Perspective Series: Nitric Oxide and Nitric Oxide Synthases

Nitric Oxide Synthases: Which, Where, How, and Why?

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The which of NOS: the NOS gene family

Although nitric oxide (NO)[1] may not modulate all cellular functions and may not be present in all mammalian cells, the sheer volume of publications on the subject might lead one to conclude that this diminutive molecule is both omnipotent and omnipresent in human biology. This series of four *Perspective* articles, in the current and following issues of *The Journal of Clinical Investigation*, will explore the limits of this assertion.[2]

NO is synthesized in mammalian cells by a family of three NO synthases (NOS). It is not known whether additional mammalian NOS isoforms exist, but the failure of homology-based molecular cloning approaches to identify novel NOS cDNAs makes it unlikely that newly discovered members of the mammalian NOS gene family will bear significant structural similarity to the current trio of isoforms (1). As for any newly described gene family, an accepted nomenclature of the NOS isoforms has evolved only as novel information becomes more generally established. The initial NOS nomenclature reflected the early observations that NO synthesis was not characteristic of unactivated inflammatory cells, but could become induced upon immunoactivation, hence the term iNOS. This prototypic "inducible" iNOS was contrasted to a "cNOS" activity that was constitutively expressed in certain characteristic cell types (neuronal, endothelial). However, it is now known that the levels of gene expression of both eNOS and nNOS may also be induced under different physiological conditions (e.g., hemodynamic shear stress or 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's being constitutive versus inducible NOS is misleading, and should be supplanted by a nomenclature that clearly identifies the specific enzyme isoform. A widely accepted nomenclature (3), which will be used in these *Perspective* articles, identifies the three mammalian enzyme isoforms as nNOS, iNOS, and eNOS, reflecting the tissues of origin for the original protein and cDNA isolates.

As denoted by its prefix, nNOS was originally purified and cloned from neuronal tissues. However, nNOS is now known to be much more widely distributed, with an important level of expression in skeletal muscle. iNOS, originally purified and cloned from an immunoactivated macrophage cell line, has since been identified in myriad mammalian tissues, and iNOS expression has been studied in cells as diverse as cardiac myocytes, glial cells, and vascular smooth muscle cells (to name only a few). eNOS, the last of the three mammalian NOS isoforms to be isolated, was originally purified and cloned from vascular endothelium, but has since been discovered in cardiac myocytes, blood platelets, brain (hippocampus), and elsewhere. To add to the confusion, the human genes for the NOS isoforms are officially categorized in the order of their isolation and characterization; thus, the human genes encoding nNOS, iNOS, and eNOS are termed *NOS1*, *NOS2*, and *NOS3*, respectively.

Clearly, the same NOS isoform may play entirely distinct biological roles when expressed in different tissues, and it must not be assumed that pathways outlined in one tissue necessarily pertain when the same isoform is expressed in a different cell. For example, differential tissue-specific splicing of nNOS mRNA generates structurally distinct protein molecules when the enzyme is expressed in neurons versus skeletal muscle (4). Another example of tissue-specific regulation is reflected in the association of eNOS with different caveolin isoforms in endothelial cells versus cardiac myocytes (5). Furthermore, the complexity of NOS catalysis, reflected in the diversity of NOS cofactors and cosubstrates, almost certainly leads to important cell-specific differences in NOS regulatory pathways.

These distinctions aside, there are important general biochemical features shared in common by the different NOS isoforms (6, 7). Indeed, the overall amino acid sequence identity for the three human NOS isoforms is $\sim$ 55%, with particularly strong sequence conservation noted in regions of the proteins importantly involved in catalysis (1). The different NOS isoforms share a similar overall catalytic scheme, involving the five-electron 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 cosubstrates, with numerous other redox cofactors including enzyme-bound heme, reduced thiols, FAD, FMN, and tetrahydrobiopterin. For all three NOS isoforms, NO synthesis depends upon the enzyme's binding of the ubiquitous calcium regulatory protein calmodulin. For eNOS and nNOS, increases in

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1. *Abbreviations used in this paper:* $[Ca^{2+}_i]$, intracellular Ca^{2+} concentration; NO, nitric oxide; NOS, NO synthase.

2. Where possible, references in support of introductory material will cite recent review articles rather than the numerous primary publications that form the basis for our current understanding.

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resting intracellular Ca^{2+} concentrations ($[Ca^{2+}_i]$) are required for their binding calmodulin and, consequently, for their becoming fully activated. By contrast, iNOS appears able to bind calmodulin with extremely high affinity even at the low $[Ca^{2+}_i]$ characteristic of resting cells. Thus, the intracellular activity of eNOS and nNOS may be 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 (7).

Recent reviews have discussed the transcriptional regulation of the different NOS isoforms (8), as well as recent advances in our understanding of NOS catalysis (9), and these important topics will not be addressed in substantive detail here. This *Perspective* explores advances in our understanding of the intracellular regulation of the NOS enzymes themselves, reflecting new insights into NOS subcellular targeting, covalent modifications, and protein–protein associations. These features of NOS intracellular regulation will be put into a broader pathophysiological context in the subsequent *Perspective* articles in this series. The accompanying *Perspective* article by David G. Harrison entitled "Cellular and Molecular Mechanisms of Endothelial Cell Dysfunction" (9a) discusses the regulation of eNOS in the vascular wall, the key role of coregulatory pathways in modulation of eNOS activity, and the metabolic fate of NO in the context of other redox pathways. The next issue of *The Journal* will contain *Perspective* articles by David Bredt and Carl Nathan focusing on the pathophysiology of nNOS and iNOS, respectively.

The where (and why) of NOS: subcellular localization and the biological roles of NO

NO is a labile molecule and may carry out important biological roles both within the cell in which it is synthesized, as well as in interactions with nearby cells and molecules (10, 11). Since 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 be expected to influence the biological role and chemical fate of the NO produced by the enzyme. Almost every conceivable intracellular organelle has been postulated as a possible site for NO synthesis, from the plasma membrane to the cell nucleus. There is considerable controversy and confusion in this area, possibly reflecting the diversity of experimental approaches made possible by a widespread availability of NOS antibodies, activity stains, and enzyme assays for these important proteins. Unfortunately, the determination of a protein's subcellular localization can be a tricky business, and conflicting conclusions have often been reached, sometimes even by the same laboratory. Below are listed most of the major subcellular compartments, all of which have been alleged as sites for NOS expression. Can the NOSs really be found everywhere? Perhaps the answer to this question is yes, but the supporting evidence varies in both quantity and quality.

NOS in the cell nucleus. Several lines of investigation have documented effects of NO on gene transcription, yet the regulatory molecules thought to serve as intermediaries in modulating the transcriptional effects of NO are, for the most part, cytosolic proteins (e.g., I-κB, guanylate cyclase) (12, 13). To date, there is no definitive evidence that any of the NOS isoforms are localized to the cell nucleus, although a handful of immunohistochemical studies have provided suggestive but inconclusive data (14). Perhaps the absence of NOS in the cell nucleus reflects that fact that NO itself can damage DNA (15), and direct transcriptional regulation by NO generated by NOS within the nucleus may lead to genotoxicity.

NOS in endoplasmic reticulum. The majority of nNOS immunoreactivity in neurons is associated with rough endoplasmic reticulum and within specialized electron-dense synaptic membrane structures (16). Although synthesis of the NOS proteins clearly involves this organelle, it is less clear that the endoplasmic reticulum serves as an ultimate target for the NOS isoforms.

NOS in mitochondria. Several studies have shown that NO derived from pharmacological sources, and administered in pharmacological doses, may inhibit mitochondrial respiration (17). Less clear are the data suggesting that NOS itself is present in mitochondria (18). Several studies provide interesting but inconclusive immunohistochemical data demonstrating mitochondrial staining for all three NOS isoforms. Although it seems plausible that NOS targeted to mitochondria could play a role in the modulation of oxidative phosphorylation, the specific NOS isoform(s) so targeted remains to be clearly identified. Clearly, more needs to be learned in this key area, and definitive studies are, no doubt, underway.

NOS in the Golgi apparatus. There have been numerous reports identifying eNOS in the Golgi apparatus (for review see reference 19). However, the interpretation of many of these studies is confounded by the experimental challenges involved in the assignment of a particular protein to a specific subcellular organelle. For example, the pattern of immunohistochemical staining for Golgi markers can easily be confused with the staining pattern characteristic of plasmalemmal caveolae (20), and earlier reports on eNOS targeting have subsequently been reinterpreted with this realization. Although it appears plausible that eNOS biosynthesis and/or recycling may involve the Golgi apparatus, the relevance of this organelle to eNOS regulation is not rigorously established.

NOS in the cytoskeleton. Although nNOS was first characterized as a soluble (cytosolic) protein, it is now clear that this protein undergoes an important association with the sarcolemma by virtue of its association with the cytoskeletal dystrophin complex in skeletal muscle (21, 22). This discovery provides an excellent example whereby a single NOS isoform may undergo tissue-specific regulation by virtue of cell-specific protein–protein associations. There are also suggestive studies that indicate eNOS may associate with cytoskeletal proteins (23, 24). In the case of eNOS, it is not known whether the enzyme undergoes direct interactions with cytoskeletal proteins, or whether these associations reflect the more general intercourse observed between the cytoskeleton and plasmalemmal caveolae. Certainly, the targeting or association of eNOS with the cytoskeleton may provide a mechanism for mechanochemical coupling of changes in cell shape (e.g., with hemodynamic shear stress or cardiac myocyte contraction) to regulation of the enzyme.

NOS in specialized intracellular organelles. Consistent with their expression in highly differentiated cells, NOS isoforms have been found in specialized intracellular organelles. In neuronal tissues, nNOS is localized in specialized postsynaptic densities, consistent with the enzyme's role in neurotransmission (22). However, the specific particulate subcellular fraction to which eNOS is targeted in hippocampal neurons has not been identified. The biochemical properties of purified, soluble iNOS have been characterized extensively, yet a substantial fraction

of iNOS activity can be found in the particulate subcellular fraction (25). This has lead some investigators to propose the existence of a distinct particulate iNOS species, an intriguing hypothesis for which there is little experimental evidence. In primary macrophages, subcellular fractionation and immunohistochemical approaches have established the presence of iNOS in intracellular vesicles (phagosomes?), possibly reflecting a locale for NO-dependent killing of opsonized intracellular microorganisms (26). The molecular mechanisms whereby iNOS is targeted to these macrophage vesicles is not clear, and it remains to be established whether iNOS is similarly targeted in other cells.

NOS in plasma membrane and plasmalemmal caveolae. The targeting of eNOS to the particulate subcellular fraction was noted in the earliest efforts to isolate the enzyme, which documented that detergents are required for eNOS solubilization (27). However, eNOS contains no hydrophobic transmembrane domain, and the association of eNOS with cell membranes is mediated principally by enzyme acylation (for review see reference 19). The identity of the specific subcellular organelle(s) to which eNOS is targeted has been a point of controversy, one now largely resolved by the discovery that eNOS is targeted to plasmalemmal caveolae (5, 28). Caveolae are small invaginations in the plasma membrane characterized by the presence of the transmembrane protein caveolin (29, 30). Plasmalemmal caveolae represent a prominent feature of the endothelial cell plasma membrane, and are also present in cardiac myocytes and many other cells. In many tissues, caveolae may serve as sites for the sequestration of signaling molecules (31) such as receptors, G proteins, and protein kinases, as well as eNOS. Recently, two G protein–coupled receptors, the muscarinic m2 and bradykinin B2 receptors, have been shown to be targeted to caveolae upon agonist stimulation (32, 33). These two receptors initiate signaling cascades leading to NO production in several cell types including endothelial cells and myocytes. 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 propinquity. Conversely, removal of eNOS from caveolae may serve as a means to uncouple or desensitize the enzyme after prolonged agonist activation, as will be discussed in detail below.

Plasmalemmal caveolae have a distinctive lipid composition, being highly enriched in cholesterol and glycosphingolipids while containing virtually no phospholipids (29–31). Alterations in cellular lipid composition may profoundly affect the structure and function of caveolae. It is intriguing to speculate that the derangement in endothelium-dependent vasorelaxation seen in hypercholesterolemia may reflect the effects of serum lipids and lipoproteins on the structure and function of plasmalemmal caveolae. Additionally, the close association between plasmalemmal caveolae and the cytoskeleton may reflect their importance in the vascular mechanotransduction mediated by NO. The targeting of eNOS to plasmalemmal caveolae might also influence the local concentration of the enzyme's substrates and cofactors, and thereby confound the interpretation of enzymological studies analyzed in cell-free systems.

What about targeting of nNOS or iNOS to caveolae? Caveolae have not yet been identified in neuronal cells, although it has been speculated that there exists a neuron-specific caveolin isoform (34). Caveolae are certainly present in skeletal muscle, and it appears possible, if not likely, that nNOS expressed in skeletal muscle may interact with the muscle-specific caveolin-3 isoform. Because the binding of caveolin and calmodulin are mutually exclusive (see below), it seems unlikely that iNOS, which binds calmodulin avidly, is regulated by interactions with caveolin or is targeted to caveolae.

The how of NOS: covalent modifications and protein associations

Phosphorylation. Phosphorylation is an important mechanism for the posttranslational regulation of diverse cellular proteins, ranging from metabolic enzymes to signaling proteins to transcription factors. Phosphorylation of the NOS isoforms (for review see reference 19) is of particular interest, as it would permit cross talk between NO and other signaling pathways, and may also serve as a modulator of other posttranslational modifications of the NOSs. Purified protein kinases can be shown to phosphorylate all three NOS isoforms, but the relevance of this observation to intracellular NOS regulation has not yet been established. The three NOS isoforms can be isolated as phosphoproteins in cultured cell systems, but the role and regulation of NOS phosphorylation is incompletely understood. It is reasonable to assume that the individual NOS isoforms may undergo phosphorylation by disparate protein kinases and for divergent reasons; even the same NOS isoform may be differentially phosphorylated when expressed in another tissue. To date, the specific amino acid residues modified by phosphorylation have not been identified for any of the NOS isoforms. This information could provide important insights into the nature of the protein kinase involved and could lead to experimental approaches that may elucidate the roles of phosphorylation in NOS regulation.

Purified nNOS serves as a substrate for a variety of several different protein kinases in vitro, but the effects of nNOS phosphorylation on enzyme activity are controversial, and the sites of serine and/or threonine phosphorylation are not yet identified. Definitive studies of nNOS phosphorylation in neurons, which have not yet been reported, may help clarify these observations. Phosphorylation of iNOS has been even less extensively characterized, but a recent report suggests that tyrosine phosphorylation of the enzyme may serve to increase its activity (35). Recently, tyrosine phosphorylation of eNOS was detected in intact endothelial cells treated with high concentrations of the phosphatase inhibitor, sodium orthovanadate (36). However, no physiological agonists for the tyrosine phosphorylation of eNOS have been identified, and other reports have failed to document any tyrosine phosphorylation of eNOS (24, 37). Certainly, many of the stimuli that activate eNOS in endothelial cells (e.g., shear stress and agonists such as bradykinin) also activate protein tyrosine kinases (23), but have not been shown to promote the tyrosine phosphorylation of eNOS itself. Phosphorylation of eNOS on serine residues has been shown in intact endothelial cells and appears to be regulated by diverse agonists (38) and by hemodynamic shear stress (37). However, the biological consequences of eNOS serine phosphorylation for the enzyme's tyrosine phosphorylation are not clearly defined. Moreover, the relative roles of the tyrosine and serine phosphorylation pathways are unclear. Serine phosphorylation of eNOS is associated with agonist-induced enzyme translocation (38). However, phosphorylation does not appear to be causal for eNOS intracellular translocation and may play a role in recycling of eNOS after its agonist-induced

eNOS translocation from plasmalemmal caveolae, as discussed below.

Acylation. eNOS is unique among the NOS isoforms in being dually acylated by the saturated fatty acids myristate and palmitate (for review see reference 19). eNOS myristoylation occurs cotranslationally on a NH_2-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 NH_2 terminus and serves to importantly stabilize eNOS 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, providing an important parallel with other reversibly palmitoylated signaling proteins such as $G\alpha_s$ (39). Depalmitoylation represents a plausible 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 a central problem in signal transduction (40). It is known that the targeting of eNOS to plasmalemmal caveolae is dependent upon palmitoylation of the protein. Therefore, it is 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 deactivation (see Fig. 1). Other reversible interactions may also modulate the subcellular localization of myristoylated proteins. For instance, it has been shown that the membrane targeting of recoverin, a myristoylated retinal protein, requires the binding of Ca^{2+} ions, which leads to the extrusion of recoverin's myristoyl group from an intramolecular hydrophobic region, permitting its interaction with biological membranes (41).

Protein–protein associations: beyond calmodulin

Calmodulin: the first NOS-associated protein. The requirement for calmodulin in NO synthesis is an essential characteristic of all three NOS isoforms, although with important differences between the NOS isoforms in the calcium dependence for their interaction with this ubiquitous Ca^{2+} regulatory protein (6, 7, 25). Calmodulin, the first NOS-associated protein thus demonstrates important isoform-specific differences in its role as an allosteric activator.

nNOS, PSD-93/95, and the dystrophin complex. The sarcolemma of skeletal muscle contains a family of intracellular and transmembrane glycoproteins associated with dystrophin, which link the extracellular matrix to the actin-based cytoskeleton. The NH_2 terminus of nNOS interacts with α1-syntrophin (a binding partner of dystrophin) through a protein motif (PDZ domain) present in both proteins (this domain is not present in either iNOS or nNOS) (21, 22). The PDZ-containing domain of nNOS also binds to related 93- and 95-kD postsynaptic density proteins (PSD-93 and PSD-95) (22). This protein interaction is important for nNOS targeting, but does not appear to directly regulate enzyme activity (22). Recent advances in this field will be discussed by David Bredt in the next issue of *The Journal.*

Other NOS-associated proteins. Stimulation of aortic endothelial cells with bradykinin produces cycles of tyrosine phosphorylation/dephosphorylation of an intriguing 90-kD protein, dubbed ENAP-1, for eNOS-associated protein-1 (24). To date, the molecular identity of ENAP-1 is unknown, yet one might already quibble with its proposed appellation: calmodulin, caveolin-1, and caveolin-3 are each eNOS-associated proteins, the identification of which antedate the discovery of ENAP-1. For nNOS, a 10-kD protein termed PIN (protein inhibitor of neuronal NOS) has been identified and appears to inactivate nNOS specifically by destabilizing the nNOS dimer (42).

eNOS and caveolin-1 and/or caveolin-3. eNOS is quantitatively associated with caveolin-1 in endothelial cells and with caveolin-3 in ventricular myocytes (6). The inhibitory effect of caveolin on eNOS activity can be completely reversed by Ca^{2+}-calmodulin (43). The interaction of eNOS with caveolin is mediated by direct protein–protein interactions that involve a 20–amino acid region within the caveolin sequence (44, 45), termed the "caveolin scaffolding domain" (46). Recently, Lisanti and colleagues (47) have identified consensus peptide sequences within the several proteins (namely H-Ras, $G\alpha_s$, Src, and eNOS) that appear to interact with the caveolin scaffolding domain. It is interesting that the region corresponding to the proposed caveolin binding sequence in eNOS (amino acids 350–358) is also present in nNOS, and it is possible that this region might also modulate nNOS–caveolin interactions. Since nNOS is known to bind to the dystrophin complex in skeletal muscle, which is in turn associated with plasmalemmal caveolae, caveolin interactions may represent a shared regulatory mechanism between eNOS and nNOS. Indeed, we have found recently that purified recombinant nNOS can be markedly inhibited by caveolin (Truss, R., J.B. Michel, O. Feron, M. Marletta, and T. Michel, unpublished observations).

A model for NOS protein–protein interactions: dynamic regulation of an eNOS–caveolin/calmodulin cycle. In the resting endothelial cell, the 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. Recently, we have found that agonist activation promotes the reversible, Ca^{2+}-dependent dissociation of the eNOS–caveolin complex (Feron, O., F. Saldana, J.B. Michel, and T. Michel, unpublished observations). Thus, transient changes in $[Ca^{2+}_i]$ consequent to agonist activation of endothelial cells are likely to be accompanied by cyclic changes in the interactions of eNOS with caveolin versus calmodulin.

A model summarizing this regulatory cycle is presented in Fig. 1. In the unactivated endothelial cell, the association between eNOS and caveolin suppresses eNOS enzyme activity (Fig. 1 *A*). After agonist activation, the increase in $[Ca^{2+}_i]$ promotes calmodulin binding to eNOS and the dissociation of caveolin from eNOS (Fig. 1 *B*). The activated eNOS–calmodulin complex synthesizes NO until $[Ca^{2+}_i]$ decreases to the point that calmodulin dissociates and the inhibitory eNOS–caveolin complex reforms. The precise molecular details and sequence of events involved in the desensitization and recycling of the enzyme, as depicted in Fig. 1 *C*, are rather murky. This speculative model incorporates several experimental observations; prolonged agonist activation leads to eNOS depalmitoylation, translocation, phosphorylation and ultimately to the rebinding of caveolin to the enzyme. However, 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 are poorly under-

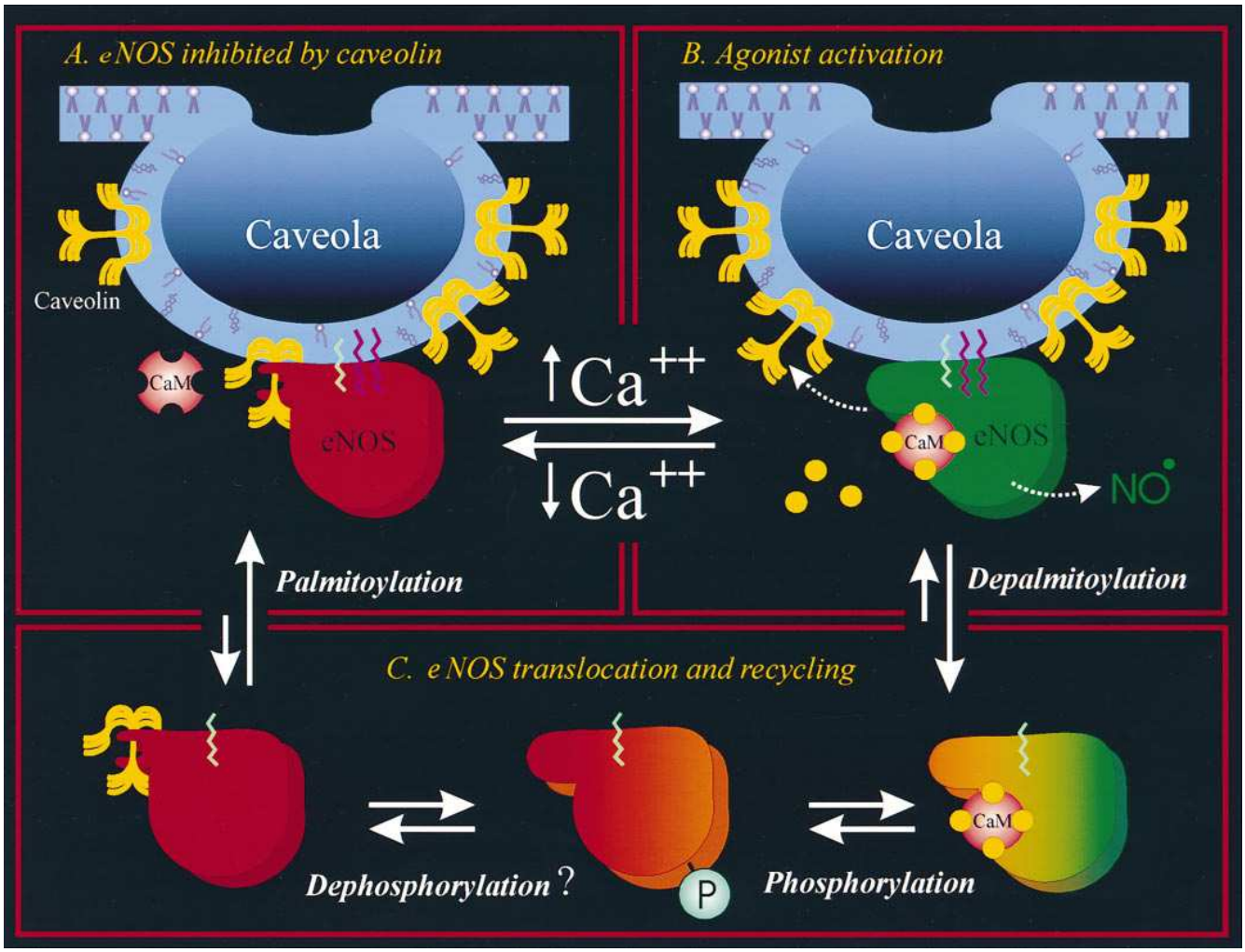

Figure 1. The cycle of eNOS activation and deactivation: reciprocal roles of Ca^{2+}-calmodulin and caveolin. (*A*) eNOS inhibited by caveolin. The interaction between eNOS and caveolin markedly attenuates enzyme activity in the resting endothelial cell. Shown is a plasmalemmal caveola characterized by its distinctive lipid content (cholesterol and glycosphingolipids vs. phospholipids in the adjacent noncaveolar plasma membrane) and by the presence of homo-oligomers of the membrane-associated scaffolding protein caveolin. The interaction of eNOS with the scaffolding domain of caveolin maintains the enzyme in its inactivated state (red). eNOS is shown as being dually acylated by *N*-myristoylation (teal) and by two molecules of palmitate (purple). The calcium regulatory protein calmodulin (*CaM*), which is enriched in the caveolar fraction of plasma membranes, does not bind to eNOS in the absence of Ca^{2+}. (*B*) Agonist activation. After agonist stimulation or other stimuli evoking a local increase in the $[Ca^{2+}_i]$ concentration, Ca^{2+}-bound CaM competitively displaces caveolin from eNOS, thereby allowing the conformational changes within eNOS required for the electron transfer between the heme protein and reductase domains (not shown), leading to the synthesis of NO by the activated enzyme (green). The binding sites for Ca^{2+}-CaM and caveolin on eNOS appear to be located in the NH_2-terminal domain of eNOS, and their binding is mutually exclusive. (*C*) eNOS translocation and recycling. The decline in $[Ca^{2+}_i]$ seen after prolonged agonist activation leads to the dissociation of calmodulin from eNOS and deactivation of the enzyme as caveolin rebinds to eNOS. Caveats concerning the molecular details, subcellular locale, and temporal sequence of this speculative pathway are discussed in the text. In the model proposed here, prolonged agonist activation leads to eNOS depalmitoylation, translocation, phosphorylation, and, subsequently, to the rebinding of caveolin as the enzyme becomes dephosphorylated and repalmitoylated on its return to caveolae. eNOS remains myristoylated throughout; this modification probably guides the retargeting of the caveolin–eNOS complex to caveolae. Illustration by Naba Bora, Medical College of Georgia.

stood. Even the identity of the intracellular compartment to which eNOS is translocated remains unclear. It is likely that some of the depalmitoylated enzyme translocates to the noncaveolar plasma membrane (28). Other plausible locales include the *trans*-Golgi system or intracellular caveolae-derived vesicles, either or both of which may represent the cytosolic component of eNOS found after agonist activation. It should be noted that the interaction between eNOS and caveolin is facilitated by, but does not require, eNOS acylation (48). Therefore, agonist-promoted depalmitoylation of eNOS is unlikely to relieve caveolin's tonic inhibition of enzyme activity. Rather, we propose that, after the sequence of eNOS depalmitoylation and translocation, enzyme palmitoylation may stabilize the eNOS on its return to plasmalemmal caveolae, and in the process may rebind caveolin and complete the cycle of activation/deactivation.

The interactions of eNOS with calmodulin versus caveolin provide a novel example of the reciprocal regulation of en-

zyme activity by competing allosteric protein–protein interactions. This close control of enzyme activity may be particularly important for eNOS in caveolae, where calmodulin is also enriched (28) and where even subtle increases in intracellular calcium could thus lead to enzyme activation if the interaction of caveolin with eNOS were not keeping the system in check. Because NO has cytotoxic as well as signaling functions, attenuation of basal enzyme "leakiness" by caveolin may be of particular importance.

Conclusions

Since the first reports six years ago describing the isolation of NOS proteins and molecular clones, thousands of publications have explored the "which, where, how, and why" of NOS. These discoveries provide a rich context for understanding the diverse roles of NO in human biology. Quantitative changes in NOS expression have been fortuitously or causally associated with disease states (49). Likewise, the abrogation of NOS expression by targeted gene inactivation or, conversely, the enhancement of NOS pathways by gene overexpression, has permitted insights to be gained by generating quantitative changes in NOS abundance (50, 51). However, it seems equally likely that qualitative changes in the NOS pathway may underlie many important aspects of NO physiology and pathophysiology. At least some of these qualitative changes may be effected by alterations in the enzymes' posttranslational modifications, subcellular targeting, or protein–protein interactions, all of which represent important areas for future investigation.

Acknowledgments

We regret that space limitations have prevented us from citing the numerous primary publications that form the basis for our current knowledge in this active field of investigation.

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