Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide

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Kone, Bruce C., Teresa Kuncewicz, Wenzheng Zhang, and Zhi-Yuan Yu. Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide. Am J Physiol Renal Physiol 285: F178–F190, 2003;10.1152/ajprenal.00048.2003.—Nitric oxide (NO) is a potent cell-signaling, effector, and vasodilator molecule that plays important roles in diverse biological effects in the kidney, vasculature, and many other tissues. Because of its high biological reactivity and diffusibility, multiple tiers of regulation, ranging from transcriptional to posttranslational controls, tightly control NO biosynthesis. Interactions of each of the major NO synthase (NOS) isoforms with heterologous proteins have emerged as a mechanism by which the activity, spatial distribution, and proximity of the NOS isoforms to regulatory proteins and intended targets are governed. Dimerization of the NOS isoforms, required for their activity, exhibits distinguishing features among these proteins and may serve as a regulated process and target for therapeutic intervention. An increasingly wide array of proteins, ranging from scaffolding proteins to membrane receptors, has been shown to function as NOS-binding partners. Neuronal NOS interacts via its PDZ domain with several PDZ-domain proteins. Several resident and recruited proteins of plasmalemmal caveolae, including caveolins, anchoring proteins, G protein-coupled receptors, kinases, and molecular chaperones, modulate the activity and trafficking of endothelial NOS in the endothelium. Inducible NOS (iNOS) interacts with the inhibitory molecules kalirin and NOS-associated protein 110 kDa, as well as activator proteins, the Rac GTPases. In addition, protein-protein interactions of proteins governing iNOS transcription function to specify activation or suppression of iNOS induction by cytokines. The calpain and ubiquitin-proteasome pathways are the major proteolytic systems responsible for the regulated degradation of NOS isoforms. The experimental basis for these protein-protein interactions, their functional importance, and potential implication for renal and vascular physiology and pathophysiology is reviewed.

calmodulin; caveolae; PDZ domains; Rac guanosine 3,5′-triphosphatase; heat shock protein 90

NITRIC OXIDE (NO) IS A GASEOUS free radical that functions as an endogenous mediator in diverse biological effects in numerous tissues. In the kidney and vasculature, these processes include the control of systemic and microvascular tone, the glomerular microcirculation, renal sodium excretion, and inflammatory responses in the glomerulus and tubulointerstitium, among many others. NO also impacts the renin-angiotensin and eicosanoid systems, endothelin, cytokines, and other key regulators of inflammation. Because of its potent chemical reactivity and high diffusibility, NO production by NO synthases (NOS) is under complex, tight control to dictate specificity of its signaling and to limit toxicity to other cellular components. Indeed, NO production from each of the three major NOS isoforms, neuronal NOS (nNOS; also termed NOS1), inducible NOS (iNOS; also termed NOS2), and endothelial NOS (eNOS; also termed NOS3), is subject to a variety of transcriptional, translational, and posttranslational controls. The posttranslational controls, which include lipid modifications, phosphorylation events, and interactions with protein partners, serve to govern the timing, magnitude, and spatial distribution of NO release.
In turn, these mechanisms specify the input signals that activate NO release and the effector functions of the molecule to target specific proteins.

Studies in recent years have uncovered an increasingly important role of physical association of the NOS isoforms with a variety of regulatory and structural proteins (Table 1). These interactions may be categorized as constitutive interactions, such as those between subunits of hemoglobin, and inducible or signal-dependent interactions, such as those between the subunits of GTP-binding proteins. Although protein-protein contacts involving each of the NOS isoforms and their functional importance have been largely observed and deciphered in other tissues, their functional importance in the control of normal or perturbed renal function has not been fully explored, and, in many cases, not yet been addressed at all. Thus there are tremendous opportunities for renal research in this area. In addition to proteins interacting directly with the NOS proteins themselves, new evidence indicates that direct interactions between transcription factors or coregulatory proteins play an important role in controlling the iNOS transcriptional response. In this review, we will first discuss the known NOS-protein interactions, their influence on NO production and intracellular locale, and the biological consequences of these interactions. We will then consider the known and potential impact of these interactions on renal and vascular function.

### Table 1. Protein partners of nitric oxide synthases

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nNOS, eNOS, and iNOS: neuronal, endothelial, and inducible nitric oxide synthase, respectively; Hsp, heat shock protein; NAP, NOS-associated protein; ID, intracellular domain; NOSIP, eNOS-interacting protein; NOSTRIN, eNOS traffic inducer; EBP, ezrin-radixin-moesin-binding phosphoprotein; PMCA, plasma membrane Ca2+/calmodulin-dependent Ca2+-ATPase; CAT, cation amino transporter; PSD, postsynaptic density; CAPON, COOH-terminal PDZ ligand of nNOS. Activators and inhibitors refer to direct effects on NOS enzymatic function. References are provided in the text.

### STRUCTURAL BIOLOGY OF NOS ISOFORMS

NO is generated from L-arginine, molecular oxygen, and NADPH by NOS enzymes. Three major NOS isoforms, which share a common basic structural organization and requirement for substrate cofactors for enzymatic activity, have been described. nNOS, primarily expressed in the brain and skeletal muscle but also expressed in the macula densa segment (104) as well as other tubule segments, is typically viewed as a Ca2+/calmodulin-dependent enzyme, but it is also subject to transcriptional and other posttranslational controls (10). A mitochondrial variant of this enzyme, which, in contrast to the nNOS of the brain, is myristoylated and phosphorylated at the COOH terminus, is widely distributed among tissues, including kidney, and may play a role in cellular energetics (19). eNOS, expressed predominantly in the endothelium, is also subject to rapid regulation by Ca2+/calmodulin as well as transcriptional, posttranscriptional, and other post-translational controls (60). iNOS is induced in virtually all tissues subjected to cytokines, endotoxin, or other proinflammatory stimuli principally at the level of transcriptional control, and it is less responsive to intracellular Ca2+ transients owing to tight calmodulin binding at ambient intracellular Ca2+ levels (1).

The NOS enzymes are bidomain proteins, in which a central calmodulin-binding motif separates an oxygenase (NH2-terminal) from a reductase (COOH-terminal) domain (Fig. 1). The oxidase domain contains a cytochrome P-450-type heme active site and a binding site for tetrahydrobiopterin (BH4), and the reductase domain contains an electron-transfer domain that binds flavin mononucleotide (FMN) and FAD (1). NO activity requires binding of calmodulin and BH4 and the formation of a homodimer. Calmodulin binding, triggered by transient elevations in intracellular free Ca2+ levels, serves as an allosteric modulator of the three major NOS isoforms (2). nNOS and eNOS contain 40–50 amino acid inserts in the middle of the FMN-binding subdomain that serve as autoinhibitory loops (87), destabilizing calmodulin binding at low Ca2+ concentrations and inhibiting electron transfer from FMN to the heme in the absence of Ca2+/calmodulin (69, 70). This insert is absent from the structure of iNOS.

Crystallographic studies have brought the structural and biochemical basis of enzymatic activity and dimerization into clearer focus. Metal binding, disulfide and
hydrogen bond formation, and three-dimensional domain swapping participate in the regulation of the assembly and activity of the three NOS isozymes. Structures of the dimeric rat eNOS oxygenase (eNOSox) domain (76) and human eNOSox and iNOSox domains (30, 59) revealed a zinc tetrathiolate center, involving two cysteines (human iNOS Cys\textsuperscript{110} and Cys\textsuperscript{115}) from each subunit, positioned at the bottom of the dimer and arranged so that interaction of the NH\textsubscript{2}-terminal “hooks” from their own subunits is favored. Site-directed mutagenesis of human iNOS Cys\textsuperscript{115} and the corresponding human eNOS Cys\textsuperscript{99} revealed that these residues are essential for dimer stability (15). Moreover, the human iNOSox domain structure with bound zinc possesses a net gain of eight hydrogen bonds, which favors dimer stability, compared with the zinc-free complex. The extensive dimer interface creates binding sites for BH\textsubscript{4}, sequesters the heme from solvent, and helps to structure the substrate-binding site and the active site. The sequestered heme makes extensive van der Waals interactions with neighboring amino acids. The BH\textsubscript{4}-binding pocket is also buried within the protein near the dimer interface. Arginine binds with the side-chain terminus nestled into the narrow part of the active site, with the guanidino group situated near the heme (30, 59, 76) (Fig. 1).

Despite the structural similarity of the three NOS isozymes, homodimers of the isozymes differ markedly in the association strength of their monomers, their full interfaces, and the influence of L-arginine and BH\textsubscript{4} on their formation and stability. These differences in dimerization represent distinguishing control points and therapeutic targets to control NOS activity. Homodimerization is dependent on the binding of L-arginine, stoichiometric amounts of heme, and BH\textsubscript{4} (93). In turn, dimerization potentiates NOS activity by creating high-affinity binding sites for L-arginine and BH\textsubscript{4}, removing heme from the solvent phase, and facilitating electron flow from the reductase domain to the oxygenase domain heme (16, 30, 59). Studies of recombinant iNOSox and nNOSox domains indicate that L-arginine and BH\textsubscript{4} facilitate dimerization of the monomers and protect both dimers against trypsin proteolysis, whereas the eNOSox dimer was resistant to proteolysis under all conditions (73). For nNOSox, L-arginine alone was more effective than BH\textsubscript{4} alone, whereas the opposite was true for iNOSox (73). Both L-arginine and BH\textsubscript{4} participate in extensive hydrogen-bonding networks that stabilize the surrounding protein. Based on urea dissociation studies (73) and studies of low-temperature SDS-PAGE (99), dimeric interaction is strongest in eNOSox, followed by nNOSox and then iNOSox.

Although the combination of L-arginine and BH\textsubscript{4} promotes the best dimer stability for all the NOS isozymes, the isozymes differ in the degree to which these two compounds individually facilitate stability. Both the NH\textsubscript{2}-terminal hooks and zinc-binding elements help to stabilize NOS dimers, and because these structures have the greatest sequence divergence among the NOS isozymes, they might confer isoform-specific differences in dimer stabilities (16, 37, 38, 73). The NOS reductase domains may also participate in the formation of a dimeric holoenzyme. Studies with isolated oxygenase and reductase domains in the yeast two-hybrid system indicate that only the oxygenase domain is involved in iNOS dimer formation, whereas interactions between the reductase domains and between the reductase and oxygenase domains are critical for dimerization of nNOS and eNOS (99).

DEGRADATION OF NOS PROTEINS

Protein-protein interactions are controlled, at the most fundamental level, by the availability of the component proteins, which, in turn, is principally controlled by the net effects of synthesis and degradation of the proteins. NOS degradation is a regulated process influenced by diverse agents, including glucocorticoids, caveolin-1, heat shock protein (Hsp) 90, neurotoxic molecules (45), and certain NOS inhibitors. The calpain and ubiquitin-proteasome pathways function as the major proteolytic systems responsible for the regulated degradation of iNOS. Studies in IFN-\gamma-stimulated RAW 264.7 macrophage cells demonstrated that dexamethasone promotes proteolytic degradation of iNOS and that the iNOS monomer is a direct substrate for cleavage by Ca\textsuperscript{2+}-dependent neutral cysteine protease calpain I (101). Access to the calmodulin-binding domain appears to be critical for substrate cleavage, because purified calmodulin inhibits calpain I-mediated iNOS degradation in vitro (102). Studies of iNOS, expressed by transfection in HEK-293 cells or induced in primary bronchial epithelial cells, A549 cells, or murine macrophages demonstrate that it is ubiquitinated and that ubiquitination is required, to a large extent, for degradation (56). Overexpression of caveo-
lin-1 in human colon carcinoma cell lines, which have low endogenous levels of this protein, results in cosegregation of a portion of cytokine-induced iNOS protein with caveolin-1 in detergent-insoluble membrane fractions and degradation there via the proteasome pathway (22). Caveolin-1 and iNOS were further shown to bind to each other in vitro (22) (Table 1). These results suggest a model of tumor biology in which downregulation of caveolin-1 may serve to promote uncontrolled iNOS activity, genotoxicity, and tumor development.

nNOS undergoes enhanced proteolytic degradation when exposed to certain neurotoxins (45), when subjected to suicide inactivation with guanidine compounds (71), or when the Hsp90-based chaperone system is inhibited with geldanamycin (7). The fact that the heme-deficient monomeric form of nNOS is preferentially ubiquitinated over that of the heme-bound homodimer suggests that ubiquitination of nNOS participates in the regulated proteolysis of the nonfunctional enzyme. The mechanisms of eNOS protein degradation have not been extensively studied. The loss of endothelial function after cardiac ischemia is associated with a loss of eNOS activity due to a combination of intracellular acidosis-dependent denaturation and proteolysis (40).

**HOW ARE PROTEIN-PROTEIN INTERACTIONS IDENTIFIED?**

Several methods for identifying protein partners have been developed and some applied to the study of NOS regulation. These include protein cross-linking (6, 66), green fluorescent protein (55, 74), phage display (85), the yeast two-hybrid system (27), chromatographic techniques (5), and fluorescence resonance energy transfer (FRET) (54). One general limitation of these methods is that they generally can screen only one bait protein at a time. One of the most commonly used methods for detecting new interactions is the yeast two-hybrid system (27). This simple and inexpensive method requires no prior knowledge about the interacting proteins. The system exploits the ability to split the functional domains of a transcription factor into a DNA-binding domain and a transcriptional activation domain. Neither domain when expressed individually can activate transcription, but they can be split the functional domains of a transcription factor to generate hybrid proteins to test for potential protein-protein interaction. Typically, investigators screen a protein of interest (“bait”) expressed as a fusion to a DNA-binding domain against a random library of DNA or cDNA fused to a transcriptional activation domain, expressed in yeast (“prey”) by genetic selection. Once the fusion proteins interact, the functional transcription factor is reconstituted and its activity can be readily monitored using reporter gene assays or nutritional selection in yeast. We used this method to identify an interaction of iNOS with Rac GTPases (58). The yeast two-hybrid system has a reputation for producing a significant number of false positives, and whereas the method can be used to map interactions within a complex, it cannot identify multiprotein complexes themselves, which are now routinely characterized by mass spectrometry (MS). In addition, because the system is based on a transcriptional readout, the protein to be studied must be largely devoid of significant transcriptional activity. Several cytoplasmic proteins have been shown to contain structural domains that exhibit transcriptional activity. Moreover, some proteins, such as cell-cycle regulators, exhibit toxicity when fused to a heterologous DNA-binding domain and expressed in yeast. Finally, the requirement of this system that the protein-protein interaction should occur at the yeast nucleus may not be appropriate for cytoplasmic or integral membrane proteins (3).

An alternative method for the identification of protein-protein interactions, which is becoming increasingly popular, is to capture proteins of interest and their interacting proteins by using high-affinity antibodies in immunoprecipitation experiments followed by polyacrylamide gel separation of the protein mixtures. Individual or mixtures of protein bands in gels are then subjected to in situ tryptic digestion, and the resultant peptide fragments are extracted and analyzed by MS. The unambiguous identification of the proteins in the mixture by MS is largely dependent on the amount, quality, and complexity of the digested peptides, the species of origin, and the strength of the corresponding genomic and protein databases (28). This methodology represents an important component of the rapidly growing field of “functional genomics,” and it was recently used to identify an association between porin and eNOS (94).

With any method, additional tests are needed both to verify the interaction independently and to establish its biological relevance. Commonly, protein pairings identified by the yeast two-hybrid method are confirmed to occur in mammalian cells by immunoprecipitation with an antibody directed against one of the protein partners, followed by immunoblotting of the resultant immunoprecipitates with an antibody recognizing the other protein. Alternatively, glutathione S-transferase (GST) fusion constructs can be generated for one of the proteins and used to retain the second protein from cell or tissue lysates or generated as a radiolabeled in vitro translation product. The demonstration of colocalization by immunolabeling or FRET methods helps to solidify the findings. FRET detects the proximity of fluorescence-labeled molecules over distances >100 A, and it can be used to map protein-protein interactions in vivo (54). Finally, the impact of the putative protein-protein interaction on target protein function, localization, or trafficking is tested in the presence and absence of the interacting protein to determine the functional consequences.

**PROTEIN-PROTEIN INTERACTIONS INVOLVING nNOS**

**PDZ Domain-Containing Proteins**

The NH2 terminus of nNOS contains a PDZ domain that participates in the formation of active NOS dimers and interacts with a variety of other proteins in specific regions of the cell. Among the NOS isoforms,
the PDZ domain is unique to nNOS. Proteins containing PDZ domains typically localize to specialized cell-cell contacts and often link components of signal transduction pathways in ternary complexes. Direct interactions of several proteins bearing PDZ domains with nNOS have been shown to influence the activity and/or the subcellular distribution of the enzyme in brain and muscle (57). By anchoring nNOS to specific targets in this manner, NO signaling is altered. In skeletal muscle, the nNOS PDZ domain scaffolds the enzyme to α1-syntrophin, which independently binds both dystro-brevin and dystrophin, which binds to glycoproteins in the membrane (11, 12). This arrangement associates nNOS with the sarcolemma, where it can generate NO to increase perfusion of adjacent blood vessels of contracting muscle. Disruption of these interactions contributes to the pathophysiology of Duchenne muscular dystrophy.

PDZ domain interactions also link nNOS to the muscle isoform of phosphofructokinase in skeletal muscle and neurons (29) and function at neuronal synapses to bring nNOS in proximity to the N-methyl-D-aspartate (NMDA) receptor, allowing glutamate-stimulated Ca\(^{2+}\) influx to specifically activate nNOS (97). For example, nNOS interacts with postsynaptic density (PSD)-95 and PSD-93 proteins in neurons (11) via direct PDZ-PDZ domain interactions. In the rat kidney, PSD-93 is expressed in the basolateral membranes of the thick ascending limb of the loop of Henle, macula densa cells, distal convoluted tubules, cortical collecting ducts, outer and inner medullary collecting duct, glomerular epithelium, and Bowman’s capsule (83, 96). In the macula densa, a site of abundant nNOS expression, a subpopulation of nNOS colocalizes with PSD-93 adjacent to cytoplasmic vesicles and the basolateral membrane (83, 96). The functional importance of this apparent association to macula densa signaling is unknown.

Another protein, the COOH-terminal PDZ ligand of nNOS (CAPON), which is highly enriched in the brain, competes with PSD-95 for interaction with nNOS in the brain (49, 50). Overexpression of CAPON results in dissociation of PSD-95-nNOS complexes in transfected cells, disrupts the proximity of nNOS to NMDA-mediated Ca\(^{2+}\) influx, and thereby may restrict NO generation. CAPON contains both a COOH-terminal domain for binding to the nNOS PDZ domain and an NH\(_2\)-terminal phosphotyrosine-binding domain that binds the small monomeric G protein Dextras1 (21). Thus CAPON serves as an adaptor protein in a ternary complex that enhances the ability of nNOS to activate Dextras1 (21) (Fig. 2). Similarly, CAPON interacts with synapsin I, II, and III through an NH\(_2\)-terminal phosphotyrosine-binding domain interaction on the synapsins to form a ternary complex comprising nNOS, CAPON, and synapsin I (49). Using affinity chromatography of brain extract with the nNOS PDZ domain, the COOH-terminal-binding protein (CtBP), a phosphoprotein first identified as a binding partner to adenovirus E1A, was identified as an nNOS-binding partner. Immunoprecipitation studies show that CtBP and nNOS associate in the brain. When CtBP is expressed in Madin-Darby canine kidney cells, its distribution is primarily nuclear; however, when wild-type but not PDZ motif-mutated CtBP is coexpressed with nNOS, its localization becomes more cytosolic. These results suggest a new function for nNOS as a regulator of CtBP nuclear localization (80).

In a yeast two-hybrid assay, Ort and co-workers (72) found that the cytoplasmic domain of islet cell autoantigen 512 of type 1 diabetes, a receptor tyrosine phosphatase-like protein associated with neuronal and endocrine secretory granules, binds the PDZ domain of nNOS. Whereas the expression of the majority of these nNOS-interacting proteins has not been reported in the kidney, it remains possible that they influence the renal nerves or that they are influenced by the metabolic consequences of uremia and contribute to neurological dysfunction in that setting.

Finally, the plasma membrane Ca\(^{2+}\)-calmodulin-dependent Ca\(^{2+}\)-ATPase serves as a regulator of Ca\(^{2+}\) homeostasis and signal transduction networks of the cell. The COOH-terminal region of plasma membrane Ca\(^{2+}\)-calmodulin-dependent Ca\(^{2+}\)-ATPase isoform 4b binds to the PDZ domain of nNOS, negatively regulating NO production in HEK-293 embryonic kidney cells and neuro-2a neuroblastoma cell models (88). Mutational analysis proved that the PDZ domains of both proteins were involved in the interactions. These results suggest an intriguing integration between Ca\(^{2+}\) and NO signaling pathways (88) that may impact renal Ca\(^{2+}\) transport.

**Protein Inhibitor of NOS**

The protein inhibitor of NOS (PIN) or dynein light chain, is an 89-amino acid polypeptide isolated from a rat hippocampal cDNA library using the yeast two-hybrid system and various fragments of nNOS as bait.

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*Fig. 2. Interaction of neuronal (n) NOS with the N-methyl-D-aspartate (NMDA) receptor (NMDAR) and PDZ domain-containing proteins at neuronal synapses. nNOS binds via PDZ-PDZ domain interactions to postsynaptic density (PSD)-95, and PSD-95 associates with the NMDAR. The resultant ternary signal complex efficiently couples Ca\(^{2+}\) entry through the NMDAR to activation of nNOS. The nNOS/PDZ domain also binds to the adaptor protein COOH-terminal PDZ ligand of nNOS (CAPON), which binds the small monomeric G protein Dextras1 and links it to nNOS. The proximity of these proteins facilitates the ability of nNOS-derived nitric oxide (NO) to S-nitrosylate and thereby activate Dextras1.*
(51). PIN was originally reported to inhibit only the nNOS isoforms by dissociating the active nNOS homodimer (51). However, these functional results have recently been challenged. Hemmens et al. (48), using recombinant proteins, demonstrated that all NOS isoenzymes form contacts with and are inhibited by PIN. This result is in keeping with the fact that the PIN recognition sequence of nNOS (residues Met228 to His244 of rat nNOS) (20) is not included in eNOS or iNOS, lies outside the catalytic core of nNOS, and is not part of the dimerization region of nNOS. Subsequent studies by Rodriguez-Crespo et al. (84) suggested that PIN neither inhibits nNOS activity nor dissociates the nNOS dimer as originally proposed. These authors suggested that PIN might function as a dynein light chain involved in nNOS axonal transport rather than as an nNOS inhibitor. Within the rat kidney, PIN immunoreactivity is evident in glomerular and vasa recta endothelial cells and apical membranes of inner medullary collecting ducts (83). Rats subjected to 5/6 nephrectomy exhibited threefold greater levels of inner medullary PIN levels compared with controls, suggesting that PIN inhibition of nNOS might serve to differentially regulate NO synthesis (83).

**Receptors**

Interactions with several receptors involved in signal transduction have been reported. The COOH-terminal four amino acids (GEEV) of the human α1A-adrenergic receptors have been reported to interact with the PDZ domain of nNOS (75). Moreover, nNOS coimmunoprecipitated with epitope-tagged α1A, α1B, and α1D-adrenergic receptors, but the functional significance is unknown (75). The serotonin 5-HT2B receptor appears to interact via its COOH-terminal PDZ domain with both nNOS and eNOS to trigger their activation (63). The bradykinin B2 receptor and nNOS were coimmunoprecipitated in human embryonic kidney cells stably transfected with human nNOS, suggesting that the bradykinin B2 receptor may functionally interact with nNOS in vivo (43). In agreement with this work, a synthetic peptide derived from the known inhibitory sequence of the bradykinin B2 receptor (residues 310–329) was found to interact with both eNOS and nNOS. Binding of this peptide blocked flavin-to-heme electron transfer of nNOS (43, 98).

**Hsp90 and Caveolin-3**

Hsp90 is a highly abundant cytosolic protein known to serve as a molecular chaperone in protein folding and maturation events, but it has received increasing recognition as an integral component of signaling networks. Formation of a NOS-Hsp90 heterocomplex that results in enhanced NO formation has also been reported (8). Mechanistic studies showed that Hsp90 increases the activity of recombinant rat nNOS, increases the binding of calmodulin to nNOS to shift the calmodulin-nNOS dose-response curve markedly to the left, and augments the maximal activity of nNOS (91). In the aggregate, these results indicate that Hsp90 directly enhances calmodulin binding and thereby increases NOS catalytic function. Caveolin-3 has been shown to interact with nNOS in skeletal muscle, where it appears to comprise a component of the dystrophin complex (100).

**PROTEIN-PROTEIN INTERACTIONS INVOLVING eNOS**

**Calmodulin, Caveolin, and Hsp90**

It has long been recognized that the Ca^{2+}-dependent activation of eNOS occurs through calmodulin binding when intracellular Ca^{2+} concentrations rise. Binding of calmodulin to its specific motif on eNOS displaces its adjoining (87) loop on eNOS and nNOS, thereby promoting NADPH-dependent electron flow from the reductase domain to the oxygenase domain of the protein. eNOS and calmodulin have been coimmunoprecipitated from human endothelial cells (86), and inhibitors of calmodulin have shown a requirement of calmodulin for eNOS activity (87). As intracellular Ca^{2+} concentrations fall, calmodulin dissociates from eNOS. This bimodal conceptualization of Ca^{2+}/calmodulin-dependent regulation of eNOS has been greatly refined to include a complex array of protein-protein interactions (Fig. 3) that was the subject of a recent, intensive review (42).

The majority of functional eNOS in quiescent endothelial cells resides in caveolae, the result of dual acylation. Myristoylation of eNOS targets the protein to the Golgi apparatus, where it undergoes palmitoylation. The myristoylated and palmitoylated eNOS is then targeted to the caveolae membrane (36, 61, 89, 92). Several groups independently reported that caveolin-1, the resident integral membrane protein of caveolae, directly interacts with and inhibits in a dynamic fashion eNOS in vitro and in endothelial cells in vivo (35, 36, 53, 67). Similarly, caveolin-3 was shown to interact with eNOS in ventricular myocytes (23). The binding regions within caveolin-1 for bovine eNOS reside on amino acids 60–101 and, to a lesser degree, amino acids 135–178 (35, 47, 53). Synthetic peptides corresponding to the caveolin scaffolding region (amino acids 82–101) disrupt immunocomplexes containing eNOS and caveolin-1 (67). Conversely, amino acids 350–358 of eNOS comprise a consensus caveolin-binding motif (90). In the resting state, eNOS appears to be tethered to caveolin-1 and inactive. However, several agonists that raise intracellular Ca^{2+} concentrations, such as bradykinin, promote calmodulin binding to eNOS and caveolin dissociation from the enzyme, resulting in an activated eNOS-caveolin complex (Fig. 3). When intracellular Ca^{2+} levels return to resting levels, the cycle is reversed, with calmodulin dissociating and caveolin reassociating with now-inactive eNOS (26, 67). This work is supported by several experimental observations in vitro and in caveolin-1 knockout mice. First, the activity of purified eNOS is suppressed by incubation with peptides derived from the scaffolding domains of caveolin-1 and -3 (35). Second, heterologous overexpression of wild-type caveolin-1 in COS-7 cells limited eNOS activity, whereas eNOS harboring...
of a mutated caveolin-binding motif was not inhibited (67). Finally, mice with targeted disruption of caveolin-1 exhibit enhanced eNOS activity, as judged by the NO-dependent relaxation of preconstricted aortic rings (18, 79).

Mechanistically, Ghosh and co-workers (39) reported that the binding of caveolin-1 to the reductase domain of eNOS inhibits NO synthesis by limiting the enzyme’s ability to bind calmodulin and to donate electrons to the eNOS heme moiety. However, others have distinguished calmodulin-dependent and-independent effects of caveolin-1 on NO function. A calmodulin-independent mechanism, characterized by an increase in the affinity of eNOS for calmodulin, may be operative at lower Ca\(^{2+}\) concentrations, whereas a calmodulin-independent mechanism apparent at high-Ca\(^{2+}\) concentrations may stimulate eNOS reductase activity and result in diminished eNOS activity.

Increased vascular flow (81) and fluid shear stress (33) also promote eNOS dissociation from caveolin and association with calmodulin to activate the enzyme in endothelial cells. Conversely, serum or its LDL fraction from hypercholesterolemic patients increases the expression of caveolin-1 and the formation of caveolin-eNOS heterocomplexes in endothelial cells and thereby limits basal and agonist-stimulated NO release (24, 25). This pathological response may represent in part the basis for endothelial dysfunction in hypercholesterolemia. Similarly, estrogen promotes increased caveolin-3 and decreased eNOS in the heart (103).

Hsp90 has been found to serve as an allosteric activator of eNOS. Garcia-Cardenas and colleagues (33) demonstrated that vascular endothelial growth factor, histamine, and fluid shear stress promote rapid association of Hsp90 with eNOS in endothelial cells and augment eNOS activity. Blockade of Hsp90-mediated signaling limits both agonist-induced NO production and vasorelaxation. Hsp90 and eNOS appear to form a ternary complex that includes the kinase Akt. Fontana et al. (31) found that the M region of Hsp90 interacts with the NH\(_2\) terminus of eNOS and with Akt. Moreover, stimulation of endothelial cells with vascular endothelial growth factor promoted recruitment of eNOS and Akt to this same domain of Hsp90, facilitating Akt-driven phosphorylation of eNOS and promoting NO release. Hsp90 appears to enhance calmodulin binding to nNOS (91), but this property remains to be firmly established for eNOS. Similarly, it remains to be established whether Hsp90 also serves as a scaffolding protein that participates in the ushering of other regulatory molecules.

Recent evidence builds on this model by suggesting that calmodulin, caveolin, Hsp90, and eNOS form part of a dynamic, integrated signaling complex (Fig. 3). Immunoprecipitation of radiolabeled protein from endothelial cells with an anti-eNOS antibody followed by immunoblotting of these immunoprecipitates revealed the presence of caveolin-1, Hsp90, and eNOS in the same complex (44). In vitro reconstitution experiments showed that eNOS interacted with the other two proteins, but Hsp90 and caveolin-1 did not interact with each other. Neither calmodulin nor Hsp90 could directly disrupt the eNOS-caveolin interaction, but Hsp90 facilitated the ability of calmodulin to displace caveolin from eNOS (44). Whether the three proteins remain bound to eNOS in the caveolar membrane and eNOS conformational transitions stimulate more efficient NO production in response to a stimulus or whether recruitment of calmodulin and Hsp90 results in weak dissociation of caveolin from eNOS but the complex is maintained in the caveolae remains unclear.

G Protein-Coupled Receptors

Multiple G protein-coupled receptors resident in caveolae appear to contribute to the eNOS-membrane complex and regulate eNOS activity. The capacity to bind and inhibit eNOS appears to be a common feature of membrane-proximal regions of intracellular domain (ID) 4 of the bradykinin B\(_2\), angiotensin AT\(_1\) receptor (52), and the ET-1 ETB receptors, but not of the ATP P2Y2 receptor (64). Ju and colleagues (52) used coimmunoprecipitation and in vitro binding assays to demonstrate that the bradykinin B\(_2\) receptor, via its
COOH-terminal ID4 (amino acids 310–329), interacts with eNOS in a ligand- and Ca<sup>2+</sup>-dependent manner. In the resting state, the receptor docks with eNOS in the caveolar membrane and participates in its inactivation. Activation of endothelial cells with bradykinin or Ca<sup>2+</sup> ionophore triggers dissociation of the eNOS-B<sub>2</sub> receptor complex and activates the eNOS enzyme (52). The sites of binding to eNOS and the mechanisms by which the bradykinin B<sub>2</sub> receptor and caveolin-1 inhibit eNOS appear to be distinct (52). Phosphorylation of serine or tyrosine residues in the eNOS-interacting region of the bradykinin B<sub>2</sub> receptor decreases the binding affinity of the receptor domain for the eNOS enzyme and relieves eNOS inhibition. Furthermore, bradykinin-induced tyrosine phosphorylation of the bradykinin B<sub>2</sub> receptor in cultured endothelial cells appears to promote a transient dissociation of eNOS from the receptor, accompanied by a transient increase in NO production. At a mechanistic level, Golser and colleagues (43) found that binding of the ID4 peptide disrupts electron transfer from flavin to heme in eNOS. Taken together, these data suggest that reversible and inhibitory interactions with G protein-coupled receptors participate in the complex regulation of eNOS activity in endothelial cells and that these interactions are regulated by receptor phosphorylation (64).

Transporters

The cation arginine transporter CAT1 represents another component of the eNOS-membrane complex in caveolae of endothelial cells. CAT1, eNOS, and caveolin-1 colocalize to plasmalemmal caveolae in pulmonary artery endothelial cells, and an eNOS-specific antibody immunoprecipitates CAT1-mediated arginine transport from solubilized plasma membrane proteins (65). The proximity of CAT1 and eNOS proteins may serve to direct arginine delivery to eNOS and thereby optimize NO release. Using coimmunoprecipitation experiments followed by MS analyses, Sun and Liao (94) identified a human voltage-dependent anion/cation channel or porin as an eNOS-binding partner. In vitro studies showed that a GST-porin fusion construct specifically retained eNOS and that this interaction augmented eNOS activity. Increasing intracellular Ca<sup>2+</sup> concentrations with Ca<sup>2+</sup> ionophore or bradykinin to activate eNOS markedly increased porin-eNOS interaction, suggesting that intracellular Ca<sup>2+</sup> transients may regulate this interaction.

eNOS Traffic Inducer

Zimmermann and colleagues (105) used the yeast two-hybrid system with the eNOSox domain as bait to isolate a novel 506-amino acid eNOS-interacting, eNOS traffic inducer protein (NOSTRIN). NOSTRIN contains an NH<sub>2</sub>-terminal cdc15 domain and a COOH-terminal SH3 domain, and its transcripts are abundantly expressed in highly vascularized tissues, including kidney, as well as vascular endothelial cells. Coimmunoprecipitation experiments confirmed eNOS-NOSTRIN interaction in vitro and in vivo and established that NOSTRIN’s SH3 domain was necessary and sufficient for eNOS binding. NOSTRIN colocalized with eNOS at the plasma membrane of human umbilical venous endothelial cells. When overexpressed, NOSTRIN triggered redistribution of eNOS from the plasma membrane to vesicle-like structures containing NOSTRIN. This redistribution correlated with a coincident inhibition of NO release. These results suggested that NOSTRIN participates in the complex regulation of eNOS trafficking, targeting, and activity (105) (Fig. 3).

eNOS-Interacting Protein

eNOS-interacting protein (NOSIP) is a 34-kDa protein that avidly binds to the COOH-terminal region (amino acids 366–486) of the eNOSox domain. NOSIP-eNOS complex formation was inhibited by a synthetic peptide of the caveolin-1 scaffolding domain, suggesting competition between NOSIP and caveolin-1 for eNOS binding, but it was not regulated by stimulation of cells with Ca<sup>2+</sup> ionophore. Although NOSIP did not alter eNOS activity in vitro, it promoted translocation of eNOS from the plasma membrane to intracellular sites when coexpressed in cells, resulting in diminished ionomycin-stimulated NO release. Thus NOSIP appears to uncouple eNOS from plasma membrane caveolae, thereby suppressing NO synthesis (17) (Fig. 3). The functional role of NOSIP in vivo and its specificity for eNOS over the other NOS isoforms is unknown.

GTPases and Kinases

The GTPase dynamin-2 is known to participate in internalization events in caveolae, vesicle formation and trafficking, and receptor-mediated endocytosis. Dynamin-2 coimmunoprecipitated with eNOS from lysates of bovine aortic endothelial cells and colocalized in these cells predominantly in a Golgi membrane distribution (13). Treatment of endothelial cells with a Ca<sup>2+</sup> ionophore enhanced the coimmunoprecipitation of dynamin, suggesting that the interaction between the proteins can be triggered by intracellular Ca<sup>2+</sup> transients (13). The proline-rich domain of dynamin-2 was found to interact with the FAD-binding region of the eNOS reductase domains and positively regulate eNOS activity, at least in part, by potentiating electron transfer between FAD and FMN of the eNOS reductase domain (14).

Several kinases, including RAF, Erk, and AKT, can be coimmunoprecipitated with eNOS from endothelial cells, and the association of these proteins can be provoked by agonists, suggesting that they may contribute somewhat to the eNOS signal (9, 68). Because these kinases affect eNOS activity through phosphorylation rather than binding per se, the physical coupling appears to be important for specifying the phosphorylation event. Bradykinin (46), VEGF, and shear stress trigger Akt-mediated phosphorylation of Ser<sup>1177/1179</sup> eNOS (bovine eNOS numbering), leading to eNOS activation in vitro and in vivo (32, 62, 68).
For the eNOS regulatory system in endothelial cells, the only obvious fact is that it is extremely complex, with multiple potential interactions, many of which can be independently regulated. Given the role of eNOS-generated NO in systemic and renal hemodynamics, however, understanding how these networks operate in different vascular beds in vivo will be extremely important.

**PROTEIN-PROTEIN INTERACTIONS INVOLVING iNOS**

*Inhibitory Proteins*

Three proteins that bind iNOS and exert inhibitory actions on it have been identified. Murine macrophages express a 110-kDa protein, termed NOS-associated protein-110 kDa (NAP110), that interacts with the NH₂ terminus of iNOS and inhibits iNOS catalytic activity by preventing dimerization (77). Expression of NAP110 may serve as a defense mechanism by which macrophages expressing iNOS protect themselves from cytotoxic levels of NO. A yeast two-hybrid screen of a hippocampal cDNA library showed that the first 70 amino acids of iNOS interact with kalirin, a neural-specific protein known to function as an interactor with a secretory granule peptide biosynthetic enzyme (78). Kalirin interacts with iNOS in vitro and in vivo and prevents iNOS dimerization, and therefore iNOS activity. Kalirin may play a neuroprotective role during inflammation of the central nervous system by inhibiting iNOS activity. As noted earlier, caveolin-1 complexes with iNOS in human colon carcinoma cells to promote iNOS proteolysis (22).

*Activating and Trafficking Proteins*

Using a yeast two-hybrid screen of a mouse kidney cDNA library with murine iNOS as bait, our laboratory recently defined a regulatory interaction of iNOS with the Rac1 and Rac2, members of the Rac family of Rho-like GTPases (58). Racs function as molecular switches in regulating a variety of important biological response pathways. The interaction was evident in coimmunoprecipitation assays in extracts harvested from activated RAW 264.7 cells and in GST pull-down assays using GST-Rac1 and GST-Rac2 fusion constructs to immobilize in vitro translated iNOS. Deletion analysis revealed that the point of interaction with Rac2 resides in the iNOS ox domain (Fig. 4). iNOS and Rac1 colocalized in activated RAW 264.7 macrophages (Fig. 4). [³⁵S]methionine-labeled iNOS was found to interact directly with GST-Rac2 in the absence of calmodulin or iNOS substrates or cofactors. Stable overexpression of Rac2 in RAW 264.7 cells augmented LPS-induced nitrite generation (~60%) and iNOS activity (~45%) without measurably affecting iNOS protein abundance and led to a redistribution of iNOS to a high-speed, Triton X-100-insoluble fraction and enhanced appearance of iNOS in Rac2-containing vesicles, suggesting a role of Rac in redistributing iNOS in the cell. In addition to increased NO production, the Rac2-overexpressing cells generated greater levels of superoxide anion, and thus greater amounts of the chemical byproduct of NO and superoxide anion, peroxynitrite, than the control cells after exposure to LPS+IFN-γ. In the aggregate, these results indicated that iNOS and Rac2 are functionally coupled through their joint effects on both iNOS-mediated NO generation and Rac2-driven NADPH oxidase activity (58).

NO is also known to modulate solute transport processes in polarized epithelial processes, suggesting that local NO delivery to its molecular targets is achieved. In cultured human proximal tubule epithelial cells, iNOS was found to localize to the apical domain within a submembranous protein complex tightly bound to cortical actin and physically interacting with the apical PDZ protein ezrin-radixin-moesin-binding phosphoprotein (EBP50). Mutational analysis indicated that this interaction was dependent on the last three COOH-terminal amino acids of iNOS, SAL, but also required the presence of additional unknown cellular proteins. The EBP50-iNOS interaction at the apical membrane apparently serves to direct vectorial NO production at this surface, facilitating NO delivery to its targets, which might include apical transporters or other proteins known to be complexed with EBP50, such as type 3 Na⁺/H⁺ exchanger (41).
HOMOCELLULAR INTEGRATION OF PROTEIN-PROTEIN INTERACTIONS: LINKING NOS ISOFORMS TO DIFFERENT SPATIAL COMPARTMENTS AND FUNCTIONS IN THE SAME CELL

Differential spatial arrangements and interactions with effector proteins of NOS isoforms in cardiomyocytes mediate independent, and at times opposite, effects on cardiac structure and function. NO is known to inhibit L-type Ca$^{2+}$ channels in the heart but also to stimulate Ca$^{2+}$ release from the sarcoplasmic reticulum via the ryanodine receptor. The molecular basis for these opposing responses appears to involve selective interactions of eNOS and nNOS with specific proteins and subcellular compartments within the same cell (4). In cardiomyocytes, caveolin-3 selectively interacts with eNOS and nNOS with specific proteins and subcellular compartments within the same cell (4). In contrast, nNOS selectively complexes with the ryanodine receptor in the sarcoplasmic reticulum, facilitating the ability of NO to promote Ca$^{2+}$ release and cardiac contractility (4). The balance of these independent and opposite roles of nNOS and eNOS serves to regulate cardiac contractility. Because several cell types and tissues express more than one NOS isoform, it will be interesting to determine whether such coordinate or discordant actions of the NOS isoforms contribute to other biological or pathobiological responses.

CONCLUSIONS

Protein-protein interactions represent an important and increasingly complex mechanism in the control of NOS activity and NO-dependent effects in the kidney and vasculature. The challenging work for the future concerns the integration of the various protein-NOS interactions into coherent models of signaling and stimulus-response coupling in normal physiology and disease. The majority of these protein-protein interactions have not yet been explored in the kidney, where important insights not only into their impact on renal function but also into the cell specificity of different interactions and their associated responses could be gained. Obviously, because all NOS isoforms are represented in the kidney and are known to contribute to renal hemodynamics, innervation, solute and water transport, inflammation, and disease progression, NOS-associated proteins in the control of NO production should be considered in attempts to understand these functions and conditions mechanistically. As the binding motifs for NOS-associated proteins are defined and functional proteomics gains more widespread use, new candidate proteins for NOS interaction and regulation will be identified and tested. The points of contact between heterologous proteins and the NOS isoforms could serve as experimental and potentially therapeutic targets to modulate NOS activity.

DISCLOSURES

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