

## Reciprocal Regulation of Endothelial Nitric-oxide Synthase by Ca<sup>2+</sup>-Calmodulin and Caveolin\*

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The endothelial nitric-oxide synthase (eNOS) is a key determinant of vascular homeostasis. Like all known nitric-oxide synthases, eNOS enzyme activity is dependent on Ca<sup>2+</sup>-calmodulin. eNOS is dynamically targeted to specialized cell surface signal-transducing domains termed plasmalemmal caveolae and interacts with caveolin, an integral membrane protein that comprises a key structural component of caveolae. We have previously reported that the association between eNOS and caveolin is quantitative and tissue-specific (Feron, O., Belhassen, L., Kobzick, L., Smith, T. W., Kelly, R. A., and Michel, T. (1996) *J. Biol. Chem.* 271, 22810–22814). We now report that in endothelial cells the interaction between eNOS and caveolin is importantly regulated by Ca<sup>2+</sup>-calmodulin. Addition of calmodulin disrupts the heteromeric complex formed between eNOS and caveolin in a Ca<sup>2+</sup>-dependent fashion. In addition, overexpression of caveolin markedly attenuates eNOS enzyme activity, but this inhibition is reversed by purified calmodulin. Caveolin overexpression does not affect the activity of the other NOS isoforms, suggesting eNOS-specific inhibition of NO synthase by caveolin. We propose a model of reciprocal regulation of eNOS in endothelial cells wherein the inhibitory eNOS-caveolin complex is disrupted by binding of Ca<sup>2+</sup>-calmodulin to eNOS, leading to enzyme activation. These findings may have broad implications for the regulation of Ca<sup>2+</sup>-dependent signal transduction in plasmalemmal caveolae.

Nitric oxide is a ubiquitous molecule implicated in diverse biological processes and is synthesized in mammalian cells by a family of Ca<sup>2+</sup>-calmodulin-dependent nitric-oxide synthase (NOS)<sup>1</sup> enzymes (1–3). Endothelium-derived nitric oxide (NO), formed by the endothelial isoform of nitric-oxide synthase (eNOS), serves as an important determinant of blood pressure and platelet aggregation. In endothelial cells, increases in intracellular Ca<sup>2+</sup> elicited by diverse extracellular signals lead to activation of eNOS. The three known mammalian nitric-oxide synthases share similar overall Ca<sup>2+</sup>-calmodulin-dependent catalytic pathways. However, the eNOS enzyme is unique among the three known NOS isoforms in being localized to the specialized cell surface signal-transducing domains termed plasmalemmal caveolae (4, 5).

Plasmalemmal caveolae are small invaginations in the plasma membrane that may serve as sites for the sequestration of signaling proteins (6, 7) including receptors, G proteins, and protein kinases, as well as eNOS. The principal protein in caveolae is the integral membrane protein caveolin, an oligomeric protein that serves as a structural “scaffold” within caveolae (8). eNOS can be quantitatively immunoprecipitated by antibodies directed against caveolin; conversely, eNOS antiserum also immunoprecipitates caveolin (5), although it has not yet been established whether the interaction between these two proteins is direct. Moreover, a functional role of the eNOS-caveolin association, beyond its postulated role in subcellular targeting of the enzyme, remains to be determined. We document in this report that the interaction between eNOS and caveolin may be regulated by Ca<sup>2+</sup>-calmodulin, and we show that caveolin can specifically inhibit eNOS enzyme activity.

### EXPERIMENTAL PROCEDURES

**Plasmid Constructs**—A plasmid construct encoding eNOS has been described previously (9, 10). cDNA clones encoding iNOS (11) and nNOS (12) were kindly provided by Carl Nathan (Cornell University Medical College) and Solomon Snyder (Johns Hopkins University), respectively. Caveolin-1 cDNA (13) in the eukaryotic expression vector pCB-7 was obtained from Michael Lisanti (Whitehead Institute). An irrelevant plasmid encoding β-galactosidase was used as a control to maintain identical amounts of DNA in each transfection.

**Cell Culture and Transfection**—Cultures of bovine aortic endothelial cells (studied between passages 4 and 10) and COS-7 cells were performed as described previously (9, 14). COS-7 cells were transfected with 10 μg of total plasmid DNA in 100-mm cell culture plates using LipofectAMINE<sup>®</sup> (Life Technologies, Inc.) according to the manufacturer's protocol.

**Immunoprecipitation, SDS-PAGE, and Immunoblotting**—Endothelial cells were lysed and solubilized either with: 1) a previously described Ca<sup>2+</sup>-free CHAPS buffer (5, 14) supplemented with 1 mM EGTA/1 mM EDTA or 2) an otherwise identical CHAPS buffer containing 1 mM CaCl<sub>2</sub> and no EDTA/EGTA. CHAPS-solubilized bovine aortic endothelial cell lysates were incubated either with a polyclonal caveolin antibody (Transduction Laboratories) at a final concentration of 4 μg/ml or with a previously characterized polyclonal antiserum directed against eNOS (9) used at a final dilution of 1:100. Immunoprecipitated complexes were then recovered, denatured in Laemmli sample buffer, separated on 12% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (5). Monoclonal antibodies directed against eNOS or caveolin-1 (Transduction Laboratories) were then used to detect

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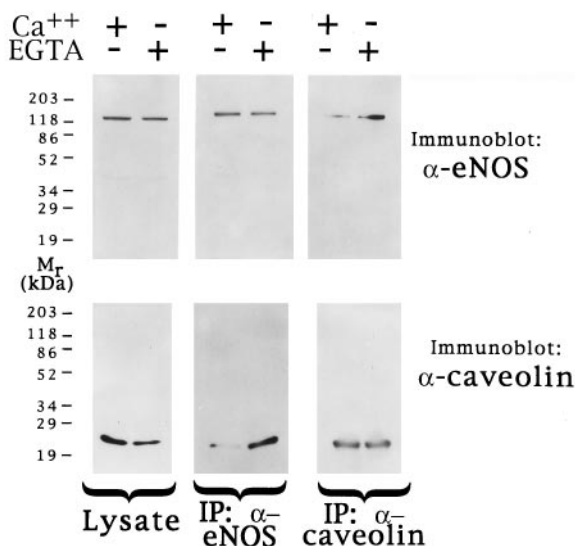
This report is dedicated to the memory of Professor Thomas W. Smith.

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<sup>1</sup> The abbreviations used are: NOS, nitric-oxide synthase; NO, nitric oxide; eNOS, endothelial isoform of NOS; iNOS, inducible isoform of NOS; nNOS, neuronal isoform of NOS; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-holamidopropyl)dimethylammonio]-1-propanesulfonic acid.



**FIG. 1. Co-immunoprecipitation of eNOS with caveolin is blocked by  $\text{Ca}^{2+}$ .** Immunoprecipitation (IP) of eNOS and caveolin was performed in endothelial cell lysates prepared in the presence (1 mM  $\text{CaCl}_2$ ) or the absence (1 mM EDTA, 1 mM EGTA) of excess calcium, as described in the text. The immunoblots in the upper panels were probed with eNOS antibody, and those in the lower panels were probed with caveolin antibody. In the left panels are shown the results of immunoblots performed directly on endothelial cell lysates prepared in the presence and the absence of added  $\text{Ca}^{2+}$ , documenting no change in the abundance of these proteins in cell lysates. In the middle panels are shown the results of immunoblot analyses following immunoprecipitations with eNOS antiserum, and in the right panels are shown the immunoblots probed following immunoprecipitation with the caveolin antibody. This experiment was repeated three times with equivalent results.

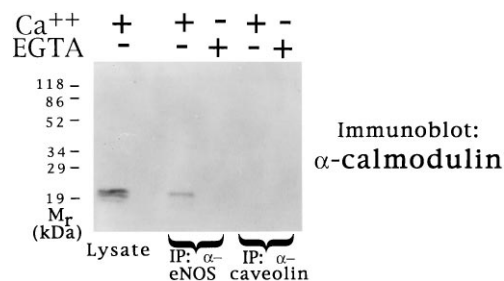
eNOS and caveolin-1 using chemiluminescence, as described previously (5). Calmodulin was detected using a previously characterized monoclonal calmodulin antibody (15). Expression of immunoblotted proteins was quantitated by laser densitometric analysis of x-ray films following chemiluminescence detection.

**NOS Enzymatic Assays**—NO synthase activity in lysates prepared from transfected COS-7 cell was determined by measuring conversion of [ $^3\text{H}$ ]L-arginine to [ $^3\text{H}$ ]L-citrulline as described previously (9, 14). In some experiments, this NO synthase activity assay was performed in washed membrane fractions prepared from endothelial cell lysates as described previously (9), except that calmodulin concentrations were varied by the addition of purified bovine brain calmodulin (Sigma) as indicated.

#### RESULTS AND DISCUSSION

In exploring the factors governing eNOS-caveolin association, we discovered that the co-immunoprecipitation of eNOS and caveolin was markedly attenuated when endothelial cell lysates were prepared in buffers containing excess free  $\text{Ca}^{2+}$  (Fig. 1). Under no conditions did the presence of  $\text{Ca}^{2+}$  affect the recovery of caveolin or eNOS from endothelial cell lysates when the proteins were directly immunoprecipitated by their cognate antibody. Only co-immunoprecipitation was abrogated by  $\text{Ca}^{2+}$ , as shown in Fig. 1.

We next investigated the role of  $\text{Ca}^{2+}$  in modulating the association of eNOS and calmodulin. This is particularly important because calmodulin, a ubiquitous  $\text{Ca}^{2+}$ -binding protein, plays a central role in nitric-oxide synthase catalysis (16). Co-immunoprecipitation experiments using eNOS antiserum to explore eNOS-calmodulin interactions in endothelial cell lysates are shown in Fig. 2. We could detect co-immunoprecipitation of eNOS and calmodulin only when free  $\text{Ca}^{2+}$  was present. This result is in striking contrast to the loss of eNOS-caveolin co-immunoprecipitation observed in the presence of  $\text{Ca}^{2+}$  (Fig. 1). Taken together, these data suggest that  $\text{Ca}^{2+}$  differentially modulates the association of eNOS with caveolin

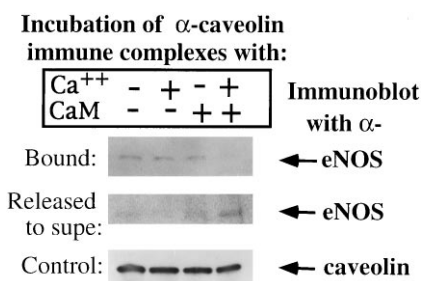


**FIG. 2. Co-immunoprecipitation of eNOS with calmodulin requires  $\text{Ca}^{2+}$ .** Shown are the results of SDS-PAGE and immunoblots probed with monoclonal calmodulin antibody. Endothelial cell lysates were solubilized with CHAPS buffer containing either EDTA/EGTA or  $\text{CaCl}_2$ , as noted above each lane. Immunoprecipitation (IP) of calmodulin by the eNOS antiserum (middle lanes) required  $\text{Ca}^{2+}$  and was not seen in the presence of EGTA/EDTA. Co-immunoprecipitation of calmodulin by caveolin (right lanes) was not detected under any conditions.

versus calmodulin in endothelial cell lysates.

We next used antibodies against caveolin to co-immunoprecipitate eNOS in  $\text{Ca}^{2+}$ -free buffers and then washed the immune complexes extensively to remove residual  $\text{Ca}^{2+}$  and calmodulin. Subsequent addition of either  $\text{Ca}^{2+}$  or calmodulin alone had no effect on the subsequent recovery of co-immunoprecipitated eNOS from the eNOS-caveolin complex (Fig. 3, upper panel). However, when  $\text{Ca}^{2+}$  and calmodulin were added together, eNOS was entirely lost from the caveolin immune complex. This “lost” eNOS could be completely recovered in the supernatant of the immune complex (Fig. 3, middle panel), indicating that the eNOS molecule had been released and not degraded following treatment of the caveolin-eNOS complex with  $\text{Ca}^{2+}$  plus calmodulin. None of these treatments affected the recovery of caveolin itself from the immune complex (Fig. 3, lower panel). The extensive washing of these immune complexes, required to remove the endogenous calmodulin, likely led to the loss of some eNOS (because the affinity of the eNOS-caveolin interaction is undoubtedly less than the affinity of the antibody for caveolin), leading to the detection of a relatively faint but highly reproducible ( $n = 4$ ) signal for eNOS released from these complexes by the combination of  $\text{Ca}^{2+}$  plus calmodulin.

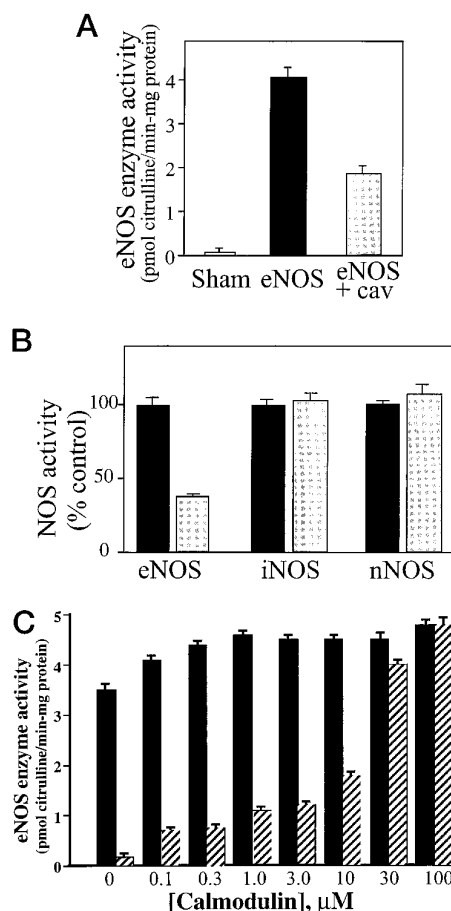
Diverse experimental approaches have shown that agonist activation of eNOS in endothelial cells is dependent on  $\text{Ca}^{2+}$ -calmodulin (1, 2). The striking effects of  $\text{Ca}^{2+}$ -calmodulin on the interactions of eNOS and caveolin therefore suggested to us that caveolin may influence eNOS enzyme activity. Indeed, caveolin has recently been shown *in vitro* to interact with other signaling proteins, including H-ras and G protein  $\alpha$  subunits, preferentially associating with the “inactive” forms of these proteins (17), but the cellular regulation of these interactions is less well understood. We explored the functional consequences of the interaction between eNOS and caveolin using transient transfection experiments in COS-7 cells and analyzed eNOS enzyme activity in transfected cells by assaying the conversion of [ $^3\text{H}$ ]L-arginine to [ $^3\text{H}$ ]L-citrulline in cell lysates, as shown in Fig. 4. In three separate experiments, each conducted in triplicate, we found that the co-transfection of a plasmid cDNA construct encoding caveolin with eNOS cDNA led to a marked attenuation of NOS activity ( $3.4 \pm 0.3$  versus  $1.6 \pm 0.1$  pmol citrulline/min-mg protein in the absence or the presence of caveolin co-expression, respectively; see Fig. 4A). There was no change in the abundance of eNOS protein associated with caveolin co-transfection, as assessed in immunoblots of these cellular lysates analyzed in each experiment (data not shown). Importantly, caveolin co-transfection failed to attenuate the enzyme activity expressed by transfected iNOS or nNOS cDNA, shown in Fig. 4B. As for eNOS, the enzyme activity of



**FIG. 3. Release of eNOS from caveolin immune complexes by Ca<sup>2+</sup>-calmodulin and its recovery.** Endothelial cell lysates were solubilized in Ca<sup>2+</sup>-free (1 mM EDTA, 1 mM EGTA) CHAPS buffer, and caveolin was immunoprecipitated as described in the text. Immune complexes were precipitated by the addition of protein G-Sepharose (16). The immune complexes bound to protein G-Sepharose beads were washed ten times in CHAPS buffer, and the beads were then equally distributed in four separate aliquots and incubated for 1 h at 4 °C as described below and then processed for SDS-PAGE and immunoblot analysis. The *first lane* shows results following incubation with CHAPS buffer alone; the *second lane* shows the results when 1 mM CaCl<sub>2</sub> is added; calmodulin (1  $\mu$ g/ml) was added for the *third lane*; in the *last lane* both CaCl<sub>2</sub> and calmodulin are added to the incubation. Following the incubation, the beads are pelleted and the supernatant (*supe*) is saved (and analyzed in the *middle panel* of the figure). After a final wash, the immune complexes are eluted from beads with SDS-PAGE sample buffer and then analyzed by SDS-PAGE; immunoblots were then probed with eNOS antibody (*top panel*) or caveolin antibody (*bottom panel*). These experiments were performed four times with similar results.

iNOS and nNOS is calmodulin-dependent (although important differences in the Ca<sup>2+</sup> dependence of the different NOS isoforms have been noted). In further contrast to eNOS, the other NOS isoforms are not targeted to caveolae. To explore the specificity of the inhibitory effect of caveolin co-expression on eNOS enzyme activity, we performed activity assays in the presence of varying concentrations of purified calmodulin added to washed membrane fractions prepared from transfected COS-7 cells. As shown in Fig. 4C, in cells transfected with eNOS cDNA alone, there is a robust NOS activity even in the absence of added calmodulin (presumably due the presence of endogenous calmodulin in these membranes); enzyme activity increases only slightly with the addition of exogenous calmodulin. By contrast, caveolin co-expression markedly inhibits eNOS activity (by >90%) in the absence of added calmodulin; addition of increasing concentrations of exogenous calmodulin relieves this enzyme inhibition in a dose-dependent fashion, documenting that the caveolin inhibitory effect may be specifically overcome by purified calmodulin.

Taken together, these studies suggest that the interaction between eNOS and caveolin is dynamically and specifically regulated by Ca<sup>2+</sup>-calmodulin and may serve as an important point of control in NO-dependent signaling. A direct interaction of caveolin with calmodulin appears unlikely to us because there was no influence of caveolin on the activity of other calmodulin-binding proteins (iNOS and nNOS) closely related to eNOS. This hypothesis is consistent with our failure to detect co-immunoprecipitation of calmodulin with caveolin (Fig. 2), under conditions in which eNOS was shown to associate with either one or the other protein. Furthermore, the amino acid sequence of caveolin isoforms show no obvious sequence or structural homologies to the known NOS isoforms (10–12) nor to any known calmodulin-binding protein sequences. Caveolin can attenuate the tyrosine kinase activity of *c-src*, an enzyme that bears no structural homology to eNOS, and is not known to be regulated by calmodulin (13). We speculate that there is a common higher order structure assumed by the inactive conformation of diverse caveolae-targeted sig-



**FIG. 4. Attenuation of eNOS enzyme activity by co-expression of caveolin in transfected cells (A); no effects on iNOS or nNOS activity (B); and reversal of inhibition by calmodulin (C).** Shown are the results of a [<sup>3</sup>H]L-arginine  $\rightarrow$  [<sup>3</sup>H]L-citrulline nitric-oxide synthase activity assay performed in lysates of transfected COS-7 cells as described above. A, COS-7 cells were transfected with an expression plasmid encoding eNOS, in a co-transfection either with caveolin (*cav*) cDNA (as noted) or with an equivalent quantity of plasmid DNA encoding an irrelevant protein ( $\beta$ -galactosidase). This experiment was conducted in triplicate three times with equivalent results. B, transfection of COS-7 cells with eNOS, iNOS, or nNOS cDNAs without (*black bars*) or with (*shaded bars*) caveolin cDNA were performed as described for A, with NOS enzyme activity measured in cell lysates and normalized to the activity seen in the absence of caveolin. Maximal NOS enzyme activities (in the absence of caveolin) in different experiments averaged  $4.2 \pm 2.1$  pmol citrulline formed/min-mg protein. The experiment shown was repeated three times in triplicate with identical results. C, NOS enzyme activity was measured in washed membranes prepared from COS-7 cells transfected with eNOS cDNA either without (*black bars*) or with (*hatched bars*) caveolin cDNA. NOS activity was assayed under conditions identical to those described above except that varying concentrations of purified calmodulin were added as noted on the abscissa. This experiment was performed twice in duplicate, yielding equivalent results.

naling proteins that forms the basis for their common interaction with caveolin.

The targeting of eNOS to caveolae is likely to facilitate the interactions of eNOS with other co-localized signaling and regulatory molecules (3). Formation of an inhibitory eNOS-caveolin heteromeric complex may serve to ensure the latency of the NO signal until calcium-mobilizing extracellular stimuli destabilize this complex and activate the enzyme. This close control of enzyme activity may be particularly important for eNOS in caveolae, where calmodulin, the enzyme's key allosteric activator, also is localized (4) and where even subtle increases in intracellular Ca<sup>2+</sup> could thus lead to enzyme activation if the interactions of caveolin with eNOS were not keeping the sys-

tem in check. Because nitric oxide has cytotoxic as well as signaling functions (1, 2), attenuation of basal enzyme "leakiness" by caveolin may be of particular importance. The reciprocal regulation of eNOS by caveolin and calmodulin may represent a novel mechanism for the concerted control of NO production in the vascular wall.

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