The Endothelial Nitric-oxide Synthase-Caveolin Regulatory Cycle*

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Nitric oxide production in the vascular endothelium is promoted by diverse agonists that transiently increase intracellular Ca\(^{2+}\) concentration and activate the endothelial nitric-oxide synthase (eNOS), a Ca\(^{2+}\)/calmodulin-dependent enzyme. eNOS is acylated by the fatty acids myristate and palmitate and is targeted thereby to plasmalemmal signal-transducing domains termed caveolae. eNOS enzyme activity is markedly attenuated by its interactions with caveolin, the structural scaffolding protein of caveolae. We have discovered that in living cells, the eNOS-caveolin heteromeric complex undergoes cycles of dissociation and re-association modulated by Ca\(^{2+}\)-mobilizing agonists. Calcium ionophore A23187 and the muscarinic cholinergic agonist carbachol both promote the dissociation of eNOS from caveolin in cultured cells, associated with translocation of eNOS from caveolae. As [Ca\(^{2+}\)]\(_i\) returns to basal levels, eNOS re-associates with caveolin, and the inhibited enzyme complex is then restored to caveolae, a process accelerated by palmitoylation of the enzyme. These data establish an eNOS-caveolin regulatory cycle, wherein enzyme activation is modulated by reversible protein-protein interactions controlled by Ca\(^{2+}\)/calmodulin and by enzyme palmitoylation. Alterations in this cycle are likely to have an important influence on nitric oxide-dependent signaling in the vascular wall.

The endothelial isoform of nitric-oxide synthase (eNOS) is robustly expressed in the vascular endothelium and in cardiac myocytes, and the cellular regulation of eNOS may represent an important determinant of cardiovascular homeostasis (reviewed in Ref. 1). In endothelial cells and in cardiac myocytes, eNOS is targeted to specialized invaginations of the plasmalemma termed caveolae (2). Plasmalemmal caveolae serve as sites for the sequestration of signaling proteins and are further characterized by the presence of caveolin, an intrinsic membrane protein that forms a structural “scaffold,” organizing both proteins and lipids within this key membrane organelle (3, 4). Caveolin directly interacts with several structurally distinct signaling proteins in caveolae, including G protein-coupled cellular oncogenes (4) as well as eNOS (2, 5–9). The activity of purified eNOS, a Ca\(^{2+}\)/calmodulin-dependent enzyme (10, 11), is markedly attenuated by its interaction with caveolin (5–9). We have also shown that purified Ca\(^{2+}\)/calmodulin can overcome the inhibitory interaction between eNOS and caveolin in vitro (5, 7, 9), but the relevance of these observations to the dynamic regulation of eNOS in endothelial cells is less well understood. In vascular endothelial cells and in cardiac myocytes, the cycle of eNOS activation and deactivation is intimately coupled to the changes in intracellular Ca\(^{2+}\) that are promoted by stimulation of diverse G protein-coupled receptors (12, 13). In this report, we describe a series of experiments that have explored the relationships between intracellular Ca\(^{2+}\) regulation and the dynamics of eNOS-caveolin interactions in living cells. We also document the role of eNOS palmitoylation in the reversible caveolar targeting of the eNOS-caveolin complex following muscarinic cholinergic stimulation.

EXPERIMENTAL PROCEDURES

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\(\) The abbreviations used are: eNOS, endothelial isoform of nitric-oxide synthase; palm, palmitoylation-deficient eNOS mutant; OG, octyl glucoside; mAChR, muscarinic acetylcholine receptor; IP, immunoprecipitation(s).

Plasmid Constructs and cDNA Transfections—cDNA constructs encoding wild-type eNOS and the palmitoylation-deficient eNOS mutant (palm) have previously been described (14). A plasmid construct encoding the muscarinic m2 mAChR cDNA was obtained from T. J. Bonner (National Institute of Mental Health, Bethesda, MD) (15). Bovine aortic endothelial cell and COS-7 cell culture conditions and cDNA transfection protocols were as described previously (2, 5, 9). The recombinant expression of eNOS and of the m2 mAChR was verified by Western blot and specific muscarinic radioligand binding, respectively, as reported (16).

Preparation of Cellular Lysates and Subcellular Fractionation—Transfected COS-7 cells or endothelial cells were extensively washed with phosphate-buffered saline, harvested, pelleted by centrifugation, resuspended in OG buffer containing 1 μM Tris-HCl, pH 7.4, 125 mM NaCl, 2 mM dithiothreitol, 50 μM EGTA, and protease inhibitors (1 mg/ml leupeptin, 1 mg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride) and sonicated as described previously (2, 5, 9). When cell fractionation was performed, cells were first lysed by sonication in a detergent-free hypotonic buffer and separated into soluble and particulate fractions by ultracentrifugation (100,000 \(\times \) g, 1 h) (9, 14). Co-immunoprecipitation—Aliquots of cell homogenates were incubated with a rabbit caveolin-1 polyclonal antibody (lot 5, Transduction Labs) at a final concentration of 4 μg/ml; antibody titration experiments (not shown) documented that this concentration led to quantitative immunoprecipitation (IP) of caveolin from cell lysates. The isofrom specificity and lack of cross-reactivity of these antibodies have been previously established (2, 5, 9). After 1 h at 4 °C, protein G-Sepharose beads (50 μl of a 50% slurry) were added to the supernatant for a further 1-h incubation at 4 °C. Bound immune complexes were washed three times with OG buffer and then once with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl. In some experiments, the supernatant fraction (remaining following pelleting of the protein G-Sepharose immune complexes) was precipitated by addition of trichloroacetic acid and buffered with a Tris-HCl solution, pH 7.4. The immunoprecipitates and/or the corresponding supernatant precipitates were then eluted by boiling in Laemmli sample buffer. SDS-polyacrylamide gel electrophoresis on 7.5% polyacrylamide gels, immunoblotting with eNOS or caveolin antibodies (Transduction Labs), and chemiluminescent detection protocols were performed as described previously (2).
RESULTS AND DISCUSSION

As shown in Fig. 1, treatment of endothelial cells with the Ca^{2+} ionophore A23187 leads to the dissociation of caveolin from eNOS. The fraction of eNOS that is liberated from caveolin by Ca^{2+} ionophore treatment can be recovered in its entirety from the supernatant fraction following the caveolin immunoprecipitation; there is no change in the recovery of caveolin in these or the other drug treatments (Fig. 1, lower panel). Following addition of the Ca^{2+} chelator EGTA to the ionophore-activated cells, the eNOS-caveolin complex re-forms, as shown by the quantitative immunoprecipitation of eNOS by the caveolin antibody after Ca^{2+} chelation (Fig. 1, lanes labeled 75). The re-formation of the inhibitory eNOS-caveolin complex may represent a mechanism whereby the enzyme can become de-activated following the return of intracellular Ca^{2+} to basal levels.

Prolonged agonist treatment of endothelial cells leads also to the translocation of eNOS from caveolae to a soluble subcellular compartment (17–19), but the role of caveolin in this enzyme translocation is entirely unknown. It seems plausible that this eNOS translocation represents a means for the desensitization of the enzyme upon prolonged agonist stimulation. We therefore examined the subcellular distribution of the eNOS-caveolin complex following treatment of endothelial cells with the Ca^{2+} ionophore. In resting endothelial cells, nearly all (>95%) of the eNOS is in the particulate subcellular fraction, and the enzyme can be almost quantitatively immunoprecipitated by antibodies directed against caveolin (Fig. 2). Following treatment of endothelial cells with Ca^{2+} ionophore and the consequent dissociation of the caveolin-eNOS complex, the newly liberated caveolin-free eNOS is now detected in both particulate and soluble subcellular fractions following differential ultracentrifugation. When the Ca^{2+} chelator EGTA is subsequently added, eNOS located in both the particulate and soluble subcellular fractions re-associates with caveolin, and the soluble complex is then re-targeted to caveolae, as shown in Fig. 2.

Taken together, these data suggest a regulatory cycle in which agonist stimulation initially leads to eNOS activation by the Ca^{2+}/calmodulin-dependent disruption of the eNOS-caveolin heteromeric complex, followed later by enzyme translocation and re-formation of the inhibitory caveolin-eNOS heteromer; finally, this inactive complex is re-targeted to caveolae, ready for another round of agonist activation. Although the agonist-evoked modulation of caveolin-eNOS binding affinity may account for changes in the hydrophobicity of eNOS and explain, in part, the reversible translocation of eNOS to and from caveolin, such a mechanism also probably involves cycles of de-palmitoylation/re-palmitoylation of the enzyme. We have indeed previously reported that eNOS is targeted to plasma-memmal caveolae by palmitoylation (20) but also that agonists promote enzyme de-palmitoylation leading to eNOS translocation from caveolae (21).

Palmitoylation is a reversible post-translational modification characteristic of diverse signaling proteins targeted to caveolae and involves the addition of the 16-carbon fatty acid palmitate to specific cysteine residues within the protein. Palmitate is attached to signaling proteins via a labile thioester bond, and for eNOS as well as some other signaling proteins, agonist activation promotes de-palmitoylation and protein translocation from caveolae (21–23). eNOS undergoes palmitoylation at two cysteine residues (Cys^{367} and Cys^{379}); mutagenesis of these residues to alanine reduces the overall affinity of the enzyme for biological membranes and attenuates the selective targeting of eNOS to caveolae (14, 20). However, the membrane-associated palmitoylated mutant is still able to bind caveolin (9), suggesting that this post-translational modification does not affect the eNOS-caveolin interaction per se, even if selective targeting of the palmitoylated eNOS mutant to caveolae is impaired. We devised a series of experiments to explore further the relationships between eNOS palmitoylation, caveolin binding, and enzyme recycling following agonist activation and exploited a heterologous expression system in transiently transfected COS cells.

To reconstitute the palmitoylated eNOS mutant with a receptor-coupled eNOS pathway in COS cells, we co-transfected cDNA encoding either wild-type or palmitoylated eNOS mutant, along with a cDNA construct encoding the m2 muscarinic cholinergic receptor (mACHR). We chose to study the m2 mACHR because (a) this receptor is well known to function in a physiologically important pathway that regulates eNOS activation in several cell types (12, 13, 16, 24) and (b) this recombinant receptor has been extensively validated as an activator of phospholipase C (25, 26) leading to transient increases in intracellular Ca^{2+} levels (27, 28). We found that the muscarinic agonist carbachol induces the dissociation of wild-type eNOS from caveolin in COS cells co-transfected with eNOS and m2 mACHR cDNAs; this process is agonist-dependent and is blocked by the cholinergic antagonist atropine (Fig. 3A). The carbachol-induced dissociation of the eNOS-caveolin complex shows an appropriate agonist dose dependence, with an EC_{50} of ~1 μM (Fig. 3, B and C). It must be emphasized that the eNOS released from the heteromeric immune complex with caveolin is recovered in the supernatant of the immunoprecipitations, indicating that
the enzyme is released and not degraded following drug treatments (as shown in Figs. 1–3).

Having validated the pharmacological characteristics of this response, we next turned to a series of time course experiments exploring the agonist-regulated association and dissociation of caveolin from wild-type and palm- eNOS using co-immunoprecipitation protocols. For the wild-type enzyme, treatment of co-transfected COS cells with carbachol led to the rapid dissociation of eNOS from caveolin; the wild-type enzyme became fully dissociated from caveolin within 5 min following the addition of carbachol, after which time the proteins were found to re-associate, with the heteromeric complex entirely reformed by 7–10 min (Fig. 4A). Although this time course parallels that seen with receptor-mediated nitric oxide release from the vascular endothelium studied in situ (29), it is difficult to directly compare the temporal sequence of these events because of the important differences in experimental conditions. For example, the NO-dependent transient hypotensive response seen following the infusion of acetylcholine in vivo is much less rapid than the cellular responses elicited by muscarinic cholinergic activation in vitro, although there is a generally similar temporal pattern (27, 28).

We next performed a series of identically configured time course experiments, now studying the palm- eNOS mutant instead of the wild-type enzyme. In contrast to the wild-type enzyme, even prior to agonist addition a significant fraction of the palm- mutant is found unassociated with caveolin, consistent with the mutant’s impaired targeting to caveolae in the resting cell. However, after the addition of carbachol, the entire fraction of the palm- mutant that had been complexed to caveolin rapidly dissociates, and within 5 min, no heteromeric caveolin-palm- eNOS complex could be found, just as for the wild-type enzyme, and the entirety of the eNOS could be recovered in the post-immunoprecipitation supernatant (Fig. 4B). However, at this point, the palm- eNOS mutant shows a dramatic divergence from the wild-type enzyme: the re-association of the caveolin-palm- complex is markedly delayed. In contrast to the wild-type eNOS, in which the entirety of the enzyme is recovered in a heteromeric complex within 7–10 min, the palm- mutant shows only a sluggish re-formation of the heteromer, a process delayed in its onset and barely at completion a full hour after the addition of drug. There is no substantive change in agonist-mediated Ca2+ transients (assessed using fura-2 epifluorescence) between COS cells transfected with the wild-type versus palm- mutant eNOS (data not shown). We therefore interpret this marked divergence in the kinetics of re-formation of the heteromeric palm- eNOS-caveolin complex to reflect the essential role for re-palmitoylation in facilitating the re-targeting of eNOS to caveolae, thereby facilitating the protein-protein interactions between eNOS and caveolin that permit the heteromeric complex to re-form.

In summary, we have shown for the first time in cells that the eNOS-caveolin complex can be rapidly disrupted and subsequently restored following agonist activation, associated with the reversible translocation of the enzyme. We therefore postulate the existence of a dynamic cycle of eNOS-caveolin interactions initiated by agonist-promoted increases in [Ca2+]i, that disrupt the caveolin-eNOS complex, leading to enzyme activation. Following more prolonged agonist stimulation, eNOS is de-palmitoylated (21) and is no longer selectively sequestered in caveolae. The translocated enzyme probably partitions both into noncaveolar plasma membrane and into more hydrophilic regions of the cell, the precise identity of which has not been established. Subsequent to the enzyme’s translocation into this more “soluble” cell compartment and following the decline in [Ca2+]i, to basal levels, caveolin may once again interact with
eNOS, leading to enzyme inhibition. The re-association of eNOS with caveolin may occur either at the membrane level or in the cytosol through which caveolin complexes may shuttle between caveolae and an internalized caveolar vesicle/trans-Golgi network (3, 30, 31). The re-association and re-targeting of the heteromeric eNOS-caveolin complex appears to be accelerated (or stabilized) by enzyme palmitoylation, which takes place either within caveolae or en route to this organelle. The re-palmitoylation of eNOS facilitates rapid and efficient stabilization of the inactivated enzyme in the caveolar environment ready for another cycle of stimulation by agonists. This dynamic cycle of eNOS intracellular regulation adds another level of complexity to the post-translational life history of this vital signaling protein and may represent an important control point for the modulation of NO-dependent signaling in the vascular wall.

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