Dissecting the Interaction between Nitric Oxide Synthase (NOS) and Caveolin

FUNCTIONAL SIGNIFICANCE OF THE NOS CAVEOLIN BINDING DOMAIN IN VIVOST

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Endothelial nitric oxide synthase (eNOS) is a dually acylated peripheral membrane protein that targets to the Golgi region and caveolae of endothelial cells. Recent evidence has shown that eNOS can co-precipitate with caveolin-1, the resident coat protein of caveolae, suggesting a direct interaction between these two proteins. To test this idea, we examined the interactions of eNOS with caveolin-1 in vitro and in vivo. Incubation of endothelial cell lysates or purified eNOS with glutathione S-transferase (GST)-caveolin-1 resulted in the direct interaction of the two proteins. Utilizing a series of GST-caveolin-1 deletion mutants, we identified two cytoplasmic domains of caveolin-1 that interact with eNOS, the scaffolding domain (amino acids 61–101) and to a lesser extent the C-terminal tail (amino acids 135–178). Incubation of pure eNOS with peptides derived from the scaffolding domains of caveolin-1 and -3, but not the analogous regions from caveolin-2, resulted in inhibition of eNOS activity and neuronal NOS (nNOS) activities. These results suggest a common mechanism and site of inhibition. Utilizing GST-eNOS fusions, the site of caveolin binding was localized between amino acids 310 and 570. Site-directed mutagenesis of the predicted caveolin binding motif within eNOS blocked the ability of caveolin-1 to suppress NO release in co-transfection experiments. Thus, our data demonstrate a novel functional role for caveolin-1 in mammalian cells as a potential molecular chaperone that directly inactivates NOS. This suggests that the direct binding of eNOS to caveolin-1, per se, and the functional consequences of eNOS targeting to caveolae are likely temporally and spatially distinct events that regulate NO production in endothelial cells. Additionally, the inactivation of eNOS and nNOS by the scaffolding domain of caveolin-3 suggests that eNOS in cardiac myocytes and nNOS in skeletal muscle are likely subject to negative regulation by this muscle-specific caveolin isoform.

Caveolae are cholesterol- and sphingolipid-rich microdomains of the plasmalemma that have been implicated in a variety of cellular functions, including transcytosis of molecules and signal transduction events (1, 2). With respect to the latter function, structurally distinct dually acylated proteins involved in signal transduction (including G-protein a subunits, Ha-Ras, Src family members, and endothelial nitric oxide synthase (eNOS)) reside in caveolae (3–6). In the case of certain Src members and eNOS, mutation of the cysteine palmitoylation sites prevents caveolae localization suggesting that palmitoylation is a “molecular zip code” for the trafficking of dually acylated proteins into glycolipid-rich microdomains of the plasmalemma (4, 6, 7).

The major coat proteins of caveolae are the caveolin family of proteins (caveolin-1, -2 and -3 (1, 8)). Besides being intimately embedded within the lipid microdomain comprising caveolae, caveolins may regulate signaling via direct interaction with other resident proteins. For example, caveolin-1 interacts with G-protein a subunits, Ha-Ras, and c-Src in intact cells and with these purified proteins as determined by co-immunoprecipitation, co-expression, or direct binding to glutathione S-transferase (GST) caveolin-fusion proteins (3, 5, 9). Utilizing the latter technique, a scaffolding domain of caveolin-1 (amino acids 82–101) is sufficient for binding G and G, and c-Src and peptides derived from this caveolin-1 region potently inhibit the auto-phosphorylation of c-Src (9). This suggests that binding to caveolin may inactivate certain signaling molecules while in caveolae and that activation of signaling would disrupt the caveolin-protein interaction either through the recruitment of additional regulatory proteins or post-translational modifications.

In this paper, we show that native eNOS from endothelial cells or recombinant eNOS purified from Escherichia coli directly interacts with caveolin-1, and the primary site of binding is the caveolin scaffolding domain. Incubation of pure eNOS, INOS, or nNOS with peptides derived from the scaffolding domains of caveolin-1 and -3, but not caveolin-2, inhibits NOS activity. Additionally, we identify the binding site for caveolin within eNOS and show that mutation of this site prevents the negative regulation of NO release mediated by caveolin co-expression. These studies demonstrate a novel role for caveolins as endogenous regulators of NOS and implicate caveolin

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1 The abbreviations used are: eNOS, endothelial nitric oxide synthase; GST, glutathione S-transferase; INOS, inducible NOS; nNOS, neuronal NOS; BLMVEC, bovine lung microvascular endothelial cells; CBD, caveolin binding domain.
proteins as generalized negative regulators of signal transduction.

MATERIALS AND METHODS

Cell Culture—Bovine lung microvascular endothelial cells (BLMVEC) and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal bovine serum, penicillin, streptomycin, and L-glutamine as described previously (6).

Purification of GST-Caveolin and GST-eNOS Fusion Proteins—Construction of the caveolin fusion proteins was performed as follows. Full-length caveolin-1 (residues 1–178, Cav-FL) and caveolin subdomains (the N-terminal domains (residues 1–61 and 61–101) and the C-terminal domain (residues 135–178)) were separately amplified by polymerase chain reaction and subcloned into the vector pGEX-4T-1 (XhoI-XhoI sites) as described previously (3). Construction of GST eNOS fusions was as follows. GST-eNOS subdomains (residues 130–310 and 310–570) were amplified by polymerase chain reaction and subcloned into the vector pGEX-Kg (BamHI-XhoI sites). All fusions were expressed in the E. coli strain BL21. Caveolin fusion proteins were purified by affinity chromatography using glutathione-agarose. Bovine eNOS, rat nNOS, and human iNOS were purified from E. coli as described previously (10–12).

In Vitro Interactions—The interaction of GST-caveolin fusion proteins with eNOS from BLMVEC cells lysates or E. coli expressed eNOS was evaluated as follows. GST or purified GST-caveolin fusion proteins bound to glutathione-agarose beads were extensively prewashed with wash buffer containing 50 mM Tris (pH 7.7), 400 mM NaCl, and 1 mM EDTA. The beads were eluted with SDS-sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (12% acrylamide) and Western blot analysis with anti-eNOS IgG (H32). The interaction of GST eNOS with caveolin was performed in an identical manner, except the BLMVEC lysates were Western blotted with an anti-caveolin-1 IgG (Transduction Labs). Horseradish peroxidase-conjugated secondary antibodies were used to visualize bound primary antibodies by ECL.

NOS Activity Assays—The conversion of 3H-labeled L-arginine to NO was measured by the assay of the manufacturer (Promega) and previously described in this laboratory (15). The mutagenic primer converting the putative caveolin binding motif of eNOS from FSAAAPFSGW to ASAAPASGA was 5′-aactctcgtgctatgtaggcgtgcgttgccgagggcgtcgaattcgcggcct-3′ incorporating a novel NheI site to facilitate mutant selection. The eNOS caveolin binding domain mutant (eNOS-CBD) was verified by DNA sequencing and analyzed by high pressure liquid chromatography and mass spectroscopy as described (9, 14).

Site-directed Mutagenesis—Oligonucleotide-mediated mutagenesis was performed with the Altered Sites mutagenesis kit as described by the manufacturer (Promega) and previously described in this laboratory (15). The mutagenic primer converting the putative caveolin binding domain of eNOS (eNOS-CBD) was verified by DNA sequencing and analyzed by high pressure liquid chromatography and mass spectroscopy as described (9, 14).

RESULTS AND DISCUSSION

Previously, we and other laboratories have shown that eNOS and caveolin-1 and -3 co-precipitate with cellular extracts (18, 19). To further investigate the molecular nature of this interaction, we examined if eNOS solubilized from microvascular endothelial cells could directly bind to caveolin-1 in the form of a GST fusion protein. Incubation of BLMVEC extracts with GST or GST-Caveolin fusion proteins (Cav-FL, Cav (1–61), Cav (61–101), or Cav (135–178)), and the binding of eNOS was assessed by Western blotting. The binding of eNOS to Cav-FL was approximately 10% of the input protein. Equivalent amounts of cell lysates, eNOS, GST, and GST fusions were used in these experiments. These data are representative of three independent experiments.

Next, we investigated if peptides derived from the caveolin scaffolding domain could influence eNOS activity in vitro. Incubation with the caveolin-1 peptide (53–81) did not influence eNOS activity; however, the caveolin-1 scaffolding domain peptide (82–101) inhibited eNOS activity in a dose-dependent manner (Fig. 2). In addition, a similar scaffolding domain peptide from caveolin-2 (64–73) did not influence eNOS activity. To map the minimal motif of the caveolin-1 peptide that could inhibit eNOS activity, we synthesized a series of N-terminal caveolin deletion mutants and assayed their effects on eNOS activity. As seen in Fig. 3, left panel, progressive deletion of the N terminus decreased the inhibitory potency of the caveolin scaffolding domain peptide, with 88–101 being almost completely inactive.

Since eNOS can interact with both caveolin-1 and caveolin-3 and nNOS localizes in plasmalemmal domains of skeletal muscle by virtue of its binding to dystrophin complexes (20), which are enriched in caveolin-3-coated caveolae (21), we tested the ability of the scaffolding domain peptides derived from both caveolin-1 and caveolin-3 on all three NOS isoforms, eNOS,
nNOS, and iNOS. As seen in Fig. 3, right panel, the scaffolding domain peptides from caveolin-1 and -3 inhibited NOS activity of all three NOS isoforms suggesting a common binding site and mechanism of action.

Recently using a phage display library, a caveolin binding motif was elucidated (14). The motif $\text{D}^a\text{d}^b\text{XXXd}^c\text{XXX}^d\phi$ (where $\phi$ represents Trp, Phe, or Tyr and $X$ represents any amino acid) is found in eNOS, nNOS, and iNOS as well as c-Src, G-protein $\alpha$ subunits, and a variety of other signaling molecules. To examine if caveolin could bind to a domain of eNOS containing this motif (amino acids 350–358 of bovine eNOS), GST-eNOS fusions were incubated with endothelial cell lysates and Western blotted for caveolin-1. As seen in Fig. 4A, incubation with GST-eNOS (310–570) but not GST alone or GST-eNOS (130–310) resulted in the binding of caveolin. This suggests that caveolin binding is likely to occur in the predicted caveolin binding domain at amino acids 350–358 of bovine eNOS ($\text{D}^a\text{SAAPFSGW}^d$). To test whether caveolin inhibits NO production from intact cells and if the caveolin binding domain (CBD) of eNOS was functionally required for this regulation, we transfected COS-7 cells with wild-type eNOS or the caveolin binding mutant eNOS (ΔCBD-eNOS) in the absence or presence of caveolin-1 and measured NO release. As seen in Fig. 4B, co-expression of eNOS with caveolin-1 markedly inhibited the basal release of NO without influencing the expression of eNOS.

Nitric Oxide Synthase Caveolin Binding Domain

nNOS, and iNOS. As seen in Fig. 3, right panel, the scaffolding domain peptides from caveolin-1 and -3 inhibited NOS activity of all three NOS isoforms suggesting a common binding site and mechanism of action.

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or caveolin. Importantly, co-expression of eNOS with ΔCBD-eNOS did not result in an attenuation of NO release. These results demonstrate negative regulation of eNOS by caveolin in vitro and identify the major site of this interaction.

Previously, we have shown that the stimulated production of NO is optimized by eNOS targeting into caveolae as mutations that block fatty acylation, and the subsequent targeting into Golgi and caveolae inhibits NO release from cells (15, 16). Here we show that caveolin, the major coat protein of caveolae, can negatively regulate eNOS in vitro and in vivo. This apparent paradox can be explained in several ways. First, perhaps eNOS is kept "less active" by binding to caveolin in caveolae or as it moves from the trans-Golgi to caveolae, and this low activation state is responsible for low level basal NO production. For maximal activation of NO release to occur locally in response to shear stress, growth factors, or calcium-mobilizing agonists, it is possible that caveolin dissociates from eNOS or that other regulatory proteins are recruited to the complex to relieve the inhibition imposed by caveolin binding. In support of this latter concept is preliminary data from our laboratory (not shown) demonstrating additional proteins that can co-precipitate with eNOS in shear stress and growth factor-treated cells. Second, in intact endothelial cells both in culture and in intact blood vessels, not all of eNOS co-localizes with caveolin or is in caveolae, suggesting that eNOS on the cytoplasmic face of the Golgi is able to produce NO. Clearly this is possible because complete mislocalization of eNOS, based on biochemical fractionation studies and confocal microscopy, only attenuates NO release by 60–70% suggesting that eNOS in non-caveolar domains is still catalytically capable of responding to intracellular signals and producing NO (15).

Another interesting observation is that peptides derived from caveolin-1 and -3 can inhibit NOS activity in vitro. Previously, it was demonstrated that these exact peptides block...
the autophosphorylation of c-Src suggesting common binding motifs for caveolin in these proteins. Using a phage display library to map the binding motif, FXAAPFXWX was found in all NOS isoforms, perhaps explaining the molecular mechanism of NOS inhibition by caveolin (14). This motif in NOS lies between the heme and the calmodulin binding domains adjacent to a glutamate residue (Glu-361) necessary for the binding of L-arginine, suggesting that caveolin may interfere with heme iron reduction, similar to L-arginine-based NOS negative regulator of eNOS transduction (1). Here, we demonstrate that caveolin is a negative regulator of nNOS in skeletal muscle (20, 26). The functional significance of iNOS inhibition by caveolin is not known.

Caveolin-1 is a major structural coat protein of caveolae (27). Recent data showing that the scaffolding domain of caveolin-1 is sufficient for the binding of Ha-Ras, c-Src, and G-protein α subunits suggested that in addition to its role as a structural protein for caveolae, specific protein-protein interactions between caveolin and other resident proteins could regulate signal transduction (1). Here, we demonstrate that caveolin is a negative regulator of eNOS in vitro and in vivo. In addition, we have identified the primary site of this interaction as the predicted caveolin binding motif, thus solidifying the regulatory role of caveolin and caveolin-derived peptides. Studies elucidating the interactions between eNOS-caveolin after stimulation of NO release and the dynamic trafficking of eNOS to and from caveolae will reveal more functional roles for this important interaction.

In summary, many structurally distinct proteins and signaling pathways are found to be localized within caveolae, suggesting that regulatory mechanisms for signal integration, amplification, and desensitization occur in these organelles. Physiological stimuli such as acute changes in blood flow, G-protein-coupled receptor activation, and growth factor signaling all cause the initial rapid release of NO, followed by a decline. Perhaps negative regulation of G-proteins, Ha-Ras, c-Src, and eNOS by direct interactions with caveolin is the molecular switch to terminate NO release in response to all forms of stimulation. Understanding how eNOS is activated in the face of caveolin binding will undoubtedly lead to the discovery of novel NOS-activating proteins.

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Note Added in Proof—While our paper was in review, Ju et al. (Ju, H., Zou, R., Venema, V. J., and Venema, R. C. (1997) J. Biol. Chem. 272, 18522–18525) published complementary data describing direct interactions between eNOS and caveolin-1.