Communications

Direct Interaction of Endothelial Nitric-oxide Synthase and Caveolin-1 Inhibits Synthase Activity*

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Endothelial nitric-oxide synthase (eNOS) and caveolin-1 are associated within endothelial plasmalemmal caveolae. It is not known, however, whether eNOS and caveolin-1 interact directly or indirectly or whether the interaction affects eNOS activity. To answer these questions, we have cloned the bovine caveolin-1 cDNA and have investigated the eNOS-caveolin-1 interaction in an in vitro binding assay system using glutathione S-transferase (GST)-caveolin-1 fusion proteins and baculovirus-expressed bovine eNOS. We have also mapped the domains involved in the interaction using an in vivo yeast two-hybrid system. Results obtained using both in vitro and in vivo protein interaction assays show that both N- and C-terminal cytosolic domains of caveolin-1 interact directly with the eNOS oxygenase domain. Interaction of eNOS with GST-caveolin-1 fusion proteins significantly inhibits enzyme catalytic activity. A synthetic peptide corresponding to caveolin-1 residues 82–101 also potently and reversibly inhibits eNOS activity by interfering with the interaction of the enzyme with Ca2+/calmodulin (CaM). Regulation of eNOS in endothelial cells, therefore, may involve not only positive allosteric regulation by Ca2+/CaM, but also negative allosteric regulation by caveolin-1.

Plasmalemmal caveolae are small membrane invaginations present in most cells of higher eukaryotes. These membrane specializations appear to function both as endocytotic carriers and as signal transduction organizing centers. In the latter case, caveolae compartmentalize a subset of signal-transducing molecules in membrane microdomains at the cell surface (1, 2). A major structural protein of caveolae is caveolin, a 21–24-kDa integral membrane protein that occurs in three homologous, but distinct isoforms termed caveolins-1, -2, and -3 (3). Full-length caveolin-1 contains three domains: a 101-residue N-terminal domain, a 33-residue membrane-spanning region, and a 44-residue C-terminal domain. The N- and C-terminal domains of caveolin-1 face the cytoplasm suggesting that the membrane-spanning region forms a hairpin loop within the membrane (4–6). A cytosolic membrane-proximal subdomain of the N-terminal domain (residues 82–101) interacts directly with Go subunits, Ha-Ras, and Src family tyrosine kinases (7–9). Interaction of these signaling proteins with this caveolin-1 scaffolding domain serves to sequester the proteins in caveolae and to inhibit or suppress their catalytic activities.

Another important signaling protein known to be localized in caveolae is endothelial nitric-oxide synthase (eNOS) (10, 11). Production of NO by eNOS in endothelial caveolae appears to play a key role in modulating vascular tone, platelet aggregation, leukocyte adhesion, vascular smooth muscle cell proliferation, and vascular lesion formation (12, 13). eNOS has a bidomain structure consisting of an N-terminal oxygenase domain and a C-terminal reductase domain (14). Located between the oxygenase and reductase domains is a Ca2+/calmodulin (CaM)-binding region (15). Association of eNOS and caveolin-1 in cultured bovine endothelial cells has been demonstrated previously in coimmunoprecipitation experiments (16, 17). It is not known, however, whether eNOS and caveolin-1 interact directly or indirectly (i.e. through an adaptor protein). Also unidentified are the interacting domains, if any, in the two proteins. Most importantly, the functional consequences of caveolin-1 binding on eNOS catalytic activity have not been determined. Each of these questions with regard to the eNOS-caveolin-1 protein-protein interaction has been addressed in the present study.

EXPERIMENTAL PROCEDURES

Materials—The glutathione S-transferase (GST)-fusion protein cloning vector, pGEX-4T-1, CaM-Sepharose 4B, and anti-GST polyclonal antibody were obtained from Pharmacia Biotech Inc. Sf9 insect cells were purchased from Pharmingen (San Diego, CA) and maintained in serum-supplemented Hink's TNM-FH media from Mediatech, Inc. (Herndon, VA). Monoclonal antibody to eNOS (clone 3) was purchased from Transduction Laboratories (Lexington, KY). L-[14C]Arginine and ECL reagents came from Amersham Corp. p6B7F9 (DNA binding domain hybrid cloning vector), pGAD424 (activation domain hybrid cloning vector), and Saccharomyces cerevisiae SFY536 were obtained from CLONTECH (Palo Alto, CA). Oligonucleotide primers for PCR, 5'-RACE kit, and TRZOL reagent were purchased from Life Technologies Inc. TA Cloning kit was obtained from Invitrogen (Carlsbad, CA). Protein assay kit was purchased from Bio-Rad. Bovine CaM came from Sigma. Synthetic peptides were obtained from Research Genetics, Inc. (Huntsville, AL) and were >95% pure as determined by high performance liquid chromatography.

Cloning of the cDNA Encoding Bovine Caveolin-1—Total RNA from cultured bovine aortic endothelial cells was isolated with Trizol reagent and subjected to reverse transcription-polymerase chain reaction. The upstream primer for PCR was based on the first 20 nucleotides of the caveolin-1 coding sequence that are identical in sequences previously cloned from dog, mouse, and human (18–20). The downstream primer used was an oligo(dT)17 oligonucleotide. PCR amplification produced a 588-base pair fragment that was subcloned into the TA cloning vector and sequenced in the Molecular Biology Core Facility of the Medical College of Georgia. Three independent PCR reactions produced identical nucleotide sequences ruling out the possibility of PCR-associated nucleotide incorporation errors. To confirm that the first 20 nucleotides of the bovine coding sequence are identical to the sequence of the

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1 The abbreviations used are: eNOS, endothelial nitric-oxide synthase; PCR, polymerase chain reaction; GST, glutathione S-transferase; RACE, rapid amplification of cDNA ends; CaM, calmodulin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonic acid.
upstream primer, 5′-RACE (rapid amplification of cDNA ends) was performed with a 5′-RACE kit (Life Technologies Inc.). The sequence obtained by 5′-RACE was identical to that obtained in the initial PCR. The nucleotide sequence has been submitted to the GenBankTVARTMS EMBL Data Bank with accession number U86639.

Construction and Purification of GST-Caveolin-1 Fusion Proteins—cDNA constructs encoding GST-caveolin-1 fusion proteins were created by subcloning into the GST-fusion protein cloning vector, pGEX-4T-1. Caveolin-1 cDNA sequences encoding full-length caveolin-1 (residues 1–178) and caveolin-1 residues 1–60, 1–101, 102–134, and 135–178 were generated by PCR amplification of the full-length bovine sequence cloned into the TA cloning vector. Primers for PCR were designed to incorporate 5′ EcoRI and SalI restriction sites for subcloning. The cDNAs encoding the fusion proteins were sequenced to confirm the creation of in-frame fusions devoid of PCR-associated nucleotide incorporation errors. Fusion proteins and a GST-nonfusion protein were expressed in Escherichia coli and purified by affinity chromatography on glutathione-agarose as described by Frangioni and Neel (21).

Expression and Purification of eNOS in a Baculovirus System—Bovine eNOS was expressed in a baculovirus/Sf9 insect cell system and purified to homogeneity as described previously (15, 22). eNOS was purified in buffers containing 2 mM EDTA, and purified enzyme was completely dependent on exogenous CaM for activity.

Interaction of Recombinant Bovine-caveolin-1 Fusion Proteins—GST-Caveolin-1 Fusion Proteins—GST-fusion proteins of 100 pmol each, quantitated by Bio-Rad protein assay) prebound to glutathione-agarose beads were washed three times in buffer containing 50 mM Tris-HCl, pH 7.4, 20% glycerol, and the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μg/ml pepstatin A, and 5 μg/ml apronitin. Equimolar amounts of fusion proteins in each condition were confirmed by immunoblotting with specific anti-GST antibody. Washed beads were incubated overnight (with shaking at 4 °C) in 1 ml of the above buffer containing 100 pmol of bovine eNOS, expressed and purified from a baculovirus system as described previously (22). Following the overnight binding reaction, beads were washed six times in 1 ml of 50 mM Hepes, pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.5% CHAPS plus the protease inhibitors listed above. Bound proteins were eluted with 100 μl of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 100 mM reduced glutathione, plus protease inhibitors. Eluted proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-eNOS monoclonal antibody as described previously (23). Binding experiments were also performed with GST or GST-caveolin-1 fusion proteins and purified bovine CaM. In one set of experiments the binding assay was carried out exactly as described above for eNOS. In another set of experiments, EDTA was omitted from all buffers and replaced with 2 mM CaCl2.

Interaction of eNOS and Caveolin-1 Domains in a Yeast Two-hybrid System—Construction of bovine eNOS oxygenase domain (residues 1–605) and reductase domain (residues 506–1205) hybrids with the GAL4 DNA binding and activation domains has been described previously (24). Bovine caveolin-1 hybrids encoding residues 1–101, 102–134 (membrane-spanning domain), and 135–178 (C-terminal cytoplasmic domain). The fusion proteins and a GST-nonfusion protein were purified by affinity chromatography on glutathione-agarose as described previously (22). Eluted proteins were eluted with reduced glutathione. Eluted proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-eNOS antibody as described previously (23).

RESULTS AND DISCUSSION

eNOS and caveolin-1 are known to be associated in cultured bovine aortic and lung microvascular endothelial cells (16, 17). It is not known, however, whether they interact directly or indirectly (i.e. through an adapter protein). To answer this question for purified, baculovirus-expressed bovine eNOS, we have isolated and sequenced the cDNA encoding bovine caveolin-1. Analysis of the deduced amino acid sequence indicates that bovine caveolin-1 shares 94, 96, 97, and 86% identity with the human, murine, canine, and chicken sequences, respectively (18–20, 25) (Fig. 1). To determine whether eNOS interacts directly with caveolin-1, we expressed full-length bovine caveolin-1 as a GST-fusion protein in E. coli. In addition, to determine which domains of caveolin-1 are involved in eNOS binding, we also expressed GST-fusion proteins of caveolin-1 residues 1–60, 1–101 (N-terminal cytoplasmic domain), 102–134 (membrane-spanning domain), and 135–178 (C-terminal cytoplasmic domain). The fusion proteins and a GST-nonfusion protein were purified by affinity chromatography on glutathione-agarose. The GST-caveolin-1 fusions or GST alone prebound to agarose beads were then used in in vitro binding assays with recombinant bovine eNOS, expressed and purified from a baculovirus system (22). Beads were incubated with eNOS at 4 °C overnight and extensively washed, and bound proteins were eluted with reduced glutathione. Eluted proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-eNOS antibody. As shown in Fig. 2, eNOS bound specifically to the full-length GST-caveolin-1 fusion protein but not to GST alone, demonstrating that eNOS and caveolin-1 interact directly. Furthermore, the eNOS bound specifically to GST-fusion protein containing only the N-terminal caveolin-1 cytoplasmic domain (residues 1–101) or only the C-terminal caveolin-1 cytoplasmic domain (residues 135–178). In contrast, GST-fusions containing only caveolin-1 residues 1–60 or the caveolin-1 membrane-spanning domain (residues 102–134) did not bind to eNOS. The eNOS-caveolin-1 association thus appears to involve binding of eNOS to both

FIG. 1. Comparison of the caveolin-1 amino acid sequences from bovine, human, murine, canine, and chicken. Data base accession numbers are bovine (U86639), human (Z18951), murine (U57645), canine (Z12161), and chicken (A46424).
Cytoplasmic tails of caveolin-1. Furthermore, either cytoplasmic domain of caveolin-1 by itself is sufficient to mediate the eNOS binding.

To verify the conclusions reached based on the in vitro binding assays and to determine whether caveolin-1 binds to either the eNOS oxygenase domain or the eNOS reductase domain, or both, we have also investigated the eNOS-caveolin-1 interaction in a yeast two-hybrid system. Hybrid cDNA constructs were prepared that encoded full-length bovine caveolin-1 (residues 1–178), the caveolin-1 N-terminal cytoplasmic domain (residues 1–101), the caveolin-1 C-terminal cytoplasmic domain (135–178), the bovine eNOS oxygenase domain (1–505), and the bovine eNOS reductase domain (506–1205) fused to either the GAL4 DNA binding domain or activation domain. Various pairwise combinations of the plasmid constructs were used to cotransform the yeast strain, S. cerevisiae. Interactions of hybrid proteins were assessed by colony lift filter assay of β-galactosidase reporter gene transcription. As shown in Table I, caveolin-1 interacted with itself in the two-hybrid system through both N- and C-terminal cytoplasmic domains, as has been demonstrated previously in in vitro binding assays with GST-fusion proteins (26, 27). Furthermore, both N- and C-terminal domains of caveolin-1 interacted with eNOS in the two-hybrid system, confirming the results obtained in the GST-fusion protein binding assays. Caveolin-1 interactions were restricted to the eNOS oxygenase domain and did not occur with the eNOS reductase domain.

To determine whether interaction of eNOS with caveolin-1 alters nitric oxide synthase activity, we incubated equal quantities of purified, baculovirus-expressed eNOS with equimolar quantities of the GST alone, GST-caveolin-1–60, GST-caveolin-1–101, GST-caveolin-1–178, GST-caveolin-1–101, and GST-caveolin-135–178 fusion proteins. eNOS activity was then determined by arginine-to-citrulline conversion assay in the presence of excess cofactors, CaCl2, and CaM. As shown in Fig. 4, the full-length caveolin-1 fusion protein inhibited eNOS activity by about 60%. Furthermore, either of the caveolin-1 cytoplasmic domains appears to be sufficient to mediate eNOS inhibition because the GST-caveolin-1–101 and GST-caveolin-135–178 fusion proteins also inhibited enzyme activity by about 60%. In contrast, the GST-caveolin-1–60 and GST-caveolin-102–134 fusion proteins were without effect on activity. To confirm that inhibition was due to binding of the fusion proteins to eNOS rather than to CaM, we also performed in vitro binding assays with CaM and GST-caveolin-1 fusion proteins under the same conditions used in the eNOS binding studies. In addition, we performed in vitro binding assays of eNOS and CaM in which 1 mM EDTA was omitted from all buffers and replaced by 2 mM CaCl2. In both sets of experiments, no CaM binding to any of the GST-caveolin-1 fusion proteins was detected.

Inhibition by GST-caveolin-1–101 but not by GST-caveolin-1–60 suggests that the inhibitory region of the N-terminal cytoplasmic domain may correspond to the caveolin-1 scaffolding domain (residues 82–101) previously shown to inhibit Go subunits, Ha-Ras, and Src family tyrosine kinases (7–9). To test this hypothesis we prepared synthetic peptides corresponding to caveolin-1 residues 61–81 and 82–101. As shown in Fig. 4, the 82–101 peptide potently inhibited eNOS activity (IC50 = 1 μM). Complete inhibition was observed at a 10 μM concentration of peptide. A 10 μM concentration of the 61–81 peptide, on the other hand, actually increased activity by about 30%. To determine whether inhibition was due to an effect of the 82–101 peptide on the eNOS interaction with Ca2+/CaM, we preincubated eNOS with and without the 61–81 and 82–101 peptides (10 μM) and then subjected the enzyme to CaM-Sepharose chromatography. Enzyme was allowed to bind to the column in the presence of 2 mM CaCl2, and was eluted with 2 mM EGTA. The amount of enzyme eluted in each condition was quantitated by immunoblotting with monoclonal anti-eNOS antibody. As shown in Fig. 5A, the 82–101 peptide reduced binding of eNOS to CaM-Sepharose by >90% (as determined by
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In summary, the results of the present study provide several important new insights into the eNOS-caveolin-1 interaction. First, eNOS and caveolin-1 interact directly rather than indirectly. Second, interaction involves both the N- and C-terminal cytoplasmic domains of caveolin-1 and is thus fundamentally different from the interaction of caveolin-1 with Go subunits, Ha-Ras, and Src family tyrosine kinases. Third, the caveolin-1 interaction with eNOS involves only the eNOS oxygenase domain and not the eNOS reductase domain. Fourth, interaction of eNOS with caveolin-1 significantly inhibits eNOS catalytic activity. Finally, inhibition appears to be due to interference with the eNOS interaction with Ca\(^{2+}\)/CaM. Regulation of eNOS activity in endothelial cells, therefore, may involve not only positive allosteric regulation by Ca\(^{2+}\)/CaM, but also negative allosteric regulation by caveolin-1. It is conceivable that interaction of eNOS with caveolin-1 provides a mechanism to deactivate the enzyme subsequent to its activation by agonist-stimulated elevation of intracellular Ca\(^{2+}\). Protein-protein interactions between eNOS and caveolin-1, however, are probably not sufficient to mediate membrane attachment. Fatty acylation of eNOS by myristate and palmitate appears to also be required. This requirement has been demonstrated in previous studies of an eNOS myristoylation-deficient mutant in which glycine 2 has been mutated to an alanine. The mutant enzyme is neither myristoylated nor palmitoylated and, as a result, is not membrane-associated (22).

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![Image](89x594 to 267x729)

**FIG. 4.** Effect of a peptide corresponding to caveolin-1 residues 82–101 on eNOS catalytic activity. eNOS activity was determined by arginine-to-citrulline conversion assay in the absence and presence of various concentrations of caveolin-1-(82–101) peptide. Results shown are the mean ± S.E. of triplicate determinations from three separate experiments.

![Image](98x347 to 258x524)

**FIG. 5.** Effect of the caveolin-1-(82–101) peptide on eNOS binding to CaM-Sepharose and reversal of eNOS inhibition by excess Ca\(^{2+}\)/CaM. A, eNOS was preincubated with and without the caveolin-1-(82–101) peptide and subjected to chromatography on CaM-Sepharose. The enzyme was eluted from CaM-Sepharose with 2 mM EGTA. The amount of eNOS eluted in each condition was quantitated by immunoblotting with anti-eNOS monoclonal antibody. Equivalent results were obtained in three experiments. B, eNOS was preincubated for 5 min with and without the caveolin-1-(82–101) peptide (10 \(\mu\)M) and then incubated for an additional 5 min with either 1.25 or 12.5 \(\mu\)M Ca\(^{2+}\)/CaM. eNOS activity was then determined by arginine-to-citrulline conversion assay. Results shown are mean ± S.E. of triplicate determinations from three separate experiments.