Reconstitution of an Endothelial Nitric-oxide Synthase (eNOS), hsp90, and Caveolin-1 Complex in Vitro

EVIDENCE THAT hsp90 FACILITATES CALMODULIN STIMULATED DISPLACEMENT OF eNOS FROM CAVEOLIN-1

Received for publication, February 29, 2000, and in revised form, April 24, 2000
Published, JBC Papers in Press, April 25, 2000, DOI 10.1074/jbc.M001644200

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The activity of endothelial nitric-oxide synthase (eNOS) is regulated by its subcellular localization, phosphorylation and through its interaction with different proteins. The association of eNOS with caveolin-1 (Cav) is believed to maintain eNOS in an inactive state; however, increased association of eNOS to heat shock protein 90 (hsp90) is observed following activation. In this study, we investigate the relationship between caveolin and hsp90 as opposing regulatory proteins on eNOS function. Immunoprecipitation of Cav-1 from bovine lung microvascular endothelial cells shows that eNOS and hsp90 are present in the Cav-1 complex. eNOS and hsp90 from the lysate also interact with exogenous glutathione S-transferase-linked caveolin-1 (GST-Cav), and the addition of calcium-activated calmodulin (CaM) to the GST-Cav complex partially inhibited the association of eNOS and hsp90. Purified eNOS associates with GST-Cav specifically through the caveolin-scaffolding domain (residues 82–101); however, the addition of CaM slightly, but nonstatistically, reduces eNOS binding to GST-Cav. When hsp90 is present in the binding reaction, the addition of increasing concentrations of CaM significantly displaces eNOS and hsp90 from GST-Cav. eNOS enzymatic activity is also less sensitive to inhibition by the caveolin scaffolding peptide (residues 82–101) when eNOS is prebound to hsp90. Collectively, our results show that the actions of CaM on eNOS dissociation from caveolin are facilitated in the presence of hsp90.

Endothelial nitric-oxide synthase (eNOS)† is the NOS isoform responsible for the physiological production of nitric oxide (NO) in the cardiovascular system. Endothelium-derived NO is an important regulator of systemic blood pressure, angiogenesis, and vascular architecture (1–3). eNOS was originally identified as a membrane-associated, NADPH- and tetrahydrobioppterin-requiring enzyme activated by calcium/calmodulin (CaM) (4). However, recent biochemical and structural studies have indicated additional regulatory controls in situ, by subcellular targeting, protein phosphorylation, and protein–protein interactions (5).

eNOS can directly interact with at least four proteins in vitro and in cellular extracts: caveolins 1 and 3 (6, 7), the intracellular domains of certain G-protein coupled receptors (8), CaM (4), and heat shock protein 90 (hsp90) (9). The first two proteins inhibit NO activity, whereas the latter two are stimulatory. In the case of caveolins, in vitro data support the concept that when eNOS is localized in the caveolae, it has the capacity to interact with caveolin. This interaction may tonically down-regulate NO production and is most likely associated with inactivation of the enzyme at rest. According to this model, agonists that promote an elevation of intracellular calcium can promote the dissociation of caveolin from eNOS via a proposed exclusive binding of either caveolin or calmodulin to eNOS, thus removing the negative regulation (10, 11). However, it is not clear if the time course of dissociation of caveolin from eNOS is consistent with eNOS activation and NO release and whether this occurs in a calcium/CaM-dependent manner with all forms of stimulation leading to NO production (12, 13). Recently, we have shown that agonists that promote NO release through distinct signaling mechanisms (histamine, estriol, vascular endothelial growth factor, and fluid shear stress) stimulate the recruitment of hsp90 to eNOS in a time frame consistent with activation of eNOS and NO production (9, 14). Moreover, the ansamycin antibiotic geldanamycin, which specifically binds in the unique ATP binding pocket of hsp90, inhibits agonist-stimulated NO production from cultured endothelial cells and endothelium-dependent relaxations of isolated blood vessels (15). Collectively, these results are consistent with the idea that hsp90 directly or indirectly modulates eNOS. This concept of hsp90 facilitating NOS activation has been extended to another NOS isoform, neuronal NOS. Neuronal NOS also interacts with hsp90 in cell lysates, and geldanamycin blocks ionomycin-stimulated NO release from cells stably expressing neuronal NOS (16).

The relationship between caveolin and hsp90 as opposing regulatory proteins that control the production of NO has been explored. Therefore, the purposes of this study are to 1) examine the interactions of eNOS with caveolin-1 and hsp90 in cells and in vitro; 2) determine the role of hsp90 in the cal-
modulin-induced dissociation of eNOS from caveolin; and 3) explore the interaction of these regulatory proteins on eNOS function.

MATERIALS AND METHODS

Cell Culture and Reagents—Bovine lung microvascular endothelial cells (BLMVEC) were cultured in high glucose Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum, penicillin, streptomycin, and l-glutamine as described previously (17). 2'-5' ADP-Sepharose 4B was obtained from Amersham Pharmacia Biotech. Peptides, corresponding to the amino acid 82–101 scaffolding domain of caveolin-1 (Cav-(82–101)), were synthesized, purified, and analyzed by reversed-phase high pressure liquid chromatography and mass spectrometry by the W. M. Keck biotechnology resource center at Yale University School of Medicine. A scrambled version of the caveolin-1 peptide (Cav-X) was also used as a negative control (19). Purified hsp90 from bovine brain was obtained from Stressgen, and purified calmodulin was from Upstate Biotechnology, Inc. (Lake Placid, NY). Recombinant bovine eNOS was purified from Escherichia coli as described previously (19). eNOS stock solutions (1 mg/ml) for all of the studies were in a buffer composed of 50 mM Tris-HCl (pH 7.5), 10% glycerol, 200 mM NaCl, 0.5 mM l-arginine, and 40 μM BH4.

Immunoprecipitation—eNOS and caveolin-1 were immunoprecipitated from cultured BLMVEC as described previously (7). Immunocomplexes were isolated, electrophoresed, and either Western blotted or exposed to autoradiography in the case of [35S]methionine-labeled BLMVEC (20). Western blots were sequentially probed with anti-caveolin-1 monoclonal Ab (Transduction Laboratories), with anti-eNOS monoclonal Ab (9D10; Zymed Laboratories Inc., Laboratories), and with anti-hsp90 monoclonal Ab (SPA 830; Stressgen).

Purification of GST-Caveolin-1 Fusion Protein and in Vitro Interactions—The caveolin fusion protein expression was performed as described previously (21). The interaction of GST-caveolin fusion protein with eNOS and hsp90 from BLMVEC cells lysates was performed as follows. Cells were lysed in a modified RIPA buffer (50 mM Tris-Cl, pH 7.4, 1% Nonidet P-40, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% SDS, 0.1% deoxycholic acid, 1 μM Pefabloc, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 μg/ml pepstatin) for 1 h at 4 °C. The samples were Dounce-homogenized (20 strokes) and centrifuged for 10 min at 16,000 × g at 4 °C. Lysates (500 μg of proteins) were incubated overnight at 4 °C with beads containing ~100 pmol of either GST alone or GST-Cav (~20 μl of packed volume) in a total volume of 400 μl. After binding, the beads were washed (three times) with wash buffer containing 50 mM Tris-Cl (pH 7.4), 125 mM NaCl, 1 mM EDTA, and 1 mM EGTA. In some experiments, the immobilized proteins were washed with 50 mM Tris-Cl (pH 7.4), 125 mM NaCl containing 1 mM CaCl2. The beads were then resuspended in the same buffer, and CaM (0.1 and 1 μM) was then added for 1 h at 4 °C. In vitro interactions of GST-Cav with purified or recombinant proteins (hsp90, eNOS, or CaM) were performed for 2 h at 4 °C in binding buffer (50 mM Tris-Cl, pH 7.4, in 100 μM l-arginine). After binding, the beads were extensively washed (five times) with a high salt wash buffer (50 mM Tris-Cl, pH 7.7, 400 mM NaCl, and 1 mM EDTA). Beads were eluted by boiling in SDS-sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. Interacting proteins were identified by colloidal brilliant blue (Sigma) staining of the gels or by Western blotting (see above).

RESULTS AND DISCUSSION

The coat protein for caveolae, caveolin-1 (6, 7), and hsp90 (9) have been shown independently to co-precipitate with eNOS from endothelial cell lysates. Therefore, we investigated if these three proteins could exist in a heterocomplex in quiescent endothelial cells. Immunoprecipitation of eNOS from [35S]methionine-labeled BLMVEC lysates revealed the presence of four major proteins in the eNOS immunocomplex: p200, p90, p60, and p20 (Fig. 1A). A 90-kDa and a 20-kDa protein were previously identified as hsp90 (9, 24) and caveolin-1 (6, 7), respectively; however, the identities of the p200 and p60 proteins are still unknown. The intensity of the radioactivity incorporated into the eNOS-associated proteins (p90, p60, and p20) compared with [35S]-labeled eNOS suggests that only a fraction of hsp90 or caveolin-1 interacts with eNOS in resting endothelial cells under these conditions. Based upon the radioactivity incorporated and taking into account that the number of labeled methionines and the turnover of labeled proteins may vary, the associated proteins are binding substoichiometrically. The substoichiometric interaction of caveolin-1 and hsp90 with eNOS is not surprising, since only a fraction of the total eNOS pool is enriched in caveolae in most endothelial cells (10–30%) (7, 25, 26), and the bulk of hsp90 is associated with cytoskeleton or in the cytosol (27). We interpret the interaction of eNOS with its associated regulatory proteins more akin to signaling complexes where the binding is rarely stoichiometric and transient.

Immunoprecipitation of caveolin-1 from BLMVEC lysates resulted in the recovery of both eNOS and hsp90 (detected by Western blotting), supporting the idea that these proteins could exist as a heterotrimeric complex in vitro (Fig. 1B). Next, to test if eNOS and hsp90 could be isolated from BLMVEC lysates using glutathione S-transferase–linked caveolin-1 (GST-Cav) as a tool to isolate the complex. As seen in Fig. 1C, eNOS and hsp90 co-associate with GST-Cav but not with GST alone. To examine if CaM, an important allosteric activator of eNOS, could influence the interaction of eNOS/hsp90 with GST-Cav, exogenous CaM was added to the GST-Cav complex.
Calmodulin Regulates the eNOS, hsp90, and Caveolin Complex

The addition of calcium-activated CaM (0.1 and 1 μM) to the GST-Cav complex partially inhibited the association of eNOS and hsp90 to the complex. These results suggest that in endothelial cells, eNOS and, interestingly, hsp90 are capable of associating with caveolin-1, thus implying that the three proteins are in a complex in resting endothelial cells and that exogenous CaM can disrupt the binding of eNOS and hsp90 to caveolin.

Next, we examined if we could reconstitute the heterotrimetric complex in vitro using purified proteins. As reported previously, the scaffolding domain of caveolin-1 (amino acids 82–101) is responsible for the interaction of caveolin with multiple proteins such as Ha-RAS, c-Src, and eNOS (28). Incubation of peptides derived from the scaffolding domain of caveolin-1 (Cav-(82–101)) completely prevented the association of eNOS with GST-Cav (Fig. 2A). However, Cav-X (10) did not affect the binding of eNOS to GST-Cav, showing the specificity of the in vitro interactions between the two proteins. Fig. 2B shows that purified eNOS and hsp90 do not interact with GST alone and that hsp90 does not interact with GST-Cav. However, in the presence of eNOS, hsp90 is detected in the complex with GST-Cav. This shows that the interaction of hsp90 with the caveolin-1 is through its association with eNOS, not via direct binding to caveolin, as described previously (29).

The association of eNOS with caveolin-1 in endothelial cells is thought to be disassembled following activation of eNOS by calcium-dependent stimuli that promote CaM binding to eNOS (11). The binding of CaM to eNOS is believed to disrupt the binding of caveolin-1 to the enzyme, thereby providing a mutually exclusive interaction with either an activator (CaM) or inhibitor (caveolin-1) of eNOS. This has been described using cell lysates for the co-purification of eNOS and caveolin-1 followed by the addition of purified CaM back to the immune-complexed proteins or by the usage of the scaffolding domain peptide as a caveolin surrogate that prevents eNOS binding to CaM-Sepharose (11, 30). Clearly, the scaffolding domain peptide is an inhibitor of eNOS activity that appears to act competitively with respect to CaM in NOS activity assays (10, 31). However, the exclusivity of caveolin-1 versus calmodulin binding to eNOS as a regulatory mechanism has not been directly examined with caveolin-1 protein. Therefore, we co-incubated eNOS and/or hsp90 with GST alone or GST-Cav in the presence of CaM (1 μM in 1 mM calcium) and analyzed the complex by Coomassie staining or Western blotting. As seen in Fig. 3A, eNOS or hsp90 does not bind to GST alone. Approximately 10% of the input eNOS (lane 6) bound specifically to GST-Cav (lane 2). The addition of CaM does not prevent eNOS binding to GST-Cav (lane 3). Co-incubation of eNOS with hsp90 results in recovery of the complex with GST-Cav, with approximately 10% of both eNOS and hsp90 input bound (compare lanes 4 and 6). Incubation of the eNOS:hsp90:GST-Cav complex with CaM results in less eNOS and hsp90 bound (lane 5). Next we performed similar experiments using semiquantitative Western blotting to analyze proteins bound to GST-Cav. As mentioned above, eNOS interacts with GST-Cav, but not GST. In the absence of concentrations of CaM up to 1 μM, CaM slightly, but non-statistically, reduces eNOS binding to GST-Cav (lanes 2–5 in Fig. 3B; quantified in Fig. 3C). When hsp90 is present in the binding reaction, the amount of eNOS bound to GST-Cav does not change (compare lane 2 with lane 6); however, the addition

![Fig. 2 Interaction of eNOS and hsp90 with GST-caveolin in cells and in vitro.](image1)

![Fig. 3 hsp90 facilitates calmodulin dissociation of the eNOS-caveolin complex.](image2)
of increasing concentrations of CaM significantly displaces eNOS from GST-Cav (lanes 7–9). Similarly, hsp90 is also markedly displaced from the caveolin complex in response to CaM addition, reinforcing the fact that hsp90 directly binds to eNOS and eNOS acts as a molecular bridge for the hsp90 association with caveolin-1 (Fig. 2B). These results suggest that the conformational changes in eNOS following the addition of CaM are not sufficient to disrupt its physical association with caveolin-1 and that the presence of hsp90 facilitates the actions of CaM in displacing eNOS from caveolin-1. The latter conditions may more accurately reflect the calcium-CaM-induced displacement of eNOS observed in endothelial cell lysates (see Fig. 1C) (11).

Since eNOS bound to caveolin-1 is believed to be in an inactive state, we monitored the reconstitution of an active eNOS complex by affinity purification of the complex using ADP-Sepharose chromatography. eNOS reversibly binds to this resin via its NADPH binding site. Purified eNOS (0.5 μM or 9.2 nM) in the absence of CaM binds to ADP-Sepharose resin (Fig. 4, lane 1), whereas hsp90 (1 μg or 27.7 nM) and CaM (1 μM) do not directly bind to the resin (lane 6). However, in presence of eNOS, hsp90 is bound to the resin via its interaction with eNOS (lane 2). Interestingly, the addition of CaM (0.01–1 μM) favors the binding of eNOS to the resin, suggesting that CaM increases the affinity of eNOS for ADP-Sepharose through increased affinity for NADPH. Enhanced binding of eNOS allows for increased recovery of hsp90 and CaM bound in the complex. These data indicate that CaM activation of eNOS and/or hsp90 increases the amount of each component in the complex.

To examine the interrelationship between hsp90 and caveolin as regulators of eNOS function, we measured NOS activity in vitro. In the presence of fixed levels of CaM (0.1 μM), the caveolin scaffolding domain peptide, Cav-(82–101) (10 μM), inhibits (Fig. 5A) and hsp90 increases NOS activity. Preincubation of eNOS with hsp90 reduces the inhibitory effects of Cav-(82–101). To examine this more thoroughly, we titrated increasing concentrations of the Cav-(82–101) on eNOS activity in the absence or in the presence of hsp90 preincubated with eNOS. As seen in Fig. 5B, Cav-(82–101) dose-dependently inhibits NOS activity with an approximate EC50 of 2.5 μM (Fig. 5B). However, in the presence of hsp90, an EC50 of 6 μM is observed, showing that the inhibitory properties of caveolin-1 are reduced when eNOS is bound to hsp90. Collectively, these data suggest that hsp90 influences the inhibitory actions of caveolin-1 on eNOS. In support of this idea are data demonstrating that 1) hsp90, eNOS, and caveolin-1 can exist as a heterotrimeric complex in endothelial cells; 2) The complex can be reassembled using lysates or purified components, with eNOS being the bridge between hsp90 and caveolin; 3) CaM weakly displaces eNOS from GST-Cav, whereas if hsp90 is included in the reaction mix, CaM exerts a stronger effect on displacing eNOS from caveolin; and finally 4) hsp90 more efficiently binds to eNOS in a CaM-dependent manner and reduces the inhibitory actions of the caveolin-1 scaffolding peptide on eNOS activity. In a cellular context, the presence of hsp90 and caveolin-1 associated to eNOS under basal conditions may modulate low level activation of eNOS and basal release of nitric oxide. Upon an increase in cytoplasmic calcium, calcium-activated CaM may aid in the further recruitment of hsp90 to the complex and facilitate the release of the caveolin inhibitory clamp. This effect was previously thought to be dependent only on CaM, whereas we suggest that hsp90, and perhaps other regulatory signals (32), will serve as active participants in the release of eNOS from caveolin-1.

The presence of a calmodulin binding site on hsp90 suggests that the interaction between eNOS and the chaperone protein can be regulated by the common binding partner, CaM (33, 34), as evidenced by CaM facilitating the effects of hsp90 on the eNOS dissociation from GST-Cav (Fig. 3) and CaM-dependent association of hsp90 and eNOS with CaM in a complex bound to ADP-Sepharose (Fig. 4). The dissociation of eNOS from caveolin in response to calcium-dependent agonists has been documented; however, our results now indicate that hsp90 plays a role in this effect. Elucidating the cellular mechanisms of eNOS interacting with CaM and hsp90 as positive modulators of eNOS activation and understanding how this regulates the caveolin-1/eNOS interaction will increase our knowledge of how endothelial cells produce NO.

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Fig. 4. Calmodulin-dependent reconstitution of eNOS-hsp90 activation complex by ADP-Sepharose chromatography. eNOS, hsp90, and increasing concentrations of CaM were incubated with ADP-Sepharose resin for 2 h at 4 °C. After washing of the resin, proteins were detected by Western blotting (wb) for eNOS, hsp90, and CaM. This experiment is representative of two independent experiments.

Fig. 5. The hsp90-bound form of eNOS is less sensitive to inhibition by Cav-(82–101) peptide. A, purified eNOS was incubated with either Cav-(82–101) peptide (10 μM) or hsp90 (1 μg; 15 min) or preincubated with hsp90 (1 μg; 5 min) followed by Cav-(82–101). B, eNOS was preincubated with hsp90 (1 μg; 15 min), and increasing concentrations of Cav-(82–101) were added prior to initiation of the reaction by the addition of NOS cofactors. NOS activity was measured as described under "Materials and Methods." These data are representative of at least three experiments performed in duplicate.
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