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Cell and Molecular Biology of Nitric Oxide Synthases

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THE MAMMALIAN NO SYNTHASE ISOFORMS

This chapter focuses on the factors influencing cellular and molecular regulation of the three known mammalian nitric oxide synthase (NOS) isoforms in the cardiovascular system. It is now known that the different NOS isoforms can be found in numerous different human tissues, including diverse locales within the cardiovascular system. The overall amino acid sequence identity for the three human NOS isoforms is approx 50–55%, with particularly strong sequence conservation in regions of the proteins involved in catalysis (1). Alignment of the amino acid sequences of the different NOS isoforms reveals two domains of amino acid sequence similarity along the length of the proteins. The NOS C-terminal domain, comprising nearly half the molecule, has been termed the “reductase domain,” as it bears striking sequence similarity to the mammalian cytochrome P450 reductase, and even shows significant sequence similarities to archetypal reductases from plants and bacteria. The N-terminal domain, variably termed the heme or oxygenase domain, shows significant sequence similarities only among the three members of the NOS family, and contains the site for binding of the enzymes’ heme prosthetic group. These striking similarities in the proteins’ primary structure are likely to be reflected in homologies in their tertiary structure, but three-dimensional structural data currently exist only for the iNOS isoform (2,3).

The three NOS enzyme isoforms are commonly denoted by prefixes that reflect the tissues of origin for the original isolation of their protein and cDNA: the nomenclature of nNOS, iNOS, and eNOS enzymes reflect their initial characterizations in neuronal tissue, immun-activated macrophages, and endothelial cells, respectively (4). The official nomenclature of the corresponding human NOS genes reflects instead the order of isolation and characterization of human genomic clones: the human genes encoding nNOS, iNOS, and eNOS are thus termed *NOS1*, *NOS2*, and *NOS3*, respectively. The three NOS genes share many features in their overall genomic structure, with striking similarity in the size of the exons and the location of the splice junctions, suggesting that the three NOS isoforms derive from a common ancestral gene. However, as might be anticipated from their distinct modes of transcriptional regulation and tissue-specific expression, there are significant divergences in the putative promoter regions among members of the NOS gene family.

The different NOS isoforms share a similar overall catalytic scheme, in which the homodimeric enzyme catalyzes the formation of NO[•] plus L-citrulline by oxidizing one of the two guanido nitrogens of the amino acid L-arginine. NOS catalysis involves the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen as cosubstrates, with the flavins adenine dinucleotide (FAD) and mononucleotide (FMN) representing key cofactors in promoting electron transfer to the NOS heme moiety; tetrahydrobiopterin

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represents another key cofactor, but the role of this compound in NOS catalysis remains less well understood. For all three mammalian NOS isoforms, binding of the ubiquitous Ca^{2+} -binding regulatory protein calmodulin (CaM) appears to be required for efficient electron transfer between the reductase and oxygenase domain of NOSs (5,6). The dependence of the different NOS isoforms on Ca^{2+} /CaM constitutes, however, a major difference in the regulatory mechanisms of NO^* production. Both nNOS and eNOS bind CaM in a reversible and Ca^{2+} -dependent manner, but iNOS avidly binds CaM even at the low ambient intracellular Ca^{2+} concentration characteristic of resting cells. iNOS activity in the cell is therefore largely independent of changes in intracellular Ca^{2+} (7), whereas the temporal pattern of nNOS or eNOS activation is closely regulated by transient changes in intracellular Ca^{2+} . The level of cellular iNOS activity appears to be closely related to the amount of iNOS protein, which, as a first approximation, is determined by mRNA abundance, which is in turn governed by the rate of iNOS gene transcription and by the stability of its mRNA. The principal form of iNOS regulation does appear to be at the level of genetic induction, hence its common appellation as an “inducible” enzyme. However, iNOS may be constitutively expressed under physiological conditions in some tissues, including pulmonary and bladder epithelia, and renal medulla (8,9). The eNOS and nNOS enzymes are often found expressed at stable levels in their characteristic tissues, and were originally denoted as “constitutive” enzymes. However, it has become clear that the expression of eNOS and nNOS genes can be regulated under different physiological and pathophysiological conditions (e.g., hemodynamic shear stress, nerve injury). In addition, posttranscriptional and posttranslational modifications importantly modulate the structure and function of all three NOS isoforms, as we discuss in detail in the following sections.

Neuronal NOS

GENOMIC STRUCTURE AND mRNA PROCESSING

The gene encoding nNOS (*NOS1*) includes 29 exons scattered over a region of 200 kb located on the human chromosome 12 (10,11). The full-length open reading frame of *NOS1* encodes a protein of 1434 amino acids with a predicted molecular mass of 160 kDa (12,13). Two major transcriptional clusters, denoted as neuronal- and testis-specific, have been identified for human *NOS1* (11,14); the identification of three potential polyadenylation sites adds to the complexity of *NOS1* posttranscriptional processing (14).

In the neuronal transcriptional cluster of the *NOS1* gene, distinct first exons of nNOS appear to splice to a common exon 2, which contains the initiator ATG codon. These different mRNA species, therefore, each encode the full-length nNOS protein, but the existence of distinct transcription initiation sites in different first exons may reflect the existence of tissue-specific or developmentally regulated *NOS1* promoters. By contrast to the neuronal transcription cluster, the site for transcription initiation from the testis-specific cluster is located between exons 3 and 4, and the predicted site for initiation of translation lies within exon 5. Translation at this site would yield a truncated protein of 125 kDa, which has been termed TnNOS. The existence of the TnNOS protein is inferred from the isolation of its cognate transcript by highly sensitive reverse transcriptase–polymerase chain reaction techniques (RT-PCR), and its corresponding cDNA can be expressed in heterologous cell systems, but the naturally occurring TnNOS protein has not itself been identified (15). The truncated TnNOS would lack key sequences at the protein’s extreme N-terminal (PDZ domain) thought to be important for nNOS targeting and protein–protein interactions in the full-length nNOS isoform. In the mouse, additional alternatively spliced nNOS transcripts have been identified: $\text{NOS}\beta$ and $\text{NOS}\gamma$ appear to be analogous to the human TnNOS in that they lack exon 2 and would encode truncated nNOS without the N-terminal PDZ domain (16). Indeed, $\text{NOS}\gamma$ and β were shown to account for the residual nNOS activity in nNOS knockout mice in which gene targeting inactivated the first coding exon but still allowed processing of

transcripts starting downstream from the site of *NOS1* gene disruption (17). Other exon deletions (exons 9/10) and insertions (between exon 16 and 17) have also been documented (11,18,19). Although it remains to be demonstrated that the exon 9/10 deleted transcript is actually translated in vivo, some of the nNOS splice variants appear to be differentially regulated in cellular models of morphine tolerance (20). Another alternatively processed nNOS transcript has been termed μ NOS, which is formed by the insertion of 102 bp between exons 16 and 17. Expression of the μ NOS protein has been established in rat and human penis as well as in rat skeletal muscle and heart (18,19), and μ NOS represents the only alternatively spliced nNOS transcript that has clearly been shown to correspond to a naturally occurring novel nNOS protein. The functional role of μ NOS remains less well understood, but the presence of a distinct nNOS enzyme generated by differential mRNA processing identifies another potential point of regulation.

CELLULAR EXPRESSION AND (POST-)TRANSCRIPTIONAL REGULATION

To date, nNOS expression has been identified in diverse neurons throughout the central and peripheral nervous systems (20), and nNOS has also been found in numerous nonneuronal tissues including skeletal muscle (13,18), lung (21), and the genitourinary tract (15,19). Increases in the abundance of nNOS mRNA have been noted following physical and mechanical stresses, including spinal cord and nerve injuries (22), ischemia (23), hypoxia (21), as well as changes in plasma osmolarity (24). Neurotransmitters and hormones also influence the spatial and temporal pattern of *NOS1* expression: upregulation has been reported in various tissues during pregnancy (25) and in cerebellar granule cells following inhibition of glutamatergic transmission (26); a development switch to μ NOS (*see above*) was recently shown to occur in skeletal muscle in the process of myotube fusion (18). Downregulation of *NOS1* expression is documented in rat brain following corticosterone treatment (27) and is also generally observed following bacterial lipopolysaccharide (LPS) and interferon- γ (28,29).

Functional characterization of the *NOS1* gene transcriptional regulatory regions has yet to be established despite the identification of several *cis*-acting and *cis*-regulatory elements in the neuronal and testis transcriptional clusters (11,14). Consensus sequences located in the putative *NOS1* promoter region suggest a possible role for diverse regulatory elements, including AP-1, AP-2, Sp-1, transcriptional enhancer factor-1/M-CAT binding factor, CREB, ATF, *cFos*, Ets, NF-1, NF- κ B motifs, GATA sites, p53 half-element, myocyte-specific enhancer factor 2 motif, and an insulin-responsive element. The presence of these putative regulatory motifs does not, in itself, establish their biological relevance, and the role, if any, of these diverse sequences for *NOS1* gene regulation remains to be defined.

“Inducible” Nitric Oxide Synthase

GENOMIC STRUCTURE AND mRNA PROCESSING

The iNOS gene (*NOS2*) is located on the human chromosome 17 and contains 26 exons spanning a region of 37 kb (30). The full-length open reading frame encodes a protein of 1153 amino acids with a predicted molecular mass of 130 kDa. Alternatively spliced iNOS transcripts have been identified using RT-PCR chain reaction techniques (31). Although they were using recombinant iNOS mutants, these authors showed that alternative splicing of exons 8 and 9 is critical for the enzyme dimerization, the endogenous expression of novel iNOS proteins derived from these transcripts has not yet been detected in tissues or cells.

CELLULAR EXPRESSION AND (POST-)TRANSCRIPTIONAL REGULATION

Since the prototypical iNOS enzyme was first characterized expressed in murine macrophages, numerous studies have documented that the *NOS2* gene can be induced in many different cell types. In human tissues, the initial report of iNOS expression in human hepatocytes (32) was followed by a plethora of studies showing iNOS expression following immunoactivation

of macrophages, monocytes, myocytes, epithelial and endothelial cells, astrocytes, fibroblasts, keratinocytes, osteoblasts, and neutrophils/eosinophils (for review *see* ref. 33). Although iNOS induction may generally reflect a pathophysiological cellular response to immunoactivation, it has been recently found that iNOS may be constitutively expressed in some tissues without any known antecedent exposure to pathological immunoactivating stimuli (8,9), suggesting that iNOS may be subserving a physiological role in some tissues.

Most commonly, however, transcriptional induction of iNOS appears to be a consequence of immunoactivation, and numerous reports in a wide variety of cell types have explored iNOS induction in response to bacterial endotoxin or following stimulation by cytokines such as interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α), and interleukin-1 (IL-1). It should be emphasized that even though a large number of different cell types have been found to express iNOS under different conditions of gene induction, one must use caution in extrapolating the transcriptional regulatory mechanisms characterized for one cellular system to another. For example, in some cell types the *NOS2* gene may be induced in response to cyclic adenosine monophosphate (cAMP)-elevating agents, by activation of protein kinase C (PKC), or by the action of various growth factors including platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) (33). Comparison of the rodent and human *NOS2* gene promoter indicates that although only 1 kb of the proximal 5' flanking region of the murine promoter is required to confer endotoxin and cytokine inducibility, other critical cytokine-responsive elements are present between 3.8 and 16 kb upstream the initiation codon of the human gene (34–36). Three γ -IRE as well as NF- κ B, NF-IL6, and TNF-RE sites have however been identified in the very proximal 5'-flanking region of the human *NOS2* gene (37–40). Among these elements, a functional role in transcriptional regulation of *NOS2* gene has been demonstrated for γ -IRE and NF- κ B sites, and these elements appear to be involved in IFN- γ - and endotoxin-stimulated gene expression, respectively (37,38). Shortly after the characterization of *NOS2* gene induction in macrophages and hepatocytes, several groups provided evidence for a similar glucocorticoid- and TGF- β -sensitive induction of iNOS by cytokine and lipopolysaccharide (LPS) in cardiac myocytes and microvascular endothelial cells (41). As reported in other cell types, induction of the *NOS2* gene by IL-1 β and IFN- γ in cardiac myocytes appears to be preceded by activation of MAPK (ERK1/ERK2) and STAT1 α phosphorylation, a finding compatible with the presence of AP-1 and GAS sequences in the *NOS2* promoter (42). Downregulation of *NOS2* gene induction is characteristically observed in various cell types following treatment with glucocorticoids or TGF- β (33). The mechanisms involved in the deactivation of iNOS are less well understood: depending on the cell type, plausible regulatory pathways might include the repression of *NOS2* transcription, alterations in iNOS mRNA stability and/or processing, as well posttranslational mechanisms.

Endothelial Nitric Oxide Synthase

GENOMIC STRUCTURE AND mRNA PROCESSING

The gene encoding for eNOS (*NOS3*) is located on human chromosome 7 and contains 26 exons spanned on a region of 22 kb (43–45). The full-length open reading frame codes for a protein of 1205 residues with a predicted molecular mass of 135 kDa. Alternative polyadenylation sites have been identified in the 3' untranslated region of the eNOS mRNA (K. Sase and T. Michel, unpublished observations; *see* ref. 46), and may influence the differential stability or subcellular targeting of eNOS transcripts. Alternatively spliced transcripts encoding novel eNOS proteins have not been described.

CELLULAR EXPRESSION AND (POST-)TRANSCRIPTIONAL REGULATION

Immunohistochemical studies have located eNOS in various types of venous and arterial endothelial cells (47), and significant endogenous expression was also reported in myocytes (48), in neuronal cells (49), in platelets (50), and in various other tissues (*see* ref. 51).

Present in the putative TATA-less promoter region of the eNOS gene are consensus sequences that potentially may serve as sites of binding for transcription/nuclear factors such as AP-1, AP-2, NF-1, IL6, as well as several putative NF- κ B sites and several half-palindromic estrogen response elements (43–45). Recently, two tightly clustered *cis*-regulatory regions were identified in the proximal enhancer of the human eNOS promoter using deletion analysis and linker-scanning mutagenesis (52): positive regulatory domains I (–104/–95 relative to transcription initiation) and II (–144/–115). Analysis of *trans*-factor binding and functional expression studies revealed a surprising degree of cooperativity and complexity. The nucleoprotein complexes that form upon these regions in endothelial cells contained Ets family members, Sp1, variants of Sp3, MAZ, and YY1. Functional domain studies in *Drosophila* Schneider cells and endothelial cells revealed examples of positive and negative protein–protein cooperativity involving Sp1, variants of Sp3, Ets-1, Elf-1, and MAZ (52). Therefore, multiprotein complexes are formed on the activator recognition sites within this 50-bp region of the human eNOS promoter in vascular endothelium.

Hemodynamic shear-stress and chronic exercise are among the stimuli that are associated with an increased abundance of the eNOS transcript (53,54). Consensus sequences present in the eNOS 5' flanking region may represent *cis*-regulatory elements responsive to shear stress, and the presence of several half-palindromic estrogen-responsive elements may provide the means for transcriptional regulation of the eNOS gene in response to physiological or pathophysiological perturbations.

Hypoxia appears to influence eNOS abundance in cultured endothelial cells, causing a decrease in steady-state eNOS transcript levels associated with a decrease in eNOS mRNA stability (55). However, in animal models, chronic hypoxia appears to be associated with an *increase* in eNOS mRNA abundance (21). Cell proliferation also profoundly influences the expression of eNOS in cultured endothelial cells: eNOS transcript and protein abundance diminishes significantly when cells reach confluence (56). Thus, any factors that influence endothelial cell growth rate may confound the interpretation of primary effects on eNOS gene expression. The nature, magnitude, and physiological relevance of studies showing altered eNOS transcript abundance analyzed in cultured endothelial cell model systems continue to be actively investigated. Among many other perturbations, sex steroids (57,58) or lipoproteins (59–61) may also influence the abundance of the eNOS transcript; findings in different experimental models have yielded mutually contradictory results and must be interpreted with caution.

Cytokines such as TNF- α are associated with a decrease in eNOS message and protein abundance in aortic endothelial cells, whereas paradoxically, NO synthesis increases (likely due to influences of the cytokine on NOS cofactor levels) (62–64). The TNF- α -induced decrease in the abundance of the eNOS mRNA in bovine aortic endothelial cells appears to be due to a decrease in eNOS mRNA stability (65); in contrast, transforming growth factor- β appears to increase the abundance of the eNOS transcript (66).

POLYMORPHISMS IN THE *NOS3* GENE

The central role of NO in blood pressure homeostasis has led to studies exploring whether polymorphisms in any of the three NOS genes, or abnormalities in NOS cellular regulation, may be associated with hypertension. To date, there have been compelling experimental or population-based studies in support of a relationship between human *NOS1* polymorphism and hypertension. Although the *NOS2* gene maps to a region in the rat genome linked to hypertension in Dahl salt-sensitive rats (67), disease association, and/or the relevance of this finding to human disease has yet to be established. Numerous studies have failed to link polymorphisms in introns of the human *NOS3* gene with cardiovascular diseases (68); yet recent studies have documented the association of the missense variant Glu298Asp in exon 7 of the eNOS gene with essential hypertension and acute myocardial infarction in two independent populations of Japanese patients (69,70). Interestingly, the same missense variant Glu298Asp

was shown to be an independent risk factor for vasospastic angina pectoris (71) in Japanese populations. In contrast, other authors failed to find a relationship between the Glu298Asp polymorphism and ischemic cerebral disease in Caucasian populations (72). Taken together, these results seem to indicate that, at least in Japanese populations, the missense variant Glu298Asp polymorphism is associated with, and could predispose to, various cardiovascular diseases. However, the functional consequences of the conservative amino acid change encoded by this eNOS genetic polymorphism have yet to be established.

Cardiovascular Phenotype of NOS Gene Knockout Mice

The characterization of mice with targeted disruption of NOS genes (NOS “knockout” mice) has provided important insights into the physiological and pathophysiological roles of the individual NOS isoforms in the cardiovascular system. Despite the ubiquitous expression of the NOS and their striking evolutionary conservation (and contrary to some expectations), mice with homozygous deletions of individual NOS isoforms are entirely viable. The roles of NO in diverse physiological processes can apparently be largely supplanted or compensated by other regulatory pathways in the cardiovascular system and elsewhere. For example, the nNOS knockout mice do not show any gross neuroanatomic abnormalities or even alteration of long-term potentiation (LTP) processes (73), but do exhibit pyloric stenosis, and have been reported to display abnormal sexual and aggressive behavior (74). iNOS knockout mice are grossly normal, but show increased susceptibility to infection (75,76). From the standpoint of cardiovascular homeostasis, it is the eNOS knockout mice that document a most dramatic phenotype: eNOS^{-/-} mice are hypertensive and show a mean arterial blood pressure approx 30% higher than wild-type littermates (77). This is a key finding: despite the wide array of compensatory mechanisms controlling vascular tone, the genetic abrogation of eNOS cannot be overcome and leads to hypertension. However, treatment of eNOS null mice with pharmacological NOS inhibitors led to a paradoxical decrease in blood pressure (77), suggesting perhaps a role for nNOS in the maintenance of blood pressure (note that nNOS^{-/-} mice present a tendency toward hypotension under anesthesia [78]). Actually, the critical role of NO[•] produced by eNOS in the regulation of vascular tone was recently specifically addressed by the generation of transgenic mice overexpressing eNOS in the vascular wall (using murine preproendothelin-1 promoter) (79). These authors reported that in agreement with the observed increase in basal NO[•] release and cGMP levels in transgenic aorta, blood pressure was significantly lower in eNOS-overexpressing mice than in control littermates.

The central role of NOS isoforms becomes much more evident when NOS knockout mice are subjected to pathophysiological perturbations. For example, a murine model of operatively induced hindlimb ischemia was recently used to investigate the impact of targeted disruption of the eNOS gene on angiogenesis (80). Laser Doppler flow analysis and capillary measurements revealed that angiogenesis was impaired and was not improved by vascular endothelial growth factor (VEGF) administration in eNOS^{-/-} mice versus wild-type. Moreover, in cerebral ischemia, NO[•] is known to rise dramatically associated with tissue damage, but the cellular source of NO[•] and its biological role were less well understood. Analyses of nNOS^{-/-} mice with experimental cerebral infarcts documented reduced infarct size when compared with age-matched wild-type animals; these changes occur independently of alterations in blood flow (81). The nNOS^{-/-} mice are also resistant to ischemic injuries (82), indicating that nNOS is importantly involved in neurotoxic damages. Interestingly, nonspecific NOS inhibitors appear to attenuate the reduced infarct size found in nNOS^{-/-} mice, plausibly by inhibiting the eNOS-mediated relaxation of pial vessels. Indeed, transgenic mice that lack eNOS show, after middle cerebral artery (MCA) occlusion, increased infarct volume and reduced cerebral blood flow (83). Furthermore, nonspecific NOS inhibitors decrease the infarct size in eNOS^{-/-} mice but not in wild-type animals, confirming both that eNOS plays a protective role by maintaining regional cerebral blood flow in the setting of

ischemia and that nNOS contributes to neurotoxicity. iNOS also appears to be involved in late neuronal injury following experimental stroke: iNOS null mice show reduced infarct volumes when compared with wild-type animals (84).

COVALENT MODIFICATIONS OF NOS ISOFORMS

Phosphorylation

The three NOS isoforms can be phosphorylated *in vitro* by purified protein kinases and can be isolated as phosphoproteins in cultured cell systems. However, to date, the roles of specific protein kinases and phosphatases have not been specifically delineated any of the isoforms and the role and regulation of NOS phosphorylation remain incompletely understood. For example, although nNOS has been shown to serve as a substrate for a variety of protein kinases *in vitro* (49,85,86), phosphorylation of nNOS in neurons has not been definitively demonstrated. In addition, different studies have observed variable effects of nNOS phosphorylation on enzymatic activity, and these *in vitro* analyses have not yet been clearly correlated with the enzyme's phosphorylation in native cells (85,86). Phosphorylation of iNOS has been even less extensively characterized (7), although a recent report suggests that tyrosine phosphorylation of the enzyme may serve to increase its activity (87). Serine phosphorylation of eNOS was shown to occur in endothelial cells subsequent to agonist-induced translocation of the enzyme to the cytosol (88), suggesting that eNOS deactivation may be regulated by phosphorylation. Treatment of endothelial cells with phorbol esters has also been shown to diminish NO[•] production, suggesting that phosphorylation may be associated with inhibition of eNOS activity (89). Possible clinical significance for these findings was suggested by studies reported by Craven et al. (90), who correlated increased PKC activity with decreased NO[•] production in glomeruli isolated from diabetic rats. These investigators further demonstrated that inhibition of PKC restored normal NO[•] production, suggesting that changes in eNOS phosphorylation may modulate the alterations in NO[•] signaling observed in diabetic vascular disease. Additionally, several groups have reported that the activation of eNOS by hemodynamic shear stress in cultured endothelial cells is influenced by reagents that modulate protein tyrosine kinase activity (91–93). Tyrosine phosphorylation of eNOS has been explored in several studies with contradictory results: several investigators failed to document any tyrosine phosphorylation of eNOS (92,94) whereas others reported either tyrosine phosphorylation (95) or dephosphorylation (93) of eNOS following incubation of endothelial cells with high concentrations of protein tyrosine phosphatase inhibitors. To date, no physiological agonists promoting the tyrosine phosphorylation of eNOS have been identified, although many agonists that modulate eNOS clearly affect tyrosine phosphorylation pathways, as well.

Acylation

MYRISTOYLATION AND PALMITOYLATION

eNOS is unique among the NOS isoforms in its being dually acylated by myristate and palmitate 14- and 16-carbon saturated fatty acids, respectively (for review *see* ref. 50), and importantly, both modifications are required for an efficient targeting of the enzyme to plasmalemmal caveolae (96). Myristoylation occurs cotranslationally on an N-terminal glycine residue within a specific consensus sequence (MGXXS) (97) and is essentially irreversible, precluding its dynamic regulation by agonists or other stimuli (98). Moreover, the stable membrane association of myristoylated proteins often requires hydrophobic or electrostatic interactions in addition to those between myristate and membrane lipids; for eNOS, several lines of evidence (99,100) suggest that this membrane-targeting role is subserved by palmitoylation.

Palmitoylation of eNOS takes place on two cysteine residues near the protein's N-terminus (Cys-15 and Cys-26) that define a novel motif for protein palmitoylation (99,101). No general consensus sequence for protein palmitoylation has been identified (98,102), although some dually acylated G protein α subunits and members of the Src family of tyrosine kinases are palmitoylated at a cysteine residue within a conserved N-terminal sequence not found in the NOS isoforms. The two palmitoylated cysteines in eNOS flank an unusual Gly–Leu repeat [(Gly–Leu)₅] not otherwise described in the protein sequence database. Mutagenesis of the palmitoylation site cysteine residues (to serine) markedly attenuates the association of eNOS with the particulate subcellular fraction, documenting a key role for this posttranslational modification in eNOS targeting (99). The myristoylation-deficient mutant eNOS also fails to undergo palmitoylation (100), plausibly because the mutant is not targeted to the plasma membrane, the presumed site for protein palmitoylation (98). The myristoylation-deficient eNOS is thus de facto an acylation-deficient enzyme, undergoing neither of the fatty acid modifications characteristic of the wild-type eNOS; this acylation-deficient enzyme is entirely cytosolic. The palmitoylation-deficient mutant eNOS still undergoes myristoylation, and, as noted earlier, its membrane targeting is reduced but not completely abrogated. Dual acylation of eNOS is thus required for efficient membrane localization, with cotranslational *N*-myristoylation and posttranslational thiopalmitoylation playing key roles in enzyme targeting.

DYNAMIC REGULATION OF PALMITOYLATION

Palmitoylation is a reversible posttranslational modification that has been shown to modulate the interaction of signaling proteins with the membrane (103). For example, agonist regulation of protein palmitoylation has previously been described for G protein-coupled receptors, such as the β -adrenergic receptor (104). For some peripheral membrane proteins, the loss of palmitate may correlate with protein redistribution to the cytosolic subcellular fraction. Agonists activating G protein α_s appear to stimulate α_s palmitate turnover, specifically accelerating depalmitoylation (102,105). There are striking parallels for eNOS: pulse-chase experiments in endothelial cells biosynthetically labeled with [³H]palmitate showed that bradykinin treatment may promote eNOS depalmitoylation (100). However, it must be noted that another study (101) failed to document agonist modulation of eNOS palmitoylation; interpretation of the negative results of this latter study are confounded by the challenges of studying intracellular modulation of biosynthetically labeled proteins under non-steady-state conditions. This controversy aside, reversible palmitoylation of eNOS represents a plausible mechanism for modulating the binding of a large hydrophilic protein such as eNOS to membranes. Depalmitoylation could therefore be part of a cellular mechanism for the release of (myristoylated) eNOS from the plasma membrane and translocation to other cellular structures in response to agonist stimulation. Conversely, repalmitoylation could then facilitate the process of retargeting to specialized compartments such as caveolae (*see* following).

The palmitoylation of several signaling proteins has been shown to influence their signaling activities as well as their subcellular localization (98,102). However, important differences have been noted in the regulatory roles of palmitoylation, even among closely related proteins. The receptor-mediated processes that regulate reversible palmitoylation of signaling proteins are not well understood, and few enzymes involved in the formation or hydrolysis of palmitoyl–protein thioesters have been extensively characterized. A protein palmitoylthioesterase was recently isolated and cloned from bovine brain (106). This palmitoylthioesterase is expressed in diverse cell types, including vascular endothelial cells (107), but its regulatory characteristics are not fully defined, and its relationship to eNOS palmitoylation is completely unknown. Another palmitoylthioesterase has been isolated that appears to be involved in the depalmitoylation of the G protein α_s (108), but the role of this enzyme in the regulation of eNOS depalmitoylation remains to be established. Another possibility is that eNOS activation itself could influence depalmitoylation, with NO production itself playing a role. It has recently been shown that NO reduces [³H]palmitate labeling of two nerve growth

cone-associated proteins (109). NO might also regulate palmitoyl thioesterase activity or directly influence eNOS palmitoylation via nitrosothiol formation at the site(s) of palmitoylation.

An understanding of the regulation of eNOS palmitoylation/depalmitoylation cycles has been facilitated by the development of heterologous cellular expression systems that permit the reconstitution of endogenous signaling pathways by cotransfecting cDNAs encoding wild-type and acylation-deficient eNOS along with constructs encoding specific cell surface receptors. Using this approach, it was shown that muscarinic cholinergic agonist stimulation rapidly induced the reversible dissociation of eNOS from caveolin. These studies revealed further that enzyme repalmitoylation markedly accelerated eNOS retargeting to caveolae following prolonged agonist stimulation (110).

FUNCTIONAL ROLE OF eNOS TARGETING

The importance of eNOS myristoylation was recently illustrated in an elegant study examining the implication of eNOS in a form of synaptic plasticity termed long-term potentiation (LTP). Kantor and colleagues (111) showed that LTP can be attenuated in brain slices by pretreating them with the myristoylation inhibitor, hydroxymyristic acid (HMA). To rule out the possibility that a myristoylated protein other than eNOS was involved, they infected brain slices with a recombinant adenovirus vector encoding a chimeric eNOS protein wherein the N-terminal glycine required for myristoylation is replaced by the extracellular and transmembrane domain of CD8. Using this fusion protein, they showed that the chimeric eNOS could be targeted to plasma membrane by the CD8 transmembrane sequence (independently of eNOS acylation), and importantly, that the HMA-induced inhibition of LTP was fully rescued by the CD8–eNOS construct. The plasma membrane targeting of eNOS therefore appears necessary for NO to fulfill its proposed role of retrograde messenger, probably by promoting its release into the extrasynaptic region and also by facilitating the enzyme activation by plasmalemmal Ca^{2+} influx.

eNOS acylation also appears to play a vital role in coupling the muscarinic cholinergic NO-mediated regulation of heart rate (112). Cardiac myocytes isolated from mice lacking a functional eNOS gene have proven valuable for the study of eNOS in myocyte function. Cardiac myocytes isolated from these eNOS gene-targeted mice lack the muscarinic cholinergic attenuation in beating rate seen in wild-type mice. The muscarinic cholinergic response can be reconstituted in these cells by the transfection of cDNA constructs encoding wild-type eNOS, but not by plasmids encoding the myristoylation-deficient eNOS mutant. In transfected cardiac myocytes expressing wild-type eNOS, the muscarinic cholinergic agonist carbachol completely abrogated the spontaneous beating rate and induced a fourfold elevation of the cyclic guanosine monophosphate (cGMP) level. By contrast, in the *myr*[−] eNOS myocytes, carbachol failed to exert its negative chronotropic effect and to increase cGMP levels. These data document an obligatory role for acylated eNOS, which is endogenously expressed in cardiac myocytes, and suggest an important role for eNOS in the modulation of heart rate control.

Although nNOS is not acylated, nNOS directly interacts with two palmitoylated proteins, i.e., PSD-95 in neurons (113) and caveolin-3 in skeletal muscle (114). In both cases, the protein–protein association was shown to account for the targeting of the enzyme in these specialized cell compartments (*see following*) and it can, therefore, be postulated that any change in the palmitoylation of nNOS partners could have, as for the eNOS protein, dramatic effects on the location and functional activity of the enzyme.

SUBCELLULAR LOCALIZATION OF NOSs

Criteria

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, serving as a reminder that rigorous criteria must be applied to the study of the subcellular targeting of proteins. For example, the interpretation of immunohistochemical approaches must be substantiated by subcellular fractionation experiments utilizing an *inclusive* (rather than selective) range of organelle-specific markers. Moreover, although quantitative approaches, such as coimmunoprecipitation methodologies, are informative for the study of NOS interactions with organelle-specific proteins, other more inherently qualitative techniques are more difficult to interpret objectively and rigorously. This applies particularly to the NADPH diaphorase assay, a commonly used tissue staining technique, which exploits the NADPH-dependent reduction of nitroblue tetrazolium as a surrogate for NOS activity.

NOS in Plasma Membrane and Plasmalemmal Caveolae

For several years, determining the specific particulate subcellular fraction to which the eNOS is targeted was an elusive goal; mutually contradictory reports have only been recently resolved by the study of Shaul and colleagues (96), documenting the localization of eNOS in plasmalemmal caveolae. The Latin term *caveola intracellularis* (115) had been introduced more than 40 years ago to describe plasma membrane invaginations identified by electron microscopy in a wide variety of cell types including epithelial and endothelial cells, as well as adipocytes and myocytes. Functionally, these 50–100 nm plasmalemmal vesicles were first shown to participate in the transcellular transport of macromolecules (transcytosis) and in the uptake of small molecules (pinocytosis). More recently, however, the discovery of a biochemical marker protein of these unusual organelles, termed caveolin, has provided the impetus for a new wave of studies suggesting that caveolae also participate in signal transduction by ensuring the compartmentalization of signaling molecules such as growth-factor and hormonal receptors, G proteins, protein kinases, as well as eNOS (for review, *see ref. 116*). Such a concentration of eNOS with other signaling proteins may facilitate, or improve the efficacy of, the coupling between the agonist stimulation and eNOS activation. We and others have, for instance, reported that G-protein-coupled receptors known to stimulate NO[•] production, such as the muscarinic and bradykinin receptors, are targeted to caveolae upon agonist stimulation (117,118). More recently, McDonald and colleagues (119) have reported the existence of a caveolar complex between the arginine transporter CAT1 and eNOS, thereby providing a mechanism for a highly efficient delivery of substrate to eNOS. In addition, the targeting of eNOS in plasmalemmal caveolae probably facilitates paracrine effects of NO[•]. In endothelial caveolae, this last statement is certainly verified, as the NO[•] produced finds most of its targets in the proximal myocyte layers or circulating blood cells such as platelets and red cells (120). The close association between plasmalemmal caveolae and the cytoskeleton could also reflect their importance in the vascular mechanotransduction mediated by NO[•].

The targeting of NOS to caveolae, which was first thought to be restricted to eNOS, is also likely to also occur for nNOS, which was recently shown to specifically interact with the muscle-specific caveolin isoform in skeletal muscle (114). In addition, caveolae were very recently identified in neuronal cells, and could therefore lead to the compartmentalization of nNOS in these specialized microdomains of the plasma membrane (121). As for iNOS, because the binding of caveolin and calmodulin are mutually exclusive, it seems unlikely that iNOS, which binds calmodulin avidly, is regulated by interactions with caveolin or is targeted to caveolae.

NOS in the Cell Nucleus and the Endoplasmic Reticulum

Although it is now clearly established that NO[•] can cause G:C→A:T transitions and mediate DNA strand breaks (122), there is no definitive evidence that any of the NOS isoforms are localized to the cell nucleus. The nucleus being a cellular target for NO[•] may by itself explain the absence of constitutive NOS, considering the high risk of inducing genotoxicity

(123). Although inconclusive, a recent immunohistochemical study has, however, provided suggestive data for the intranuclear localization of iNOS (124). Again, this could correspond to a pathological situation leading to cell death following DNA damage.

A large proportion of nNOS immunoreactivity in neurons is associated with rough endoplasmic reticulum (125), but the functional implication of this compartmentation is still unknown. More generally, although synthesis of the NOS proteins clearly involves the nucleus and the endoplasmic reticulum, it is less clear that these locales constitute ultimate targets for the NOS isoforms.

NOS in Mitochondria

In two successive papers, Giulivi and colleagues have documented the existence of a functionally active mitochondrial NOS (126,127). These reports succeeds to several studies providing interesting but inconclusive immunohistochemical data demonstrating mitochondrial staining for all three NOS isoforms. Giulivi and colleagues (127) have demonstrated, by using two different spectroscopic techniques, that rat liver mitochondria produce NO[•]: most of the enzymatic activity appears localized in the mitochondrial inner membrane and is sensitive to NOS inhibitors. In addition, Tatoyan and Giulivi (126) also reports the purification to homogeneity of the rat liver mitochondrial isoform (mtNOS). Monoclonal antibodies against iNOS are the only to show a crossreactivity with the mitochondrial NOS isoform. Furthermore, despite an apparent constitutive expression in the mitochondrial membranes, mtNOS exhibits kinetic parameters, molecular weight, and the requirement of a tightly bound calmodulin similar to the inducible NOS isoform. The authors, however, mention a distinctive proteolytic pattern of mtNOS, which suggests the existence of a splice isoform containing mitochondrial-targeting sequences. Thus, the exact identity of mtNOS remains to be established, but the production of NO[•] directly by the mitochondria opens new perspectives in the understanding of regulatory processes modulating oxidative phosphorylation in different biological systems. In this connection, a recent report by Clementi and colleagues (128) suggests that, beside the physiological regulation of the mitochondrial respiratory chain by NO[•] via its action on complex IV, long-term exposure to NO leads to persistent inhibition of mitochondrial complex I and may be of pathophysiological importance.

NOS in the Golgi Apparatus

There have been numerous reports identifying eNOS in Golgi (129,130). The interpretation of many of these studies is confounded by the experimental challenges involved in the assignment of a (recombinant) protein to a specific subcellular organelle. Using green fluorescence protein (GFP) technology, Sessa and colleagues (129) reported that in more than 90–95% of transfected NIH 3T3 cells, GFP-eNOS was absent from the plasma membrane borders and exclusively located in the Golgi (130). As acknowledged by these authors, this observation is completely different when using native microvascular endothelial cells for expressing the GFP-eNOS, as 80% of these endothelial cells are positive for eNOS in plasmalemmal caveolae. These experimental differences aside, the dynamic nature of protein trafficking is very likely to account for differences in eNOS location noted between different cell types and experimental conditions. Prabhakar and colleagues (131) recently used a quantitative approach exploiting immunofluorescence microscopy to show that bradykinin stimulation of aortic endothelial cells promotes the translocation of eNOS from the cell membrane to intracellular structures, suggesting that transport of eNOS from plasmalemmal to intracellular structures is part of a physiological cycle highly sensitive to the state of cell activation (*see following*). Thus, even though it appears plausible that eNOS biosynthesis and/or recycling may involve the Golgi, the relevance of this organelle for the critical step of NOS activation and NOS release remains to be rigorously established.

NOS in the Cytoskeleton

There are suggestive studies that indicate eNOS may associate with cytoskeletal proteins (93,114). Intuitively, 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. However, whether eNOS undergoes direct interactions with cytoskeletal proteins remains to be established by rigorous methodology. By contrast, the cytoskeleton association is much more clearly established for nNOS by virtue of its association with the cytoskeletal dystrophin complex in skeletal muscle (16,132) (see following). Furthermore, members of the postsynaptic-density 95 family of cytoskeletal proteins, which are known to interact with nNOS, have been shown to mediate receptor clustering at excitatory synapses in the brain (133).

NOS in Specialized Intracellular Organelles

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 (134). 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.

REGULATION OF NOS BY PROTEIN-PROTEIN ASSOCIATIONS

Calmodulin: The First NOS-Associated Protein

The requirement for calmodulin in nitric oxide synthesis is an essential characteristic of all three NOS isoforms, although this ubiquitous Ca²⁺ regulatory protein demonstrates important isoform-specific differences in its role as an allosteric activator. Indeed, nNOS and eNOS are low-output NOS whose activity is regulated by Ca²⁺/calmodulin, increasing as Ca²⁺ rises and decreasing as Ca²⁺ falls. By contrast, iNOS is a high-output NOS whose activity is essentially Ca²⁺-independent, as calmodulin is very tightly bound to the enzyme.

Moreover, in the case of eNOS, and probably for nNOS in skeletal muscle, calmodulin activation of the enzyme involves not only the binding to the calmodulin binding motif within the NOS sequence but also the allosteric displacement of caveolin from NOS, thereby reversing the inhibitory effect of the scaffolding protein.

nNOS, PSD-93/95, and the Dystrophin Complex

The sarcolemma of skeletal muscle contains a family of intracellular and transmembrane glycoproteins associated with dystrophin, linking the extracellular matrix with the actin-based cytoskeleton. The N-terminus of nNOS interacts with α 1-syntrophin, a binding partner of dystrophin through a PDZ/GLGF protein motif of approx 100 amino acids present in both proteins (132). The PDZ-containing domain of nNOS also binds to PDZ repeats in postsynaptic density 95 (PSD-95). In certain nonneuronal cells, including developing chromaffin cells of the adrenal gland and secretory cells of salivary gland, nNOS is coexpressed with the related protein PSD-93 (16). Binding interaction between PDZ domains are selective. Though nNOS binds to the PDZ motif in α 1-syntrophin and to the second PDZ motif of PSD-95, nNOS does not associate with the first and third PDZ motifs in PSD-95 (16). Certain PDZ domains are capable of binding to the extreme C-terminus of a family of receptors and ion channels, including *N*-methyl-D-aspartate (NMDA) receptors, Shaker-type K⁺ channels, and FAS. Brenman and colleagues found that nNOS and NMDA receptors compete for common or nearby binding sites within the second PDZ repeat of a PSD-95 subunit. The PDZ consensus sequence is present in a diverse family of enzymes and structural proteins. Many of

these proteins are found concentrated at specialized cell–cell junctions, such as neuronal synapses, epithelial zona occludens, and septate junctions. PDZ domains may therefore be important elements of interactions required for signal transduction at the membrane. Furthermore, the finding that PDZ/GLGF is present in a heterogeneous family of enzymes has motivated suggestions that the Gly-Leu-Gly-Phe (GLGF) domain may regulate enzyme activities. However, Bredt and colleagues (12) found that deletion of the GLGF domain of nNOS does not alter NOS catalytic activity in transfected cells.

PIN and CAPON

By means of yeast two-hybrid system screening, a 10-kDa protein was identified that physically interacts with and specifically inhibits the activity of nNOS (135). These authors named this protein PIN (for protein inhibitor of neuronal NOS) and presented evidence that the PIN/nNOS interaction leads to the destabilization of the nNOS dimer. Contradictory results (136; I. Rodriguez-Crespo, personal communication) were recently reported according which PIN inhibits all isoforms of NOS and has no effect on nNOS dimerization; stoichiometric analysis also shows that an approx 300-fold molar excess of PIN are required to inhibit nNOS by 50%. Moreover, PIN was simultaneously discovered as a light chain (LC) of dynein and myosin, with a highly conserved sequence over a wide spectrum of different organisms (137), and the role of the NOS inhibitor therefore appears as secondary to its other functions in myosin and dynein complexes. Clearly, the relevance of the PIN/nNOS remains to be rigorously established and considering that the stoichiometry represents a limiting factor, it may be most interesting to explore physiological or pathological conditions where high levels of PIN/dynein LC expression are observed. In this perspective, Gillardon and colleagues (138) recently reported that following global ischemia, mRNA expression of PIN/dynein LC was rapidly induced in pyramidal neurons of the hippocampal CA3 region and granule cell of the dentate gyrus which are resistant to ischemic damage. In vulnerable CA1 pyramidal neurons however, PIN/dynein LC remained at basal level after global ischemia and was associated to neuronal cell death.

CAPON (for carboxyl-terminal PDZ ligand of nNOS) is a recently identified cytosolic protein highly enriched in brain that competes with particulate PSD95 for interaction with nNOS (139). The interaction CAPON/nNOS is highly specific, and although CAPON does not inhibit nNOS activity by itself, CAPON is thought to reduce the accessibility of nNOS to NMDA receptor-mediated calcium influx, thus diminishing the capacity of nNOS to acutely produce NO[•]. Although phosphorylation of nNOS by various kinases failed to alter the CAPON/nNOS interaction, phosphorylation of CAPON in its C-terminal region could be involved through disruption of its specific β -sheet conformation sequence in the regulation of the interaction (139). Clearly, more needs to be learned and studies are, no doubt, underway to examine the native CAPON/nNOS association in its physiological environment and to address specific questions such as the stoichiometry of the protein–protein interaction.

ENAP-1 or Hsp90

Stimulation of aortic endothelial cells with bradykinin produces cycles of tyrosine phosphorylation/dephosphorylation of a 90-kDa protein, termed ENAP-1 (for eNOS-associated protein-1) by Venema and colleagues (94). This protein, recently identified as the heat-shock protein 90 (Hsp90), appears to facilitate eNOS activation in endothelial cells by forming a heterocomplex with the enzyme following stimulation with a Ca²⁺-mobilizing agonist or shear stress (140). In the specific case of shear stress, the eNOS/Hsp90 association appears to be somewhat delayed relative to that seen following agonist stimulation (94) and may correspond with the slower change in detergent solubility of eNOS seen in endothelial cells following exposure to shear stress (93).

Caveolin-1, Caveolin-3 and Their Regulatory Cycles

Following the original observation of the caveolar localization of eNOS in plasmalemmal caveolae (96), several lines of evidence in endothelial cells and cardiac myocytes revealed that eNOS is quantitatively associated with caveolin, the structural protein within caveolae (141). Further experiments revealed that this association leads to the inhibition of the enzyme activity, and that a stable protein–protein interaction takes place between both proteins (142–146). Consensus sequences were identified within both proteins: the *scaffolding domain of caveolin*, a juxtamembrane region of 20 amino acids in the C-terminal moiety of caveolin (143,145), and a putative *caveolin-binding motif*, a peptide sequence rich in aromatic residues localized in the oxygenase domain of eNOS (114,144,146); more recently, sites for caveolin inhibition of eNOS have been identified within the reductase domain of the enzyme (147) and within the caveolin N-terminal region (145). Like other modular protein domains, the scaffolding domain of caveolin appears to function by providing frameworks for the assembly of preassembled oligomeric signaling proteins, but, in addition, these structures maintain these diverse signaling proteins in their “off” state. The caveolin/eNOS interaction constitutes a new biological framework within which to understand the regulation of eNOS, yet many details still remain to be addressed to attribute to each interaction level the corresponding effect on the catalytic properties and/or targeting of eNOS.

In order for eNOS to be fully activated, caveolin must dissociate from the enzyme. The ubiquitous Ca^{2+} regulatory protein calmodulin (CaM) disrupts the heteromeric complex formed between eNOS and caveolin in a Ca^{2+} -dependent fashion (142): caveolin serves as a competitive inhibitor of CaM-dependent eNOS activation (143). The CaM binding consensus sequence is located at the border of the NOS reductase and heme domains, and CaM binding to this site activates NO synthesis by enabling the reductase domain to transfer electrons to the heme domain. Caveolin appears to attenuate this electron transfer, and CaM apparently rescues the caveolin-inhibited eNOS at this level, probably by binding to sequences in the eNOS reductase domain (147). This close control of enzyme activity may be particularly important for eNOS in caveolae, where CaM is also largely enriched (96) and could thus lead to undesired enzyme activation if the interaction of caveolin with eNOS was not keeping the system in check. The relevance of the caveolin/CaM reciprocal regulation of eNOS was recently demonstrated in intact cells wherein transient increase in $[\text{Ca}^{2+}]_i$ consequent to agonist activation was shown to promote the dissociation of eNOS from caveolin, associated with translocation of eNOS from caveolae (110,144). Such agonist-induced disruption of the caveolin/eNOS heterocomplex promotes the dissociation of the enzyme from proximity to the transporter of arginine (119) and thus, may serve as a feedback mechanism for eNOS activation (*see earlier*).

An obvious question raised by the findings of the counterbalancing modulation of eNOS by caveolin and CaM is related to the cellular regulation of eNOS/caveolin interaction in the context of enzyme acylation. We have observed that the *myr*[−] and *palm*[−] eNOS mutants may both interact with caveolin in the cytosol (110,148); this association also leads to a marked inhibition of enzyme activity, which is completely reversed by addition of CaM (144). The regulatory caveolin/eNOS association therefore appears independent of the state of eNOS acylation, indicating that agonist-evoked Ca^{2+} /CaM-dependent disruption of the caveolin–eNOS complex, rather than agonist-promoted depalmitoylation of eNOS, relieves caveolin’s tonic inhibition of enzyme activity. Thus, we propose that caveolin may serve as an eNOS chaperone regulating NO[•] production independently of the enzyme’s residence within caveolae or its state of acylation (110,148).

These data suggest a dynamic cycle of eNOS–caveolin interactions initiated by agonist-promoted increases in $[\text{Ca}^{2+}]_i$ that disrupt the caveolin–eNOS complex, leading to enzyme activation. Following more prolonged agonist stimulation, eNOS is likely to be depalmitoylated, and is no longer selectively sequestered in caveolae. The translocated enzyme then

partitions into noncaveolar plasma membrane and also in the perinuclear region of the cell (131), the precise identity of which has not yet been established. Furthermore, several lines of evidence indicate that subsequently to the enzyme's translocation, and following the decline in $[Ca^{2+}]_i$ to basal levels, eNOS may once again interact with caveolin and is then retargeted to caveolae, a process accelerated (or stabilized) by enzyme palmitoylation (110, 144). The reassociation of eNOS with caveolin could occur either at the plasma or perinuclear membrane levels or even in the cytosol through which caveolin complexes may shuttle between caveolae and an internalized caveolar vesicle/*trans*-Golgi network.

G-Protein-Coupled Receptors

A direct interaction between eNOS and seven-transmembrane-segment receptors has recently been described by Ju and colleagues (149): the bradykinin B2 receptor appears to be able to physically associate, through its fourth intracellular domain, with eNOS. Similar results were obtained using peptides derived from the angiotensin II receptor AT-1. This intriguing signaling paradigm was explored principally by studying purified proteins and synthetic peptides, and the role of these protein-peptide interactions remains to be established in intact cell systems. Although a small fraction of cellular eNOS appears to be physically associated with the B2 receptor (<5%), the agonist dependence of this reversible interaction increases the likelihood that these interactions may have physiological relevance. Clearly, more experiments need to be done to understand the role of this nonconventional model of direct receptor-effector coupling.

THE BASEBALL CATCHER'S MITT

Recently published analyses of the crystal structure analysis of NOS have begun to reveal how enzyme dimerization and cofactor/substrate binding combine to form the catalytic center for NO[•] synthesis (2,3). The structure of the NOS oxygenase dimer has been compared to a left-handed baseball catcher's mitt, with heme clasped in the palm. Interestingly, dimerization creates a 3-nm-deep, funnel-shaped, active-center channel by refolding and recruiting the pterin, thereby exposing a heme edge and the adjacent Trp residue for reductase domain interactions. This Trp residue may form part of the caveolin binding motif, and it can therefore be hypothesized that caveolin binding may biologically regulate NOS by blocking the reductase domain of NOS from supplying electrons to the heme. This finding provides an excellent example of how crystal structure determination may help dissecting the regulatory mechanisms of NOS activation. Perhaps even more exciting is the unexpected finding revealed by crystallography of a Zn²⁺ atom bound at the protein-protein interface of the eNOS dimer (150), a finding that opens intriguing perspectives of investigations for the biochemist, the pathologist, and the drug designer.

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