Interaction between Caveolin-1 and the Reductase Domain of Endothelial Nitric-oxide Synthase

CONSEQUENCES FOR CATALYSIS*

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Endothelial nitric-oxide synthase (eNOS) is targeted to caveoli through interaction with caveolin-1 (cav-1). cav-1 binding to a consensus site in the eNOS oxygenase domain is proposed to antagonize calmodulin (CaM) binding and thereby inhibit eNOS nitric oxide (NO) synthesis. To study the mechanism, we examined how cav-1 scaffolding domain peptide (amino acids 82–101; cav-1P) would affect NO synthesis, NADPH oxidation, cytochrome c reduction, and ferricyanide reduction by full-length eNOS or its isolated oxygenase and reductase domains. Cav-1P equivalently inhibited NO synthesis and NADPH oxidation by full-length eNOS in a manner reversible by CaM but did not affect NADPH-independent NO synthesis by full-length eNOS or its oxygenase domain, indicating inhibition required the reductase domain. Similar concentrations of cav-1P inhibited cytochrome c reduction by full-length eNOS or the reductase domain (amino acids 492–1205) in a CaM-reversible manner, indicating cav-1P interaction with reductase or full-length eNOS are equivalent. Ferricyanide reduction was unaffected by cav-1P in all cases. Immunoblotting showed that full-length eNOS, eNOS oxygenase, and eNOS reductase all bound to an immobilized glutathione S-transferase-cav-1 fusion protein. Thus, cav-1 interacts independently with both oxygenase and reductase domains of eNOS. The reductase interaction occurs independent of a cav-1 binding motif, is CaM-reversible, and is of sufficient affinity to match cav-1P inhibition of NO synthesis by full-length eNOS. We propose that cav-1 binding to eNOS reductase compromises its ability to bind CaM and to donate electrons to the eNOS heme, thereby inhibiting NO synthesis.

Nitrile oxide (NO) is a widespread mediator in physiology

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‡ The abbreviations used are: NO, nitric oxide; L-Arg, L-arginine; BSA, bovine serum albumin; CaM, calmodulin; cav-1, cavolin-1; cav-1P, cavolin scaffolding domain peptide; iNOS, nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; eNOSFL, full-length eNOS; eNOSox, endothelial oxygenase domain; eNOSr, eNOS reductase domain; EPPS, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GST-cav-1, glutathione S-transferase cavolin-1 fusion protein; H2B, (H6R)2-6-amino-4-hydroxy-6-(1-erythro-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropteridine; iNOSFL, full-length inducible NOS; iNOSr, iNOS reductase domain; Ni-NTA, nickel nitrilotriacetate; and functions in the circulatory system as the endogenous vasorelaxant (1–3). The NO synthase present in vascular endothelium (eNOS) is a multifrom enzyme consisting of an N-terminal oxygenase domain (amino acids 1–491) that contains binding sites for heme, t-arginine (t-Arg), and tetrahydropyropterin (H4B) and a reductase domain (492–1205) containing binding sites for FMN, FAD, NADPH, and calmodulin (CaM) (4, 5). During NO synthesis, NADPH-derived electrons pass into the reductase domain flavins and then must be transferred to the heme located in the oxygenase domain so that the heme iron can bind O2 and catalyze stepwise NO synthesis from t-Arg (6–10). The CaM binding consensus sequence (amino acids 493–512) is located at the N terminus of the reductase domain, and CaM binding to this site activates NO synthesis by enabling the reductase domain to transfer electrons to the eNOS oxygenase domain (6, 11). Besides providing reducing equivalents to the oxygenase domain, the eNOS reductase domain can directly transfer electrons to artificial acceptors like cytochrome c and ferricyanide (6–9) independent of the oxygenase domain (8). Moreover, the rates of these reductase activities are stimulated 2- or 3-fold by CaM binding and are not affected by t-Arg analogs like N^6-nitro-l-arginine methyl ester (NAME) that block eNOS heme reduction (6), indicating that CaM directly activates the eNOS reductase domain.

In addition to being controlled by Ca^2+-dependent CaM binding, eNOS catalysis and cellular localization are influenced by a surprising number of post-translational modifications. For example, myriostylolation, palmitoylation, and/or acylation cause eNOS to associate with cellular membrane components such as the plasmalemma caveoli (12–15), which are invaginations in the membrane that function to bind and organize a variety of signal proteins (16). The chief structural component of caveoli are a group of 21–24-kDa integral membrane proteins designated caveolin-1, caveolin-2, and caveolin-3. Caveolin-1 (cav-1) is abundant in endothelium and consists of N- and C-terminal cytosolic domains separated by a 33-residue membrane-spanning region. A component of its C-terminal membrane proximal segment termed the scaffolding domain (amino acids 82–101) is responsible for attachment of a number of signal proteins to the caveoli, including G subunits, Ha-Ras, the Sre family tyrosine kinases, and eNOS (16). Besides locating eNOS to caveoli, interaction of eNOS with cav-1 or its scaffolding domain peptide 82–101 (cav-1P) specifically inhibits NO synthesis (17–20), suggesting the interaction may control eNOS function in cells.

Significantly, the inhibition of eNOS NO synthesis by cav-1 or cav-1P can be completely reversed by CaM, even though
cav-1 does not interact with CaM itself or with the eNOS CaM binding site (17, 19, 21). On this basis, a cav-1 binding consensus sequence (22) that is present in the oxygenase domain of bovine eNOS (amino acids 350–358) has been suggested to be the site of cav-1 binding, which then presumably interferes with CaM binding to the enzyme. Although other possible modes of cav-1 inhibition remain to be explored, and it is unclear how cav-1 binding to the oxygenase domain could affect CaM binding at a separate site, the proposed mechanism is attractive because loss of CaM binding should prevent the reductase-oxygenase domain interactions that enable electron transfer to the heme iron, and thus inhibit NO synthesis.

Our laboratory has a long standing interest in how CaM activates NOS (6, 11, 23–25). We therefore explored the basis of cav-1 inhibition by determining its effect on various domain-specific catalytic activities, utilizing both full-length eNOS (eNOSFL) and its individual oxygenase (eNOSox) and reductase (eNOSr) domains. Our results show that a specific interaction occurs between cav-1 and the reductase domain of eNOS that reversibly inhibits its function and is likely to be of primary importance in regulation of NO synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glutathione-agarose was obtained from Amersham Pharmacia Biotech. All other common chemicals, resins, and reagents were obtained from sources as previously reported (6, 25). Peptides corresponding to the scaffolding domain of cav-1 (D82GIWKASFTTFTVTKYWFYR101, termed cav-1P) or a rearranged version of cav-1P (WGIDKAFITTSTITYKWFRY, termed cav-x) were synthesized by the Peptide Synthesis Core facility of the Cleveland Clinic Foundation and purified by high pressure liquid chromatography to a purity of ≥98% as confirmed by mass spectrometric analysis. Stock solutions of peptides (1 or 4 mM) were dissolved in MeSO.

**Preparation of GST-Caveolin-1—**GST-caveolin-1 (GST-cav-1) cDNA cloned into the pGEX-4T-1 expression vector was the generous gift of Dr. Takashi Okamoto, Dept. of Neuroscience, Cleveland Clinic Foundation. The GST-cav-1 fusion protein was expressed in Escherichia coli strain DH5, and purified by affinity chromatography using glutathione-agarose as described by Frangioni and Neel (26). Purified GST-cav-1 was run in SDS-polyacrylamide gel electrophoresis and was identified by Coomassie staining and also by Western blotting using an anti-cav-1 IgG obtained from Transduction Laboratories. Herosadish peroxidase-conjugated secondary antibody was used to visualize bound primary antibodies by enhanced chemiluminescence.

**Preparation of eNOSFL, eNOSr, eNOSs, Full-length Inducible NOS and iNOS Reductase Proteins**—All proteins except eNOSox were expressed in E. coli BL21(DE3) using the pCWori expression vector by methods as detailed previously (10, 27). CaM was coexpressed with each NOS construct to assure its proper expression in E. coli (27). The eNOSFL, full-length inducible NOS (iNOSFL), eNOSox, and iNOS reductase (iNOSr) contained a 6-histidine tag on their N terminus. Both eNOSr and iNOSr also contained functional CaM binding sites (4, 23, 28). The eNOSFL and iNOSFL were purified by ammonium sulfate precipitation followed by Ni-NTA and 2′,5′-ADP affinity chromatographies essentially as described (27), whereas the iNOSr and eNOSr were purified only by ammonium sulfate precipitation and 2′,5′-ADP chromatography. The eNOSox was obtained by limited trypsin hydrolysis of pure eNOSFL as described (4, 28). This procedure cleaves eNOSFL at a point between its oxygenase domain and CaM binding sequence (4, 28). The eNOSox bound an alkaline-phosphatase Ni-NTA conjugate (Qiagen), indicating its N terminus was intact, and exhibited the expected molecular mass when run on an SDS-polyacrylamide gel. All purified proteins were judged homogeneous by gel electrophoresis. Activities of full-length and oxygenase proteins were quantitated based on hemoglobin content, and reductase proteins were quantitated based on the Bio-Rad protein assay.

**NO Synthesis Activity**—The initial rate of NO synthesis was quantitated at 37 °C using the oxyhemoglobin spectrophotometric assay as described previously (6). The NO-O2-mediated conversion of oxyhemoglobin to methemoglobin was monitored at 410 nm, assuming an ε410 = 38 mmol−1 cm−1 for the transition. The assay solution (0.7 ml final volume) consisted of 40 mM EPPS (pH 7.4), 0.3 mM diithiothreitol, 25 mM NOS protein, 1.0 mM mg/ml bovine serum albumin (BSA), 10 units/ml superoxide dismutase (SOD), 100 units/ml catalase, 4 mM FAD, 4 mM FMN, 4 mM H2B, 0.7 mM CaCl2, 0.5 mM EDTA, 15 μg/ml CaM, 1.4 mM L-Arg, 0.3 mM NOS, and 1.0 mM NOHA. In most cases, NOS protein with or without CaM plus various amounts of cav-1P, cav-x, or vehicle were added to the reaction mix, incubated for 10 min at room temperature, and then for 5 min at 37 °C prior to initiating the reaction by adding NADPH. Initial experiments showed this incubation time was sufficient to reach equilibrium regarding cav-1P inhibition of eNOS activities.

**Product Formation from NOHA**—Catalysis of nitrite production by eNOSFL or eNOSox from the reaction intermediate N′-hydroxy-L-arginine (NOHA) in an NADPH-independent, H2O2-supported reaction was assayed in 96-well microplates at 37 °C as described previously (29, 30) with modifications. Assays (100 μl final volume) contained 60 mM EPPS, pH 7.5, 250 mM NOS protein, 1 mM NOHA, 1 mM dithiothreitol, 30 mM H2O2, 25 units/ml SOD, 0.5 mg/ml BSA, 0.5 mM EDTA, and 4 μM H2B. All incubations also contained 0.7 mM CaCl2 and 10 μg/ml CaM. NOS proteins, CaM, and various concentrations of cav-1P, cav-x, or vehicle were preincubated as described for the NO synthesis assay prior to addition to the assay. The reaction was initiated by adding NOHA (0.1 mM final concentration), incubated for 10 min at 37 °C, and then for 5 min at 37 °C to ensure complete NOS activity. The reaction was terminated by the addition of 0.4 M H2SO4 (final concentration 0.2 M) to a final volume of 1 ml. Nitrite production was assayed at 540 nm using an ε414 = 2.6 mmol−1 cm−1 for the transition. The assay solution (0.7 ml final volume) consisted of 0.5 M sulfuric acid, 4% metaphosphoric acid, and 1% sulfosalicylic acid.

![Fig. 1. Inhibition of eNOS NO synthesis and NADPH oxidation by Cav-1P and dependence on the reductase domain. A, eNOSFL protein was preincubated with cav-1P (0–10 μM) in the presence of 0.5 μM CaM, and then its NADPH-dependent NO synthesis (●) and NADPH oxidase (●) activities (nmol/min/mg) were determined as described under “Experimental Procedures.” The rate of nitrite formation from NOHA (mol of nitrite/mol of cytochrome P450) in a NADPH-independent, H2O2-supported reaction was also determined for eNOSFL in the presence (○) or absence (▲) of CaM, and for eNOSox in the presence (●) or absence (▲) of CaM, after the proteins had been preincubated with the indicated concentrations of cav-1P. Results shown are the mean ± S.E. (n = 6) of triplicate determinations from two separate experiments. B, reversal of cav-1 inhibition of eNOSFL NADPH oxidase activity by added CaM. eNOSFL was preincubated for 10 min with 5 μM cav-1P and with 0.5 μM CaM and then incubated for an additional 5 min with 0.1–10 μM added CaM. NADPH oxidation activities (light bars) were then determined relative to the activity of a control receiving CaM but no cav-1P (100%) and to the rate of enzyme-independent NADPH oxidation (0%). Results shown are the mean ± S.E. (n = 6) of triplicate determinations from two separate experiments. In a replicate experiment, 1 mM NAME was also added to an assay containing eNOSFL, 5 μM cav-1P, and 10 μM CaM (dark bar).
Caveolin-1 Interaction with eNOS Reductase Domain

**RESULTS**

**Effect of Cav-1P on Heme-Dependent Catalytic Activities of eNOS—Cav-1P (residues 82–101) represents the scaffolding domain of cav-1 and has been shown to inhibit eNOSFL NO synthesis (17–19). Using pure recombinant eNOS, we confirmed the ability of cav-1P to inhibit NO synthesis in a con-
Caveolin-1 Interaction with eNOS Reductase Domain

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**Fig. 3. Interaction of eNOSFL, eNOSr, and eNOSox with a GST-cav-1 fusion protein.** Purified samples of eNOSFL, eNOSr, and eNOSox were incubated with immobilized GST or GST-cav-1 fusion protein. After washing the beads, the bound proteins were eluted and run on SDS-PAGE as detailed under “Experimental Procedures.” The figure shows the position of the bound proteins in relation with molecular weight standards following their Western transfer onto a polyvinylidene difluoride membrane. The eNOSFL and eNOSr proteins were visualized using an anti-eNOS antibody, and the eNOSox was visualized using a Ni-NTA-alkaline phosphatase conjugate.

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concentration-dependent manner (Fig. 1A), with EC_{50} ~ 3 \mu M in the presence of Ca^{2+} and 0.5 \mu M CaM. We also observed a similar inhibition profile for eNOSFL NADPH oxidation by cav-1P under identical assay conditions (Fig. 1A). Neither activity was inhibited by the cav-x control peptide (data not shown). This indicates cav-1P does not uncouple eNOSFL NADPH oxidation in the process of inhibiting NO synthesis and instead inhibits both NADPH oxidation and NO synthesis together. Because both of these reactions depend on electron transfer to the heme, cav-1P may act by blocking this process. Indeed, inhibition of NADPH oxidase activity was almost completely reversed by adding excess CaM (Fig. 1B), which triggers electron transfer from the reductase domain to the heme in eNOSFL (6). NAME, an l-Arg analog that inhibits eNOSFL heme reduction by lowering the iron redox potential (6, 31), antagonized rescue of activity, confirming CaM functioned to restore reductase domain-mediated heme iron reduction.

The mechanism of cav-1P inhibition was further examined in experiments that measured NO synthesis from NOHA by either eNOSox or eNOSFL in an assay that is independent of reductase domain-mediated heme reduction. In this system, cav-1P was completely unable to inhibit NO synthesis either by eNOSFL or eNOSox in the presence or absence of added CaM (Fig. 1A). Its inability to affect oxygenase domain catalysis held even though this domain contains a consensus binding site for cav-1 and therefore is thought to be the site of its binding (16, 19, 20). We conclude that cav-1P does not directly inhibit the activity of the eNOS oxygenase domain but instead can only inhibit eNOSFL catalysis when it depends on electron transfer from the reductase domain to the heme, consistent with cav-1P antagonizing the CaM-dependent electron transfer function of the reductase domain.

**Effect of Cav-1P on Reductase Domain-specific Catalytic Activities—**We next examined the effect of cav-1P on two reductase domain-catalyzed reactions, NADPH-dependent cytochrome c or ferricyanide reduction (4, 6–9). Because a consensus cav-1 binding motif is not present in the eNOS reductase domain, we expected that cav-1P would not affect these activities. However, as shown in Fig. 2, A and B, cav-1P significantly inhibited cytochrome c reduction by eNOSFL and its isolated reductase domain (eNOSr) in the presence of Ca^{2+} and 0.5 \mu M CaM, with an EC_{50} of ~3 \mu M for both proteins. The CaM-independent cytochrome c reduction activities of eNOSFL and eNOSr were also inhibited by cav-1P over the same concentration range (Fig. 2, A and B), indicating the inhibition need not involve CaM. Cav-x peptide did not inhibit in any case, indicating specificity of action. In contrast to its potent effect on eNOS cytochrome c reduction, cav-1P did not inhibit ferricyanide reduction by eNOSFL or eNOSr at any concentration tested (Fig. 2, A and B). Because ferricyanide likely receives electrons from the FAD or FMN group of eNOSr, while cytochrome c only receives electrons from the terminal FMN group (4, 6–9, 23), this finding suggests that cav-1P inhibits electron transfer only from the terminal FMN of the reductase.

We explored the basis for the action of cav-1P by comparing the K_m for cytochrome c reduction by eNOSr in the presence and absence of 2.5 \mu M cav-1P. This gave apparent K_m values of 21 and 28 \mu M, respectively (data not shown), indicating that cav-1P does not antagonize eNOSr interaction with cytochrome c. Thus, an interaction of cav-1P with the eNOS reductase domain inhibits its rate of electron transfer to cytochrome c, and this occurs at cav-1P concentrations equivalent to those that inhibit NO synthesis and NADPH oxidation by eNOSFL.

Cav-1P inhibition of eNOSFL or eNOSr cytochrome c reduction could be fully reversed by adding excess CaM (Fig. 2, B and D), matching the characteristic pattern observed for CaM rescue of eNOSFL NO synthesis and NADPH oxidation (Fig. 1B) (21, 22). That reductase domain catalysis is under opposing control by cav-1P and CaM was further evidenced by cav-1P only slightly inhibiting cytochrome c reduction by iNOSFL or its isolated reductase domain (iNOSr) (Fig. 2, A and C), which both contain tightly bound CaM (32).

**In Vitro Binding of eNOSFL and Its Domains to Immobilized Cav-1—**To confirm that cav-1 affects eNOSFL catalysis through a direct binding interaction, we examined binding of eNOSFL and its isolated domains to a GST-cav-1 fusion protein (17–19, 26) immobilized on glutathione-agarose beads. As shown in the Western blot of Fig. 3, left panel, eNOSFL bound to the immobilized GST-cav-1 fusion protein, as indicated by the band at ~78 kDa, but did not bind to immobilized GST alone. Specific binding was also observed for eNOSFL (135-kDa band, left panel)

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\[ \text{NO synthesis was not inhibited over the same cav-1P concentration range} \] (S. Ghosh, unpublished results).
and for eNOSox (50 kDa band, right panel), as previously reported (17–19). Thus, in addition to interacting with the eNOS oxygenase domain, cav-1 specifically binds eNOSr, implying a direct binding interaction is responsible for cav-1 inhibition of reductase domain catalysis.

**DISCUSSION**

Our data show that cav-1 specifically interacts with both the reductase and oxygenase domains of eNOS. Its interaction with eNOSr was unexpected, because the reductase domain does not contain a consensus binding motif for cav-1 that is present in the bovine eNOS oxygenase domain (F350XXXFXFXW) and in several other proteins known to interact with cav-1 (16, 22). The eNOSr interaction with and response to cav-1P (or cav-1) occurred in the absence of the oxygenase domain, indicating they are independent of the cav-1 binding motif in eNOSox. Our data appear to contradict a yeast 2-hybrid study that suggested no interaction occurs between eNOSr and cav-1P (17). The basis for this discrepancy is unclear.

Although the mechanism by which cav-1 regulates eNOS NO synthesis is still unclear (20), the following data reveal that cav-1 interaction with eNOSr is of primary importance. 1) The concentration of cav-1P required for 50% inhibition of eNOSr cytochrome c reduction, as well as the extent of inhibition, match the concentration response for cav-1P inhibition of eNOSFL NO synthesis observed here or reported by others (17–19). 2) Cav-1P reversibly inhibits eNOSr cytochrome c reduction by antagonizing CaM activation of eNOSr, identical to the mechanism by which cav-1P reversibly inhibits eNOSFL NO synthesis (17–19) and NADPH oxidation (Fig. 1). Thus, the cav-1-CaM antagonism that is characteristic of eNOS inhibiting NOS activity of excess CaM to reestablish normal electron flow to the oxygenase domain of eNOSFL would be expected to slow electron transfer from the reductase, thus inhibiting heme iron reduction and NO synthesis, whereas cav-1 interaction with the oxygenase domain may help target the eNOSFL-cav-1 complex to caveoli (20), and have as yet unspecified effects on catalysis. Although many details remain to be addressed, our model provides a starting point for experiments that further define cav-1 interactions with both eNOS domains and its mechanism of inhibition.

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