or PM.

Methods

Cell Culture and Transfection

COS-7 cells and bovine aortic ECs (BAECs) were grown in DMEM containing 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% FCS. BAECs were harvested in-house and passaged from primary cultures. COS-7 cells and BAECs were transfected using Lipofectamine 2000 (Invitrogen).

RNA Interference Screening

RNA interference (RNAi) targets were selected, and small interfering RNA expression cassettes were generated by polymerase chain reaction (Ambion). Six targets were selected using the following primers: (1) 5′-AACTCACAAAGTTCTTCACGCGGAGG-GAACCCTTCTCTTCTTTCCACAAATGTCCTCGTGATAGCGC-3′; (2) 5′-ATGTCACAAAAAGATGACATTGAGAGCAAAGGCACCGCT-3′; (3) 5′-GCCCTACAAAGGCGTACAGGATGTGTTGTGCCTGCTTGGTGGTGGTGTGTTGTTTGTTACCTCAACTGGCTTGATAGCGC-3′; (4) 5′-TCTCAGCAAAAAATGTCCTCGTGATAGCGC-3′; (5) 5′-GCCAGCAGACCTTTCACAAAAGACT-3′.

The mutated eNOS fragment was subsequently subcloned into WT, S17, or calcium-insensitive eNOS constructs. BAECs expressing different eNOS constructs were stimulated with the agonists thapsigargin (100 nmol/L), angiopeptin (50 ng/mL), or insulin (200 nmol/L) for 25 minutes. Net NO release was calculated by NO-specific chemiluminescence after subtracting unstimulated basal release as described previously.

Generation of Subcellular eNOS-Targeting Fusion Proteins

The cDNAs encoding the eNOS fusion proteins that target specifically the Golgi or the PM have been described previously. The eNOS construct impervious to the RNAi (Δ45/Δ14 eNOS) identified by Chen et al. was generated in the eNOS construct containing the mutated regions were generated by polymerase chain reaction using the following primers: 5′-GAAGCGATCTTTGACCTTCAATGCACTCCATCGAGGGAGAAGCTTTTTCCAAAAAAAGATGACATTGAGAGCAAAGGCGACCGCTTCTCAGCAAAAAATGTCCTCGTGATAGCGC-3′ and 5′-GATTTCAACAAGATCCTAGGCTTCTCAGGTTGTTCTGTGTGTTGTTGGTGGTGTGTTGTTTACCTCAACTGGCTTGATAGCGC-3′ for the Δ45 mutation; 5′-GATGCGGCTCCTGCGAGGAAACGTTGAGGGGAAAAAATGTCCTGAAGGCTTTTTCCAAAAAAAGATGACATTGAGAGCAAAGGCGACCGCTTCTCAGCAAAAAATGTCCTCGTGATAGCGC-3′; 5′-CGGCGAACCTTTCACAAAAGACT-3′.

The mutated eNOS fragment was subsequently subcloned into WT, S17, or calcium-insensitive eNOS constructs. BAECs expressing different eNOS constructs were stimulated with the agonists thapsigargin (100 nmol/L), angiopeptin (50 ng/mL), or insulin (200 nmol/L) for 25 minutes. Net NO release was calculated by NO-specific chemiluminescence after subtracting unstimulated basal release as described previously.

Retroviral Generation and Transduction

Retroviruses were generated encoding either an irrelevant sequence (Negative) or 3122 (Clonetics), BAECs were seeded at a density of 1.3×10⁶ cells/100-mm dish and infected with ~10⁶ cfu/mL of active retrovirus. Twenty-four hours later, cells were selected for viral uptake using the antibiotic puromycin (0.8 μg/mL) for 10 to 14 days. During the process of puromycin selection, BAECs were maintained in a subconfluent state.

NO Release

Thirty-six hours after transfection or viral transduction, cells were incubated with serum-free medium for 45 minutes (iNOS- and calcium-insensitive eNOS constructs). BAECs expressing different eNOS constructs were stimulated with the agonists thapsigargin (100 nmol/L), angiopeptin (50 ng/mL), or insulin (200 nmol/L) for 25 minutes. Net NO release was calculated by NO-specific chemiluminescence after subtracting unstimulated basal release as described previously.

Live Cell Imaging

Retroviral “knockdown” BAECs were seeded at a density of 2.5×10⁵ cells/cm² well dish and transfection with cDNAs encoding fusion proteins of enhanced green fluorescent protein (EGFP) and monomeric RFP (Roger Tsien, UCSD) as described above. Twenty-four to 48 hours later, cells were reseeded onto glass-bottomed culture dishes (MatTek). All imaging was performed using the LSM 510 Meta 3.2 Confocal Microscope (Zeiss). Magnification power was set at ×40 with oil.

Cholesterol Extraction and Supplementation

Cholesterol was prepared as a 50 mmol/L stock in 100% ethanol. Methyl-β-cyclodextrin (CD) was dissolved in warm DMEM, and cholesterol dissolved in ethanol was added to make a final concentration of 4.5 mmol/L CD +0.5 mmol/L cholesterol (9:1) solution. COS cells or BAECs were seeded at a density of 2.5×10⁴ cells/3.8 cm², and 36 hours after transfection or after viral transduction, cells were incubated in serum-free medium containing 5 mmol/L CD to extract membrane cholesterol or CD cholesterol complex (4.5 mmol/L CD +0.5 mmol/L cholesterol; 9:1) to load cholesterol into membranes for 1 hour. After incubation, cells were washed with warm medium and stimulated with ionomycin (1 μmol/L) as described above.

LDL Oxidation

Modified LDL (modLDL) was prepared by incubating fresh human LDL (EMD Biosciences) with 10 μmol/L of CuSO₄ at 37°C for 16 hours as described previously. BAECs were seeded at a density of 2.5×10⁴ cells/3.8 cm². Thirty-six hours after viral transduction, cells were incubated in serum-free medium containing 50 μg/mL
modLDL for 40 minutes, then washed with warm medium and
stimulated with ionomycin (1 μM) as described above.

Statistical Analysis
Data are expressed as means ± SEM. Comparisons were made using
ANOVA with a post hoc test. Differences were considered signifi-
cant at *P < 0.05.

Results
Screening and Specificity of RNAi Sequences
To determine the most effective sequence for silencing eNOS expression, we screened 6 potential RNAi sequences. COS-7 cells were cotransfected with a fixed concentration of eNOS (100 ng) and increasing concentrations of RNAi expression cassettes (10 to 300 ng). The relative inhibition of eNOS expression was determined via Western blotting. As shown in Figure 1A, 1 sequence (3122) was significantly more effec-
tive at silencing eNOS expression. This sequence at a
concentration of only 30 ng inhibited eNOS expression by
50% and was comparable to 300 ng of the other effective
RNAi sequences (234, 1545, and 2304; data not shown). The
3122 sequence (Figure 1B) is also compatible with published
human and porcine eNOS sequences. To control for possible
off-target effects of this sequence, we performed a series of
basic local alignment search tool (BLAST) searches using the
full-length 21-nt sequence and truncated 5’ and 3’ ends and
found that virtually all of the sequences returned were eNOS
genes from other species. To generate an eNOS construct
imperious to RNAi-mediated gene silencing, several silent
mutations were generated in the open reading frame of
WT-eNOS. These mutations, while changing the nucleotide
sequence, do not alter the amino acid coding for eNOS
(Figure 1B). To verify that the most effective anti-eNOS
RNAi sequence (3122) does not inhibit the expression of
mutated eNOS, the 2 constructs were coexpressed as de-
scribed above. As shown in Figure 1C, the expression of the
mutated eNOS was not inhibited by 10 to 300 ng of RNAi
3122. Therefore, having shown that it is feasible to circum-
vent the inhibition of endogenous eNOS with RNAi, our next
goal was to restore eNOS expression in BAECs.

Generation of a Stable Population of
“Knockdown” ECs
To generate a stable population of BAECs with greatly
diminished levels of endogenous eNOS (“knockdown”), we
used a retroviral RNAi expression system to deliver RNAi
sequences for eNOS (3122) to BAECs. As shown in Figure 2A, ECs expressing the
3122 RNAi sequence exhibit significantly less eNOS expres-
sion relative to cells transduced with an irrelevant sequence (Negative). Consistent with these findings, the
ability of thapsigargin to elicit NO release from these cells
was virtually abolished (Figure 2A). These cells retained the
cobblestone EC morphology (Figure I, left versus right
panels, available online at http://atvb.ahajournals.org) and
phenotype as determined by the equivalent presence of an EC
marker, the Tie-2 receptor (Figure 2A, bottom panel). Fur-
thermore, these cells retained the ability to respond to
different endothelial specific agonists, as shown by the ability
of both vascular endothelial growth factor (VEGF) and the
Tie-2 ligand angiopoietin to stimulate equivalent Akt phos-
phorylation (Figure 2B).

It has also been reported that RNAi sequences can induce
the intracellular interferon-triggered Jak/Stat signaling path-
way. To address this, we determined the activation state of

the Jak/Stat signaling axis by measuring the levels of Jak/Stat phosphorylation using Western blotting in ECs stably expressing RNAi. We found no difference in the phosphorylation of these molecules between BAECs exposed to a control retrovirus encoding GFP or RNAi 3122 (data not shown).

Subcellular Targeting and Activation of Golgi and Plasma eNOS in ECs

Subconfluent eNOS “knockdown” ECs were transfected with cDNAs encoding eNOS-GFP, GFP-eNOS-CAAX, or GFP-eNOS-S17 together with a nuclear-driven RFP fusion protein, and live cells were visualized for GFP or RFP using confocal microscopy (A through C). A, WT eNOS is present at the PM and Golgi (arrow). B, Golgi targeting of eNOS-S17. C, eNOS-CAAX displays prominent peripheral membrane staining. D, Cells were exposed to calcium-dependent (thapsigargin, 100 nmol/L) and Akt-dependent (angiopeptin 50 ng/mL and insulin 200 nmol/L) agonists for 25 minutes, and NO release was measured via chemiluminescence. Relative expression of eNOS constructs were determined via Western blot with hsp90 and caveolin-1 (cav-1) used as loading controls (bottom panel). The data are presented as mean ± SE (n = 6). *P < 0.05 vs the unstimulated control; +P < 0.05 vs the WT enzyme.

Mechanisms Underlying Differences Between Golgi (S17)- and PM (CAAX)-Targeted eNOS in BAECs

To identify mechanisms that account for the differences between Golgi and PM eNOS, we first investigated whether there are differences in eNOS phosphorylation. As shown in Figure 4A, the PM eNOS is more heavily phosphorylated on serines (S) 116, 617, 635 and S1179 and threonine (T) 497 was determined via Western blot as shown in A, B, “Knockdown” BAECs were transduced with viruses encoding Golgi and PM eNOS with or without constitutively active Akt or control LacZ (50 mois). NO release was measured by chemiluminescence, and the relative expression of phosphorylated and nonphosphorylated proteins were determined via Western blot. The data are presented as mean ± SE (n = 6). *P = 0.05. C, “Knockdown” BAECs expressing either Golgi (S17) or PM (CAAX) eNOS were lysed, and the various forms of eNOS were immunoprecipitated (IP), immunoprecipitated proteins were immunoblotted with antibodies against eNOS, hsp90, or caveolin-1. Results are representative of 2 independent experiments.

Figure 3. Subcellular targeting and agonist activation of Golgi- and PM-targeted eNOS in “knockdown” BAECs. Retroviral eNOS “knockdown” BAECs were cotransfected with WT eNOS-GFP, GFP-eNOS-CAAX, or GFP-eNOS-S17 together with a nuclear-driven RFP fusion protein, and live cells were visualized for GFP or RFP using confocal microscopy (A through C). A, WT eNOS is present at the PM and Golgi (arrow). B, Golgi targeting of eNOS-S17. C, eNOS-CAAX displays prominent peripheral membrane staining. D, Cells were exposed to calcium-dependent (thapsigargin, 100 nmol/L) and Akt-dependent (angiopeptin 50 ng/mL and insulin 200 nmol/L) agonists for 25 minutes, and NO release was measured via chemiluminescence. Relative expression of eNOS constructs were determined via Western blot with hsp90 and caveolin-1 (cav-1) used as loading controls (bottom panel). The data are presented as mean ± SE (n = 6). *P < 0.05 vs the unstimulated control; +P < 0.05 vs the WT enzyme.

Figure 4. Mechanisms underlying the differences in activity of Golgi and PM eNOS. Retroviral eNOS “knockdown” BAECs were transduced with adenoviruses encoding Golgi (S17) or PM (CAAX), and the degree of eNOS phosphorylation on serines (S) 116, 617, 635 and S1179 and threonine (T) 497 was determined via Western blot as shown in A, B, “Knockdown” BAECs were transduced with viruses encoding Golgi and PM eNOS with or without constitutively active Akt or control LacZ (50 mois). NO release was measured by chemiluminescence, and the relative expression of phosphorylated and nonphosphorylated proteins were determined via Western blot. The data are presented as mean ± SE (n = 6). *P < 0.05 vs the unstimulated control; +P < 0.05 vs the WT enzyme.

The equal expression of eNOS transgenes was confirmed by Western blot as shown in Figure 4D (bottom panel).
Effect of Cholesterol and modLDL on the Activity of Golgi and PM eNOS

Retroviral eNOS “knockdown” ECs were reconstituted with adenoviruses encoding Golgi and PM eNOS fusion proteins. Thirty-six hours later, cells were treated with regular medium containing 5 mmol/L CD to extract membrane cholesterol or CD–cholesterol complexes to load more cholesterol into cellular membranes. Manipulation of cellular cholesterol levels had the greatest impact on the PM eNOS. As shown in Figure 5A, CD significantly reduced NO release from Golgi (S17)- targeted eNOS (40% inhibition), whereas cholesterol supplementation enhanced its activity 2-fold. In contrast, the Golgi pool of eNOS was not significantly affected. Golgi- and PM eNOS–reconstituted BAECs were also incubated in serum-free medium containing 50 μg/mL modLDL for 40 minutes followed by agonist stimulation (1 μmol/L ionomycin). As shown in Figure 5B, NO production from the PM targeting eNOS construct (CAAX) was significantly decreased after short-term exposure to modLDL compared with the Golgi (S17)- targeted eNOS. To address potential mechanisms underlying the increased vulnerability of the PM eNOS, we determined the relative phosphorylation state of Golgi and PM eNOS under the various conditions. Manipulation of membrane cholesterol did not modify the phosphorylation of S1179 on either the Golgi- or PM-targeted eNOS in unstimulated cells or in the presence of active Akt (Figure IIIA and IIIB, available online at http://atvb.ahajournals.org). We also examined whether cholesterol modifies the relative binding of hsp90 to PM eNOS. As shown in Figure IIIC, there was no significant difference in the association of hsp90 with eNOS in cholesterol-loaded or depleted BAECs. In Figure IIID, we determined whether cholesterol affects the subcellular targeting of PM eNOS to cholesterol-enriched membrane microdomains using sucrose gradient centrifugation. However, there was no significant redistribution of PM eNOS to light or heavy membrane fractions.

Manipulation of Membrane Cholesterol Modifies Calcium-Dependent Activation of PM eNOS

Given that the PM eNOS is highly sensitive to transmembrane calcium fluxes, we next addressed whether cholesterol selectively modifies the activity of PM eNOS by altering the levels of intracellular calcium. BAECs were transduced with adenoviruses encoding cytosolic and PM-targeted aequorin calcium sensors. Cholesterol loading or depletion in BAECs did not influence the relative exposure of cytosolic or PM probes to calcium under basal conditions (data not shown) or after ionomycin stimulation (Figure 6A and 6B). To address whether the inherent calcium sensitivity of the PM eNOS had changed, “knockdown” BAECs were transfected with cDNAs encoding novel calcium/calmodulin-insensitive eNOS fusion proteins targeted to both the Golgi and PM. The calcium-insensitive eNOS produces NO constitutively, and activity is not significantly modified by chelation of extracellular calcium with EGTA or elevation with ionomycin.20 These eNOS constructs also contained the silent mutations described in Figure 1 to avoid RNAi. Thirty-six hours after reconstitution of eNOS, CD or CD–cholesterol complexes did not significantly influence NO production from Golgi- or PM-targeted calcium-insensitive eNOS (Figure 6C). Identical results were obtained with the calcium-independent NOS isoform iNOS (data not shown).

Discussion

The presence of eNOS has been reported within PM caveolae and also the perinuclear/Golgi region both in vivo and in vitro.11–13,17 Because of the existence of these 2 distinct enzyme pools, it is difficult to dissect the regulation and significance of each pool of eNOS to overall NO release in ECs. The central dogma is that for eNOS to be fully functional it must reside within caveolae or lipid rafts at the PM. The function, if any, of the perinuclear/Golgi pool of eNOS in ECs has remained an enigma. To address this
hypothesis, the goal of this study was to functionally separate eNOS into Golgi and PM pools in ECs.

Our initial approach was to use cultured ECs from eNOS−/− mice. However, in our hands without immortalization, these cells do not grow well in culture and rapidly lose phenotypic markers and EC morphology. Therefore, we adopted an alternative approach by generating BAECs that were devoid or had greatly reduced levels of endogenous eNOS. To achieve this, we created stable populations of ECs “knockdown” ECs using retrovirus-mediated integration of anti-eNOS RNAi. To replace the endogenous eNOS with a Golgi- or PM-restricted eNOS fusion protein, we created several silent mutations that circumvented the RNAi-mediated eNOS inhibition. The mutated eNOS constructs, which have the same amino acid sequence as the endogenous eNOS, were then expressed in “knockdown” ECs using adenovirus.

In these reconstituted ECs, we found that the PM-restricted eNOS was much more responsive to transmembrane calcium fluxes compared with the WT- and Golgi-restricted pools of eNOS. These results are consistent with those reported in COS cells13 and support the concept that the PM is an optimal location for eNOS activity. However, the Akt-dependent agonists angiopoietin and insulin did not show a preference for the Golgi pool of eNOS and released the most NO from the PM eNOS. Previously in COS cells, we reported that constitutively active Akt preferentially activated only the Golgi pool of eNOS. To determine whether BAECs respond similarly to Akt, we coexpressed constitutively active Akt with Golgi and PM eNOS. We found that in the “knockdown” BAECs, Akt elicits the greatest amount of NO release from PM eNOS. Although these findings are at odds with those reported in COS cells, they provide an explanation for the greater ability of angiopoietin and insulin to activate the PM eNOS. Interestingly, we also found that PM-restricted eNOS binds to significantly more hsp90 than the Golgi eNOS.

Previous studies have shown that depletion of membrane cholesterol impairs caveolae structure, induces eNOS translocation from caveolae to intracellular locations, and inhibits eNOS activity.18 In addition, oxidized LDL can also modify eNOS subcellular targeting and inhibit NO release.18,19 However, the mechanisms by which modification of membrane cholesterol and oxidized LDL influence eNOS activity are not fully understood, particularly in light of the ability of Golgi or intracellular eNOS to produce equivalent amounts of NO.13 Therefore, eNOS “knockdown” ECs were reconstituted with Golgi- and PM-targeted calcium/calmodulin-independent eNOS (C) in the presence and absence of CD or cholesterol for 1 hour. NO release (45 minutes) was measured by chemiluminescence and the relative expression of eNOS and hsp90 determined by Western blotting. The data are presented as mean±SE (n=4 to 8). Retroviral “knockdown” ECs were transfected with Golgi- and PM-targeted calcium/calmodulin-independent eNOS (C) in the presence and absence of CD or cholesterol for 1 hour. NO release (45 minutes) was measured by chemiluminescence and the relative expression of eNOS and hsp90 determined by Western blotting. The data are presented as mean±SE (n=6).

Figure 6. Changes in membrane cholesterol do not affect cytosolic or membrane calcium levels or activity of a calcium/calmodulin-independent eNOS construct. A and B, CYT-AEQ (HA-AEQ) and PM-AEQ (CD8-AEQ) were expressed in BAECs via adenovirus. AEQ was reconstituted by incubation with coelenterazine (5 μmol/L; 1 hour) in the presence and absence of 5 mmol/L methyl-β-CD or a CD–cholesterol complex (Cholesterol, 4.5 mmol/L CD +0.5 mmol/L cholesterol), and cells were stimulated with ionomycin (1 μmol/L) in DMEM containing 1.5 mmol/L free Ca2+. Depletion of the coelenterazine pool was measured by determining Lmax at 0, 5, or 30 minutes. Data are presented as means±SE (n=4 to 8). Retroviral “knockdown” ECs were transfected with Golgi- and PM-targeted calcium/calmodulin-independent eNOS (C) in the presence and absence of CD or cholesterol for 1 hour. NO release (45 minutes) was measured by chemiluminescence and the relative expression of eNOS and hsp90 determined by Western blotting. The data are presented as mean±SE (n=6).
tivity of the PM eNOS to calcium/calmodulin. Because we detected no significant difference in calcium levels under these conditions, we hypothesize that changes in the association of calmodulin must account for these differences.

In summary, we successfully used an RNAi strategy to generate a stable population of eNOS “knockdown” ECs and reconstituted these cells with eNOS fusion proteins that specifically target the Golgi and PM. In response to the Akt-dependent agonists insulin and angiopeitcin, ECs produced very little NO and did not show a preference for the Golgi pool of eNOS. In response to thapsigargin, which elevates intracellular calcium, the PM eNOS produced substantially more NO than WT- or Golgi-localized eNOS. However, the PM eNOS was more vulnerable to changes in membrane cholesterol and to modLDL. CD significantly reduced and CD–cholesterol complexes significantly increased NO release from PM eNOS but did not affect the activity of Golgi eNOS. Similarly, modLDL substantially reduced the activity of PM eNOS but not Golgi eNOS. The molecular mechanisms by which eNOS activity is impaired in the endothelial dysfunction associated with atherosclerosis are not fully understood. However, the ability of cholesterol and modLDL to selectively influence the activity of PM eNOS elevates the significance of eNOS subcellular targeting in vivo and raises the important question of whether a Golgi-targeted eNOS would offer more vascular protection than a PM eNOS in models of endothelial dysfunction.

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References

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Supplemental Fig. I.
Representative phase-contrast pictures of confluent native (top panel, A) and retrovirus transduced eNOS "knockdown" endothelial cells (lower panel, B).
Supplemental Fig. II.
Activation of eNOS by different agonists. eNOS "knockdown" endothelial cells were reconstituted with WT, Golgi (S17) and PM (CAAX) restricted eNOS via adenoviruses (MOI of 100) and stimulated with VEGF (50ng/ml) and bradykinin (1mM). The data are presented as mean +/- S.E. (n=6).
Supplemental Fig III.
eNOS "knockdown" BAEC were transduced with adenoviruses for Golgi(S17, A) or PM(CAAX, B) eNOS with or without myr-Akt in the presence or absence of cholesterol or CD. The relative level of phosphorylated eNOS (S1179), Akt and hsp90 (loading control) were determined by Western blot. In C, "knockdown" BAECs were transduced with PM (CAAX) eNOS and eNOS was immunoprecipitated from cells treated as described and then immunoblotted for eNOS, hsp90 and caveolin-1. In D, subcellular fractionation of "knockdown" BAEC expressing PM (CAAX) eNOS. BAECs were processed as described using sucrose gradient centrifugation and equal volumes of each fraction were immunoblotted for eNOS and caveolin-1 (cav-1).