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. Within endothelial cells (ECs), eNOS is localized to the plasma membrane (PM) caveolae and lipid rafts and is also found on intracellular membranes such as the Golgi complex. Mislocalization of eNOS attenuates both agonist-stimulated NO release and eNOS phosphorylation, suggesting that the proper subcellular localization is critical for stimulus-dependent phosphorylation and activation of the enzyme. Within the endothelium of blood vessels, eNOS is also present at both the Golgi and PM, and across vascular beds, differences in eNOS abundance between Golgi and PM suggest a functional role of subcellular targeting.

In a reconstituted system in COS-7 cells, we have recently shown that the subcellular location of eNOS has a profound effect on its ability to produce NO. When restricted to the PM, 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;26:1015-1021.)

Key Words: eNOS † cholesterol † Golgi † plasma membrane † RNAi
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) 234: Sense: 5'-AACCTACACAAATGTCCTACCAGGG-GAACCGGTGTTTCGTTCC TACCAAG-3' Antisense: 5'-CG-GGCAAGCTTTTTCCAAAAAACGCTATCA CGAGGAC-3'; (2) 1590: Sense: 5'-CGCGGAACGTCTTTTTCCAAAAAGATG-3' Antisense: 5'-CGCGGAACGTCTTTTTCCAAAAAGATG-3'; (3) 3122: Sense: 5'-GCCCTACCAAAAGGCGTACAGGATGGTT- GCTCGGGTTGGTTCGTT TTCCACAAG-3' Antisense: 5'-CGCGGAACGTCTTTTTCCAAAAAGATG-3'; (4) 2308: Sense: 5'-GTCTCTACACAAAGGA- CTTGGGCTGAAATCCACCTTTGCTCT TTCCAACAG-3' Antisense: 5'-CGCGGAACGTCTTTTTCCAAAAAGATG-3'; (5) 3122: Sense: 5'-GCATGACATTGAGAGCAAAGGCGAACCTTTGCTC- ATATCGTGC-3' Antisense: 5'-GCATGACATTGAGAGCAAAGGCGAACCTTTGCTC- ATATCGTGC-3'; (6) 3493: Sense: 5'-GCCCTACCAAAAGGCGTACAGGATGGTT- GCTCGGGTTGGTTCGTT TTCCACAAG-3' Antisense: 5'-CGCGGAACGTCTTTTTCCAAAAAGATG-3'. The negative control (NEGATIVE) was obtained from Ambion (Silencer validated Negative small interfering RNA Control 1).

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.12 To generate an eNOS construct impervious to the RNAi 3122, several silent mutations, which do not change the amino acid sequence, were generated in the wild-type (WT) eNOS using the following primers: Sense: 5'-GCATGACATTGAGAGCAAAGGCGAACCTTTGCTC- TCAATGTCA TGC-3' Antisense: 5'-GCATGACATTGAGAGCAAAGGCGAACCTTTGCTC- TCAATGTCA TGC-3' 

Calcium-Insensitive eNOS Fusion Proteins
Calcium-insensitive eNOS constructs were based on constructs (Δ45/Δ14eNOS) identified by Chen et al.21 Autoinhibitory control elements present on eNOS (594-606/614-645 [Δ45] and 1165-1178 [Δ14]) but not inducible NOS (iNOS) were deleted. cDNA fragments containing the mutated regions were generated by polymerase chain reaction using the following primers: 5'-GAGG-CGATCTTGTAACTCTTCATCTCCATCGAGGAGCAG-3' and 5'-GAGTACAAAGTACGGCCTTCAGGTGTGTTTGC- CGGACTGGGC-3' for the Δ45 mutation; 5'-GATGCAGGCTCCTGCGAGGGAAACCTGAGGCCAAATGGTC-3' and 5'-CCTCAAGTTCTTCCACTGAGGCAACGCTC-3' for the Δ14 mutation.

Generation of Golgi- and PM-Targeted Calcium-Insensitive eNOS Fusion Proteins
Cytosolic- and PM-targeted (CD8-aequorin) aequorin proteins encoding the various eNOS constructs were generated using the Ad-Easy system. DNA sequences were verified by automated DNA sequencing (Genomics Core Facility; Medical College of Georgia).

Ca²⁺ Measurements Using Aequorin
These measurements were obtained using methods described previously.20 Cytosolic- and PM-targeted (CD8-aequorin) aequorin proteins were expressed in knockdown BAECs via adenovirus, and the relative exposure of these probes to calcium over time was calculated as a ratio to the total amount of aequorin remaining in each well under the different conditions (Lmax). Aequorin and its cofactor coelenterazine react in the presence of calcium to emit a photon. This reaction results in the permanent oxidation of coelenterazine to coelenteramide, and therefore the emission of light in response to calcium is a once-only reaction. Thus, the size of the available pool of luminescence decreases constantly over time in direct proportion to the amount of calcium present. We therefore interpreted the decrease in Lmax as being indicative of greater exposure to calcium over time.

Retroviral Generation and Transduction
Retroviruses were generated encoding either an irrelevant sequence (Negative) or 3122 (Clonetech). 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 μmol/L), angiopeitin (50 ng/mL), or insulin (200 mmol/L) for 25 minutes. Net NO release was calculated by NO-specific chemiluminescence after subtracting unstimulated basal release as described previously.7

Live Cell Imaging
Retroviral “knockdown” BAECs were seeded at a density of 2.5×10⁴ cells/3.8 cm² well dish and transfected 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 replated 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 2.5×10⁶ 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.22 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.
hours as described previously. BAECs were seeded at a density of 2.5 × 10⁵ 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 μmol/L) as described above.

Statistical Analysis
Data are expressed as means±SEM. Comparisons were made using ANOVA with a post hoc test. Differences were considered significant 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. A, Cells were cotransfected with 10 to 300 ng of RNAi vs a fixed concentration of eNOS cDNA (100 ng/3.8 cm²). Relative expression of eNOS and GAPDH was determined via Western blotting. B, Strategy for bypassing RNAi inhibition: silent mutations were generated in the eNOS cDNA, which do not change the amino acid sequence. C, COS cells were cotransfected with RNAi 3122 (10 to 300 ng) vs a fixed amount of exogenous eNOS cDNA containing the silent mutations (100 ng/3.8 cm²).

Figure 1. Inhibition of eNOS expression by RNAi. RNAi sequences designed to specifically recognize endogenous eNOS mRNA were screened in COS-7 cells. A, Cells were cotransfected with 10 to 300 ng of RNAi vs a fixed concentration of eNOS cDNA (100 ng/3.8 cm²). Relative expression of eNOS and GAPDH was determined via Western blotting. B, Strategy for bypassing RNAi inhibition: silent mutations were generated in the eNOS cDNA, which do not change the amino acid sequence. C, COS cells were cotransfected with RNAi 3122 (10 to 300 ng) vs a fixed amount of exogenous eNOS cDNA containing the silent mutations (100 ng/3.8 cm²).

Figure 2. Retroviral knockdown of endogenous eNOS in BAECs with retention of EC phenotype. A, BAECs were treated with retroviruses (10⁵ cfu/mL) encoding RNAi sequences for eNOS (3122) or an irrelevant sequence (negative). Thapsigargin-stimulated NO release was measured via chemiluminescence (*P<0.05 vs negative; +P<0.05 vs unstimulated controls). The relative expression of eNOS, hsp90, and Tie-2 were determined via Western blot (bottom panels). B, Serum-starved ECs were exposed to angiopoietin and VEGF (50 ng/ml) for 10 and 5 minutes, respectively, and the phosphorylation level of Akt was determined via Western blot.

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 3122 to BAECs. As shown in Figure 2A, ECs expressing the 3122 RNAi sequence exhibit significantly less eNOS expression than untransduced cells. 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). Furthermore, 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 angioptoinetin to stimulate equivalent Akt phosphorylation (Figure 2B).
It has also been reported that RNAi sequences can induce the intracellular interferon-triggered Jak/Stat signaling pathway. 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 (WT), GFP-eNOS-CAAX (PM), and GFP–eNOS-S17 (Golgi) together with RFP-NLS (nucleus) cDNA. As shown in Figure 3A, the WT eNOS is found at both the PM and Golgi region. The PM-targeted eNOS-CAAX is found predominantly at the cell periphery (Figure 3C), and the Golgi-targeted eNOS-S17 is exclusively perinuclear (Figure 3B). The ability of these 2 separate pools of eNOS (PM versus Golgi) to respond to different stimuli was determined by stimulating the reconstructed “knockdown” BAECs with calcium-elevating agonists (thapsigargin) or agonists that activate Akt (angiopeptin, insulin). The PM-targeted eNOS-CAAX was much more sensitive to transmembrane calcium fluxes compared with WT- or Golgi-targeted S17 (Figure 3D). However, the Akt-dependent agonists did not show a preference for the Golgi pool of eNOS and angiopeptin significantly activated the PM eNOS to a greater extent (Figure 3D). The equal expression of eNOS transgenes was confirmed by Western blot as shown in Figure 3D (bottom panel).

Mechanisms Underlying Differences Between Golgi and PM eNOS
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 Serine (S) 1179, S617, and S1179 and threonine (T) 497 was determined via Western blot as shown in A. B, “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 adenoviruses encoding Golgi and PM eNOS with or without constitutively active Akt or control LacZ (50 MOI). NO release was measured by chemiluminescence, and the relative expression of phosphorylated and nonphosphorylated proteins was 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.

It has also been reported that RNAi sequences can induce the intracellular interferon-triggered Jak/Stat signaling pathway.23 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).

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Mechanisms Underlying Differences Between Golgi and PM eNOS
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 S1179, S617, and S116 compared with the Golgi eNOS. To assess whether Akt can preferentially activate the Golgi or PM eNOS, we transduced BAECs with adenoviruses encoding 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.
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Figure 5. Manipulation of membrane cholesterol levels selectively influences PM-targeted eNOS. A, “Knockdown” ECs were reconstituted with Golgi (S17)- and PM (CAAX)-restricted eNOS via adenoviruses (100 moi). Thirty-six hours after transduction, these cells were incubated in serum-free medium with 5 mmol/L methyl-β-CD or a CD–cholesterol complex (cholesterol [Chol.], 4.5 mmol/L CD + 0.5 mmol/L cholesterol) separately for 1 hour. Cells were stimulated with ionomycin (1 μmol/L; 25 minutes). NO release was measured by chemiluminescence, and relative expression of eNOS and hsp90 was determined via Western blot. The data are presented as mean ± SE (n = 6). *P < 0.05 vs the respective S17 response; ++P < 0.05 vs the control-treated CAAX cells.

B

4C, there was significantly more hsp90 bound to the PM eNOS versus the Golgi eNOS. However, there was no difference in the relative association of caveolin-1.

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 the PM 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 IID, 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 eNOS “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 cells12 and support the concept that the PM is an optimal location for eNOS activity.18 In addition, oxidized LDL can also modify the location from caveolae to intracellular locations, and inhibits calcium/calmodulin-independent (calcium/calmodulin-45/14) eNOS 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 means±SE (n=4 to 8). C, Retroviral “knockdown” ECs were transfected with Golgi- and PM-targeted calcium/calmodulin-independent (Δ45/Δ14) eNOS 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).

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 caveola 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.12 Therefore, eNOS “knockdown” ECs were reconstituted with Golgi- and PM-restricted eNOS and were exposed to CD or CD–cholesterol complexes and modLDL. We found that the PM location of eNOS was most sensitive to changes in membrane cholesterol. Removal of cholesterol with CD greatly attenuated NO release, whereas cholesterol supplementation increased activity 2-fold. In contrast, the Golgi-targeted S17 eNOS was not affected by manipulation of membrane cholesterol.

To address the mechanisms by which cholesterol modification influences the activity of the PM eNOS, we investigated a number of different post-translational controls on eNOS activity. Alterations of membrane cholesterol did not influence the phosphorylation state, protein–protein interaction, or overall membrane association of PM eNOS. Because the increased activity of PM-restricted eNOS is highly dependent on transmembrane calcium fluxes, our next hypothesis was that cholesterol was influencing the calcium-dependent activation of PM eNOS. To test this, we compared the effect of cholesterol modification on cytosolic versus PM calcium concentrations. We found no change in the basal or ionomycin stimulated calcium levels with either the PM or cytosolic aequorin probe. However, because we were seeing clear changes in the calcium-dependent activation of the PM eNOS, we next targeted a calcium/calmodulin-insensitive eNOS construct to the PM and Golgi and treated ECs with CD or CD–cholesterol complexes. No significant difference...
in NO production was observed in the calcium-independent eNOS mutants or iNOS constructs targeted to either the Golgi or the PM. Therefore, the mechanism by which cholesterol modifies PM eNOS activity relates to changes in the sensitivity 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 angiopoietin, 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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Functional Relevance of Golgi- and Plasma Membrane-Localized Endothelial NO Synthase in Reconstituted Endothelial Cells

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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).