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Targeting and translocation of endothelial nitric oxide synthase

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Abstract

This review explores advances in our understanding of the intracellular regulation of the endothelial isoform of nitric oxide synthase (eNOS) in the context of its dynamically regulated subcellular targeting. Nitric oxide (NO) is a labile molecule, and may play important biological roles both within the cell in which it is synthesized and in its interactions with nearby cells and molecules. The localization of eNOS within the cell importantly influences the biological role and chemical fate of the NO produced by the enzyme. eNOS, a Ca^{2+}/calmodulin-dependent enzyme, is subject to a complex pattern of intracellular regulation, including co- and post-translational modifications and interactions with other proteins and ligands. In endothelial cells and cardiac myocytes eNOS is localized in specialized plasmalemmal signal-transducing domains termed caveolae; acylation of the enzyme by the fatty acids myristate and palmitate is required for targeting of the protein to caveolae. Targeting to caveolae facilitates eNOS activation following receptor stimulation. In resting cells, eNOS is tonically inhibited by its interactions with caveolin, the scaffolding protein in caveolae. However, following agonist activation, eNOS dissociates from caveolin, and nearly all the eNOS translocates to structures within the cell cytosol; following more protracted incubations with agonists, most of the cytosolic enzyme subsequently translocates back to the cell membrane. The agonist-induced internalization of eNOS is completely abrogated by chelation of intracellular Ca^{2+}. These rapid receptor-mediated effects are seen not only for "classic" eNOS agonists such as bradykinin, but also for estradiol, indicating a novel non-genomic role for estrogen in eNOS activation. eNOS targeting to the membrane is labile, and is subject to receptor-regulated Ca^{2+}-dependent reversible translocation, providing another point for regulation of NO-dependent signaling in the vascular endothelium.

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eNOS and the family of NOS genes

Nitric oxide (NO) is synthesized in mammalian cells by a family of three nitric oxide synthases (NOS) (see review in Ref. 1). The initial NOS nomenclature reflected early observations that NO synthesis was not found in unactivated inflammatory cells, but could become induced upon immunoactivation, hence the term iNOS. This "inducible" iNOS was contrasted to a "cNOS" activity, constitutively expressed in certain cell types (neuronal, endothelial). It is now known that gene expression of both eNOS and nNOS may

also be "induced" under different physiological conditions (e.g., hemodynamic shear stress, nerve injury), and, conversely, that iNOS may function as a "constitutive" enzyme under physiological conditions in some cells (2). Thus, the designation of a NOS isoform being "constitutive" *vs* "inducible" NOS is inappropriate, and should be supplanted by a nomenclature that clearly identifies the specific enzyme isoform. A widely accepted nomenclature (3) identifies the three mammalian enzyme isoforms as nNOS, iNOS and eNOS, reflecting the tissues of origin for the original protein and cDNA isolates. The human genes for the NOS isoforms are officially categorized in the order of their isolation and characterization; the human genes encoding nNOS, iNOS and eNOS are thus termed *NOS1, NOS2* and *NOS3,* respectively.

There are important general biochemical features shared by the different NOS isoforms (3-5). The overall amino acid sequence identity for the three human NOS isoforms is ~55%, with stronger sequence conservation noted in regions of the proteins importantly involved in catalysis (1). The NOS isoforms share a similar overall catalytic scheme, involving the oxidation of the terminal guanido nitrogen of the amino acid L-arginine to form NO plus L-citrulline, in a complex reaction involving molecular oxygen and NADPH as co-substrates, plus other redox cofactors including enzyme-bound heme, reduced thiols, flavin adenine dinucleotide, flavin mononucleotide and tetrahydrobiopterin (3,6). For all three NOS isoforms, NO synthesis depends upon the enzyme's binding the ubiquitous calcium regulatory protein calmodulin. For eNOS and nNOS, increases in resting intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) are required for binding calmodulin and, consequently, for their becoming fully activated. By contrast, iNOS appears able to bind calmodulin with high affinity even at the low $[Ca^{2+}]_i$ characteristic of resting cells. Thus, the intracellular activity of eNOS and nNOS is closely modulated by transient changes in $[Ca^{2+}]_i$, whereas the activity of iNOS in immunoactivated cells is no longer temporally regulated by intracellular calcium transients (5).

In different tissues and for different NOS isoforms, NO synthesis has been identified within a variety of subcellular organelles (reviewed in Ref. 7). In contrast to the other NOS isoforms, the targeting of eNOS to the particulate subcellular fraction was observed in the initial characterizations of the purified enzyme, which documented that detergents are required for eNOS solubilization (8). When molecular clones for eNOS were identified, it was noted that eNOS contains no hydrophobic transmembrane domain, and it was subsequently established that the association of eNOS with cell membranes is mediated principally by enzyme acylation (reviewed in Ref. 9). Nitric oxide is a labile molecule, and may play important biological roles both within the cell in which it is synthesized and in its interactions with nearby cells and molecules (10,11). NO may be either stabilized or degraded through its interactions with diverse intracellular or extracellular chemical moieties. The localization of NOS within the cell might therefore be expected to influence the biological role and chemical fate of the NO produced by the enzyme.

eNOS acylation and targeting to caveolae

eNOS is unique among the NOS isoforms in its being dually acylated by the saturated fatty acids myristate and palmitate (9). These acylations are required for the subcellular targeting of eNOS to specific regions within the plasmalemma caveolae (*vide infra*). eNOS myristoylation occurs cotranslationally on an N-terminal glycine residue within a specific consensus sequence that is not present in nNOS and iNOS. Palmitoylation (which has not been reported

for the other NOS isoforms) takes place on two cysteine residues near the eNOS N-terminus and stabilizes the enzyme's association with the membrane. Myristoylation, which is required for eNOS targeting to the endothelial cell membrane (caveolae), is essentially irreversible. By contrast, eNOS palmitoylation is reversible: agonists such as bradykinin promote eNOS palmitate turnover (12), providing an important parallel with other reversibly palmitoylated signaling proteins such as $G\alpha_s$ (13). Depalmitoylation represents a mechanism for the release of signaling proteins from the membrane in response to agonist stimulation. The receptor-mediated processes that regulate reversible palmitoylation of signaling proteins are not well characterized, and a deeper understanding of this pathway is an important problem in signal transduction (14). It is known that the targeting of eNOS to plasmalemmal caveolae is dependent upon palmitoylation of the protein (15). It is therefore plausible that agonist-induced depalmitoylation of eNOS promotes the dissociation of the enzyme from proximity to activating molecules (or substrate or cofactors) localized in caveolae, and may serve as a feedback mechanism leading to eNOS de-activation. Other reversible ligand interactions may further modulate the subcellular localization of eNOS as well as other myristoylated proteins (16).

Plasmalemmal caveolae are present in the endothelial cell plasma membrane, and are also prominent in cardiac myocytes and many other cells (17). Caveolae, which are characterized by the presence of the transmembrane protein caveolin (18), may serve as sites for the sequestration of signaling molecules (19) including receptors, G proteins and protein kinases, as well as eNOS. At least two G-protein-coupled receptors that lead to eNOS activation, namely the muscarinic m2 and bradykinin B_2 receptors, have been shown to be targeted to caveolae upon agonist stimulation (20,21). The presence within caveolae of these receptors may facilitate the activation of eNOS by establishing local caveolar domains in which NOS-coupled signaling molecules are in close proximity. Conversely, removal of eNOS from caveolae may serve as a means to uncouple or desensitize the enzyme following prolonged agonist activation.

Plasmalemmal caveolae have a distinctive lipid composition, being highly enriched in cholesterol and glycosphingolipids while containing virtually no phospholipids (17-19). Alterations in cellular lipid composition may affect the structure and function of caveolae; indeed, the abnormalities in endothelium-dependent vasorelaxation seen in hypercholesterolemia may reflect the effects of serum lipids and lipoproteins on the structure and function of plasmalemmal caveolae. The close association between plasmalemmal caveolae and the cytoskeleton may reflect their role in the vascular mechanotransduction mediated by NO. Targeting of eNOS to plasmalemmal caveolae might also affect the local concentration of the enzyme's substrates and cofactors.

The eNOS regulatory cycle: reversible caveolin association, reversible translocation

In unstimulated endothelial cells, the eNOS enzyme is tonically inhibited by its protein-protein interactions with caveolin, the resident scaffolding protein in caveolae (22-24). Cell stimulation with Ca^{2+}-mobilizing agonists such as bradykinin promotes calmodulin binding to eNOS and caveolin dissociation from the enzyme, rendering the enzyme active; as intracellular Ca^{2+} returns to basal levels, calmodulin dissociates from the enzyme and the inhibitory eNOS-caveolin complex reforms (25). Upon more prolonged cell stimulation with bradykinin, eNOS is redistributed from particulate to more soluble cellular fractions, concomitant with depalmitoylation (12) and increased phosphoryla-

tion of the enzyme (26). It should be noted that the interaction between eNOS and caveolin is facilitated by, but does not require eNOS acylation (27); agonist-promoted depalmitoylation of eNOS is therefore unlikely to relieve caveolin's tonic inhibition of enzyme activity. Rather, following the sequence of eNOS depalmitoylation and translocation, enzyme palmitoylation appears to stabilize the eNOS on its return to plasmalemmal caveolae, in the process rebinding caveolin and completing the cycle of activation/de-activation (25).

Recent imaging studies have provided additional information on eNOS cellular targeting, revealing that Ca^{2+}-mobilizing agonists such as bradykinin induce translocation of eNOS from the plasmalemma to intracellular sites close to the nucleus (28). Bradykinin B_2 receptors are coupled through G proteins to the activation of phospholipases C and A_2 (29), leading to the transient increases in $[Ca^{2+}]_i$ characteristic of the physiological response to bradykinin. Bradykinin-activated increases in intracellular calcium arise from regions of the endothelial cell membrane enriched in caveolin, and quickly lead to increases in $[Ca^{2+}]_i$ throughout the cell. Increases in $[Ca^{2+}]_i$ appear to be both necessary and sufficient for eNOS translocation (28,30). The dynamic equilibrium of eNOS membrane targeting in cultured endothelial cells is thus exquisitely sensitive to changes in intracellular calcium concentration. The heterogeneity of eNOS immunostaining, and its lability in the face of diverse agonists and under different conditions, may also help to explain the discordance of recent observations with several earlier reports.

More recent studies have shown that estradiol induces translocation of eNOS by a Ca^{2+}-dependent, receptor-mediated mechanism, temporally associated with an estrogen-induced rise and fall in intracellular Ca^{2+} (30). Classically, estrogen and other steroid hormones are thought to bind to an intracellular receptor which, upon ligand binding, acts as a transcription regulatory factor (31). However, the finding that eNOS rapidly and reversibly translocates in response to estradiol is incompatible with a genomic mechanism of estrogen action. Rather, these effects of estradiol are better explained by a mechanism of action involving cell surface receptors. The first suggestive evidence for specific binding sites for estrogen at the cell membrane was provided by Pietras and Szego (32) more than twenty years ago. Since then, the concept of a non-genomic mechanism of steroid action has emerged from numerous studies on the rapid effects of steroid hormones (reviewed in Ref. 31). The identity and regulation of the receptor(s) that modulates the non-genomic effects of estrogen remain unknown, although a recent report has provided evidence that the classic estrogen receptor-alpha can activate eNOS in transfected cells (33). However, the molecular mechanisms underlying eNOS translocation in response to estradiol remain less well understood. The temporal pattern of eNOS translocation by estradiol resembles that of eNOS translocation in response to bradykinin (28), suggesting that these two events could be mediated by similar signaling pathways. Both estrogen and bradykinin-induced eNOS translocation are strictly Ca^{2+}-dependent processes (28,30); the temporal patterns of bradykinin- or estradiol-induced intracellular Ca^{2+} changes and eNOS translocation suggest that the Ca^{2+} spike precedes eNOS translocation.

We have proposed a model for the eNOS-caveolin regulatory cycle (7) wherein the association between eNOS and caveolin suppresses eNOS enzyme activity in the unactivated endothelial cell. Following agonist activation, increases in $[Ca^{2+}]_i$ promote calmodulin binding to eNOS and lead to the dissociation of caveolin from eNOS. The activated eNOS-calmodulin complex synthesizes NO until $[Ca^{2+}]_i$ decreases to the point that calmodulin dissociates and the

inhibitory eNOS-caveolin complex re-forms. Prolonged agonist activation leads to eNOS depalmitoylation, translocation, phosphorylation and ultimately to the re-binding of caveolin to the enzyme. The enzymes involved in the palmitoylation and depalmitoylation of signaling proteins are almost entirely unknown, and the protein kinases and phosphatases germane to eNOS regulation remain poorly understood. The identity of the intracellular compartment to which eNOS is translocated remains unclear; plausible locales include the nuclear membrane, the endoplasmic reticulum, trans-Golgi system or intracellular caveolae-derived vesicles, either or all of which may represent the "cytosolic" component of eNOS found after agonist activation.

The most plausible physiological consequence of eNOS redistribution following agonist activation is the enzyme's sequestration away from its proximal activators and concomitant attenuation of the extracellular release of NO. The time course of eNOS activation is much more rapid than the time course of enzyme translocation in the endothelial cell. The activation and translocation processes reflect distinct components of the agonist response, with translocation plausibly representing a mechanism to down-regulate or uncouple enzyme activation from receptor occupancy. Alternatively, translocation of the enzyme could play a role in redirecting the formation and release of NO to specific, as yet unidentified, intracellular sites. Taken together, our results establish that eNOS targeting to the cell membrane is labile and subject to receptor-regulated Ca^{2+}-dependent reversible translocation, thereby providing another point for regulation of NO-dependent signaling in the vascular endothelium.

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