Nitric oxide signaling specificity – the heart of the problem

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Summary

Nitric oxide (NO) is a gaseous free radical that functions as an endogenous mediator in numerous tissues. Because NO is both reactive and highly diffusible, its formation must be tightly regulated to control its synthesis and to specify its signaling. Indeed, molecular studies of the NO synthase (NOS) family of enzymes have elaborated a variety of mechanisms, including protein interactions, lipid modifications and protein phosphorylation cascades that spatially and temporally control NO biosynthesis. These mechanisms determine both the upstream cellular signals that stimulate NO formation and the downstream molecular targets for NO. Understanding these cellular pathways that control NOS will help us to elucidate the functional roles of NO and provide novel strategies to treat diseases associated with NO abnormalities.

Key words: Nitric oxide, Heart, Protein targeting, PDZ domain

Introduction

The free radical gas nitric oxide (NO) is now recognized as a major messenger molecule that has diverse functions throughout the body. Although originally identified as the endothelial-derived relaxing factor (Furchgott and Zawadzki, 1980; Hobbs and Ignarro, 1996; McDonald and Murad, 1995), NO also has prominent signaling functions in macrophages, neurons, endocrine cells, skeletal muscle fibers and numerous other cell types (Hibbs et al., 1987; Snyder and Bredt, 1992; Stamler et al., 2001). In fact, a very recent study identified particularly important functions for cardiomyocyte derived NO in regulating heart structure and function (Barouch et al., 2002). Unlike most other endogenous chemical mediators, which are stored in vesicles or are secreted in a controlled fashion, NO is a diffusible gas that readily permeates cell membranes. Because NO can not be stored, its signaling specificity must be controlled at the level of synthesis. Indeed, the members of the NO synthase (NOS) family are amongst the most highly regulated of known enzymes.

The primary mechanism controlling the neuronal NOS (nNOS) and the endothelial NOS (eNOS) enzymes is increased levels of intracellular calcium (Knowles et al., 1989; Palmer and Moncada, 1989), which activates these NOS isoforms by binding to calmodulin (Bredt and Snyder, 1990; Pollock et al., 1991), which in turn interacts with NOS. To enhance specificity for responses to calcium, NOS enzymes concentrate in specific subcellular domains. This allows NOS activity to respond selectively to calcium mobilization from localized calcium pools. Furthermore, diverse protein interactions and post-translational modifications, including protein phosphorylation and lipid modification of the enzyme, modify NOS isoforms to control their activation by upstream signal transduction events. The reactive and diffusive nature of the NO signal also requires that the synthase be localized in proximity to its downstream targets. This enhances signaling specificity for the reactive NO mediator and minimizes toxicity that arises from the free radical nature of NO.

Here, we highlight recent studies showing the importance of subcellular targeting and protein interactions of NOS in controlling both upstream and downstream signaling by the unique mediator NO.

nNOS association with dystrophin skeletal muscle

The first evidence for protein interactions regulating a NOS isoform came from studies of skeletal muscle, which showed that nNOSμ, the alternatively spliced muscle isoform (Silvagno et al., 1996), is specifically localized at the skeletal muscle plasma membrane or sarcolemma (Kobzik et al., 1994). This distribution was striking, because nNOS lacks transmembrane domains or lipid modifications that could target the synthase to the plasma membrane (Bredt et al., 1991). Cell biological and genetic studies showed that the sarcolemmal localization of nNOS is due to its interaction with the dystrophin glycoprotein complex (Brenman et al., 1995), which comprises a group of proteins that are mutated in Duchenne and several other inherited muscular dystrophies (Bonnemann et al., 1996; Campbell, 1995).

NO derived from nNOSμ in skeletal muscle fibers plays a major role in dilating blood vessels adjacent to contracting skeletal muscle (Thomas and Victor, 1998). This physiological response, functional hyperemia, plays an important role in increasing blood flow to contracting skeletal muscles to support their enhanced metabolic needs. Targeting nNOSμ to the sarcolemmal dystrophin complex specifies both upstream and downstream signaling by NO in this pathway (Fig. 1) – that is, localization of NO at the sarcolemma is critical for coupling calcium influx associated with muscle contraction to NO synthesis. Formation of NO at the muscle membrane facilitates its diffusion to the adjacent vascular smooth muscle

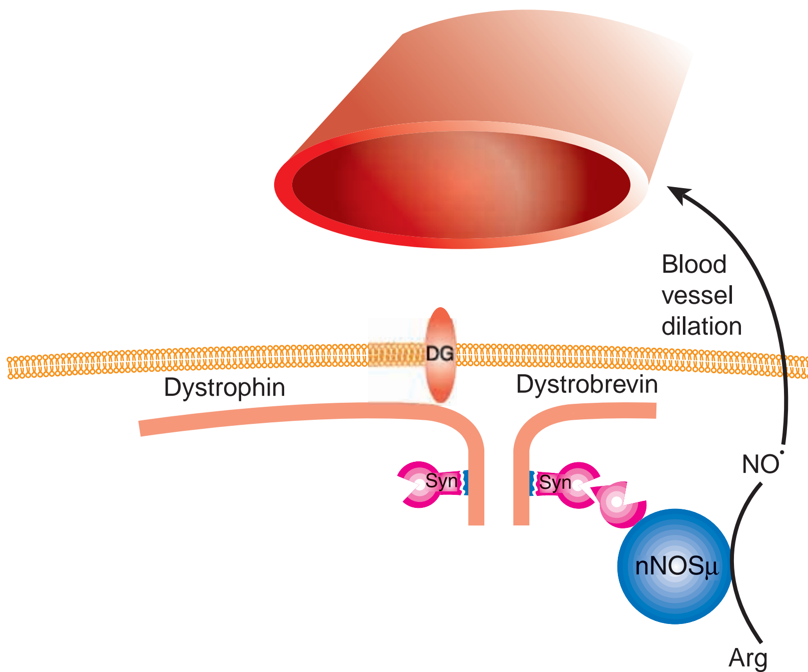

Fig. 1. Skeletal-muscle-derived NO enhances blood flow to contracting muscle. nNOSμ occurs at the skeletal muscle plasma membrane as a component of the dystrophin glycoprotein complex. The PDZ domain (pink) from nNOS binds to the PDZ domain of syntrophin (Syn), which independently binds to both dystrophin and dystrobrevin. Dystrophin itself adheres to the membrane by binding to glycoproteins such as dystroglycan (DG). Skeletal-muscle-derived NO diffuses to adjacent blood vessels to increase perfusion of contracting muscle.

(Thomas et al., 1998). The target for NO in smooth muscle is the soluble isoform of guanylyl cyclase, whose activity is increased 100-fold by its binding to NO; the elevated cGMP levels then mediate blood vessel dilation (Hobbs and Ignarro, 1996; McDonald and Murad, 1995).

The molecular mechanism for association of nNOS with the sarcolemmal dystrophin complex involves an N-terminal PDZ domain of nNOS (Brenman et al., 1996). PDZ domains are small modular protein–protein interaction interfaces that mediate assembly of protein complexes at cell junctions. These domains are ubiquitous and occur in a variety of dissimilar enzymes and cytoskeletal adaptor proteins. In fact the recently sequenced human genome boasts over 150 PDZ containing proteins (Schultz et al., 2000). Amongst the NOS isoforms, nNOS is unique in containing a PDZ domain, and this domain plays a major role in targeting the nNOS to specific cellular membranes (Brenman and Bredt, 1997).

Structurally, PDZ domains are compact globular modules that contain a single binding pocket that typically interacts with protein ligands that end with a specific PDZ-binding C-terminal consensus sequence (Craven and Bredt, 1998; Doyle et al., 1996; Garner et al., 2000; Harris et al., 2002; Kornau et al., 1997; Sheng, 2001). However, biochemical studies showed that the N-terminal PDZ domain of nNOS binds to a similar PDZ domain of syntrophin (Fig. 1), a dystrophin-associated protein (Brenman et al., 1996). This association of nNOS with syntrophin differs from canonical C-terminal PDZ interactions in that it involves two PDZ domains that form a heterodimer. X-ray crystallographic studies of the nNOS–syntrophin complex showed that a β-finger projection from the nNOS PDZ domain fits into the syntrophin PDZ binding pocket and makes contacts that resemble those formed by a typical C-terminal peptide ligand (Hillier et al., 1999). This prototypical PDZ heterodimer structure of nNOS–syntrophin helps to explain numerous examples of PDZ domains binding to internal protein binding sites (Hillier et al., 1999).

This nNOS–syntrophin PDZ–PDZ complex is essential for sarcolemmal association of nNOS, since mutant mice lacking syntrophin show a selective loss of nNOS from the skeletal muscle plasma membrane (Adams et al., 2001; Kameya et al., 1999). Sarcolemmal localization of nNOS is also lost in patients with Duchenne muscular dystrophy, which is due to genetic disruption of dystrophin (Brenman et al., 1995). The loss of dystrophin in muscular dystrophy prevents assembly of the dystrophin glycoprotein complex. As a result, NO signaling in response to muscle contraction is disrupted (Brenman et al., 1995), and, the blood vessel dilation of contracting skeletal muscle that is normally mediated by NO is abolished (Thomas et al., 1998).

Recent studies show that disruption of NO signaling plays a major role in muscular dystrophy pathophysiology. Transgenic overexpression of nNOS in skeletal muscle of *mdx* mice, which lack dystrophin, rescues much of the muscle pathology and enhances muscle function (Wehling et al., 2001). Furthermore, certain mutations of the dystrophin complex that cause a mild muscular dystrophy uniquely disrupt sarcolemmal nNOS (Bredt, 1999). In patients with Beckers muscular dystrophy due to mutations in the spectrin-like domains of dystrophin, and in mutant mice lacking the dystrophin-associated dystrobrevin, nNOS but not other components of the dystrophin complex are lost from the sarcolemma (Chao et al., 1996; Grady et al., 1999). Curiously however, mutant mice lacking nNOS do not show muscular dystrophy (Chao et al., 1998; Crosbie et al., 1998). Taken together, these studies suggest that whereas loss of skeletal muscle NO does not itself cause dystrophy, the lack of nNOS in Duchenne muscular dystrophy augments the damage caused by absence of the structural dystrophin glycoprotein complex. Because restoring nNOS ameliorates muscle injury in muscular dystrophy (Wehling et al., 2001), NO donors may offer a potential avenue for therapy.

nNOS association with NMDA receptors

The major location of nNOS is in brain, where the synthase is highly enriched in specific neuronal populations (Bredt et al., 1990). Again, protein interactions involving the nNOS PDZ domain in neurons regulate coupling to upstream calcium stores and specify downstream targets of NO (Brenman and Bredt, 1997). Most NO synthesis in brain is coupled to calcium influx through the N-methyl-D-aspartate (NMDA) type glutamate receptor, which was first shown to increase cGMP levels via neuron-derived NO (Bredt and Snyder, 1989; Garthwaite and Boulton, 1995). NMDA receptors occur

selectively at the postsynaptic density of excitatory synapses and are critical for synaptic plasticity and learning and memory (Moriyoshi et al., 1991). Although there are several distinct calcium pools at the synapse, only calcium influx through the NMDA receptor efficiently activates nNOS (Kiedrowski et al., 1992). Physical association of nNOS with the NMDA receptor determines this specificity (Fig. 2). Although nNOS does not directly bind to the receptor, nNOS and NMDA receptors associate with the postsynaptic density protein PSD-95/SAP-90 (Brenman et al., 1996), a membrane-associated guanylate kinase (Cho et al., 1992; Kistner et al., 1993). PSD-95 contains a set of modular protein–protein interaction domains, including three PDZ domains, an SH3 domain and a region homologous to guanylate kinase. PDZ domains from PSD-95 mediate binding to nNOS and the NMDA receptor (Christopherson et al., 1999). The C-terminal tail of the NMDA receptor 2 subunit binds to a PDZ domain from PSD-95 (Kornau et al., 1997) and nNOS forms a PDZ–PDZ interaction with a distinct PDZ domain from PSD-95 (Christopherson et al., 1999). These interactions occur simultaneously to form a ternary signaling complex. This complex is essential for the efficient upstream coupling of calcium influx through the NMDA receptor to formation of NO (Christopherson et al., 1999). This complex tethers the nNOS to the postsynaptic density, which also determines downstream signaling specificity. Targets for synaptic NO are ion channels at the synapse as well as soluble guanylyl cyclase, which is concentrated in the adjacent presynaptic nerve terminal.

The nNOS PDZ domain binds PSD-95 through the same β-finger region that mediates binding to syntrophin in skeletal muscle (Tochio et al., 2000). In addition, the nNOS PDZ domain but has its own binding groove that can associate with specific C-terminal protein ligands to assemble more elaborate protein complexes (Stricker et al., 1997). One prominent binding partner for the nNOS PDZ domain is CAPON (Jaffrey et al., 1998), an adaptor protein that contains both a C-terminal region that binds to the PDZ domain nNOS and an N-terminal phosphotyrosine binding (PTD) domain. This PTD domain binds to the small monomeric G protein Dexras1 (Wehling et al., 2001). Importantly, NO can activate Dexras1 via S-nitrosylation, and this pathway (Fig. 2) for downstream signaling requires the adaptor protein CAPON (Fang et al., 2000).

Some nNOS is also present at the presynaptic nerve terminal, where NO may regulate neurotransmitter release (Meffert et al., 1996). In nerve terminals of parallel fibers from cerebellar granule cells, nNOS is apparently regulated by presynaptic NMDA receptors to regulate Purkinje cell long-term depression, a cellular model for motor learning (Casado et al., 2002; Daniel et al., 1998; Lev-Ram et al., 1997). Protein interactions with CAPON have also been shown to target the enzyme to the nerve terminal, where CAPON interacts with synapsins I, II and III (Jaffrey et al., 2002).

Whereas small amounts of NO formed in association with synaptic activity mediate physiological functions, the overproduction of NO that occurs in association with specific toxic processes, such as cerebral ischemia, cause brain injury (Dawson et al., 1991). Again, the stimulus for NO production in these conditions is activation of the NMDA receptor, which occurs to excess in such ‘excitotoxic’ conditions (Coyle and Puttfarcken, 1993). The NMDA receptor–nNOS pathway is a major pharmaceutical target, and a plethora of NMDA receptor and nNOS antagonists have been characterized and evaluated as possible therapies for excitotoxicity (Lipton and Rosenberg, 1994). However, NMDA receptors and NOS mediate diverse effects; so simple receptor or enzyme antagonists show severe side effects (Olney et al., 1989). The functional nNOS–PSD-95–NMDA complex described above provides a new target for preventing neurotoxicity. Drugs that target this complex could provide neuroprotection from strokes and other neurodegenerative diseases while minimizing peripheral side effects. Indeed, downregulation of PSD-95 with antisense oligonucleotides protects cultured neurons from NMDA-mediated damage (Sattler et al., 1999). It will now be important to test whether compounds that block assembly of the PSD-95–nNOS–NMDA complex can provide neuroprotection in vivo.

Subcellular targeting and regulation of eNOS

Although it lacks a PDZ domain, eNOS is also found in association with cell membranes and is targeted to specific protein complexes. The first identified mechanism for membrane association of eNOS was lipid modification: eNOS undergoes both myristoylation and palmitoylation. Myristoylation is a permanent modification that adds a 14-

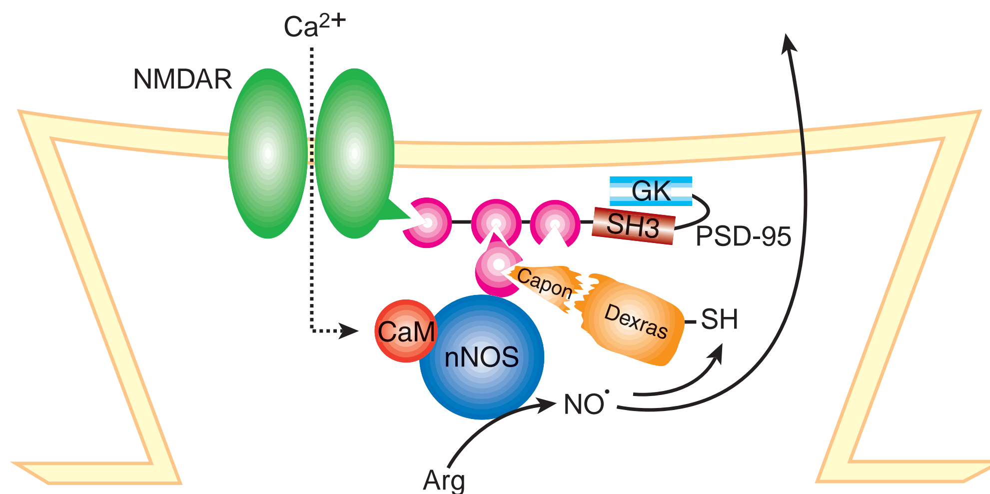

Fig. 2. Protein interactions regulating NO signaling at neuronal synapses. PDZ domain interactions (pink) function to bring nNOS to the NMDA receptor, allowing specific activation of nNOS in response to glutamate-induced calcium influx. Binding of nNOS to PSD-95 leaves the nNOS PDZ domain peptide-binding pocket free to interact with other proteins such as Capon. The NO produced in association with NMDA receptor activity diffuses to presynaptic nerve terminals and nitrosylates Dexras associated with Capon. Abbreviations: Arg, arginine; CaM, calmodulin; GK, guanylate kinase domain.

carbon lipid to the N-terminus of eNOS through a stable amide bond (Busconi and Michel, 1993; Sessa et al., 1993). In contrast, palmitoylation involves a labile thioester bond that links 16 carbon palmitates to a pair of cysteine residues at positions 15 and 26 of eNOS (Garcia-Cardena et al., 1996b; Robinson et al., 1995). These lipid modifications of eNOS are added sequentially. Myristoylation of eNOS occurs co-translationally and targets eNOS to cellular membranes, where eNOS is then palmitoylated. Mutations that block eNOS myristoylation prevent its subsequent palmitoylation. These lipidation promote eNOS association with cell membranes and are essential for linking upstream signal transduction pathways to eNOS activity in cells (Busconi and Michel, 1993; Sessa et al., 1993). Interestingly, increases in intracellular calcium and calmodulin binding stimulate depalmitoylation of eNOS by acyl protein thioesterase, displacing eNOS from the membrane (Yeh et al., 1999). This may be a homeostatic mechanism to down-regulate NO production following intense stimuli.

Rather than being homogeneously distributed at the plasma membrane, eNOS is specifically targeted to the Golgi complex and to plasma membrane caveolae (Garcia-Cardena et al., 1996b; Shaul et al., 1996), flask-shaped invaginations of the plasma membrane. Caveolae are specifically enriched with signaling molecules, including G-protein-coupled receptors as well as ion channels and pumps specifically involved in regulating intracellular calcium (Anderson, 1998; Lisanti et al., 1995). Dual lipidation of eNOS by the long chain palmitate fatty acids mediates targeting to caveolae (Garcia-Cardena et al., 1996b; Shaul et al., 1996), which have a specialized lipid composition rich in cholesterol and sphingomyelin that is favorable for incorporation of dually acylated proteins.

Multiple protein interactions in caveolae regulate eNOS activity and its coupling to extracellular stimuli (Fig. 3). eNOS interacts with the major protein of caveolae, caveolin, which inhibits eNOS (Feron et al., 1996; Garcia-Cardena et al., 1996a; Ju et al., 1997). Mutant mice lacking caveolin-1 have an absence of plasmalemmal caveolae and show enhanced eNOS activity, which fits with inhibition of eNOS by caveolin (Drab et al., 2001). Caveolin inhibition of eNOS is relieved by calmodulin, which causes dissociation of eNOS from caveolin. This regulatory mechanism is further modified by heat shock protein 90 (Hsp90), which binds to eNOS and facilitates displacement of caveolin by calmodulin (Gratton et al., 2000). Hsp90 associates with eNOS at rest, and stimulation with vascular endothelial growth factor (VEGF), histamine or shear stress increases the hsp90–eNOS interaction (Brouet et al., 2001; Garcia-Cardena et al., 1998), which further promotes its dissociation from caveolin and enhances its calmodulin-dependent activity.

In addition to these protein interactions that modulate calmodulin binding, other cellular signaling cascades also regulate eNOS activity. Shear stress, isometric vessel contraction, insulin and VEGF activate eNOS without increasing intracellular calcium (Ayajiki et al., 1996; Fleming et al., 1999). A role for phosphorylation in these pathways was first suggested by pharmacological studies showing that inhibitors of phosphoinositide 3-kinase (PI-3K) block insulin and VEGF stimulation of eNOS (Papapetropoulos et al., 1997; Zeng and Quon, 1996). These effects are explained by activation of the protein kinase Akt by the 3-phosphorylated inositol lipids generated by PI-3K. Akt directly phosphorylates eNOS at Ser1177 and activates the enzyme 15-20-fold (Dimmeler et al., 1999; Fulton et al., 1999). Mutation of Ser1177 to aspartate to mimic the negative charge of phosphorylation yields an eNOS that is constitutively active (Dimmeler et al., 1999) at low levels of calcium (10 nM), whereas mutation of Ser1177 to alanine prevents Akt-dependent regulation of eNOS (Fulton et al., 1999).

Cellular mechanisms controlling NO signaling in heart

These diverse cellular mechanisms for differential targeting of nNOS and eNOS isoforms to regulate both upstream and downstream signaling are especially critical for control of cardiac function by NO. NO has diverse effects and sometimes even opposing influences on cardiac function. A recent study shows that understanding NO actions on the heart requires careful consideration of the distinct cellular mechanisms that regulate eNOS and nNOS (Barouch et al., 2002). In cardiac myocytes, eNOS localizes to caveolae (Feron et al., 1996; Garcia-Cardena et al., 1996b), where it is compartmentalized with β-adrenergic receptors and L-type calcium channels (Schwencke et al., 1999). β-agonists such as isoproterenol increase L-type calcium channel function by stimulation of

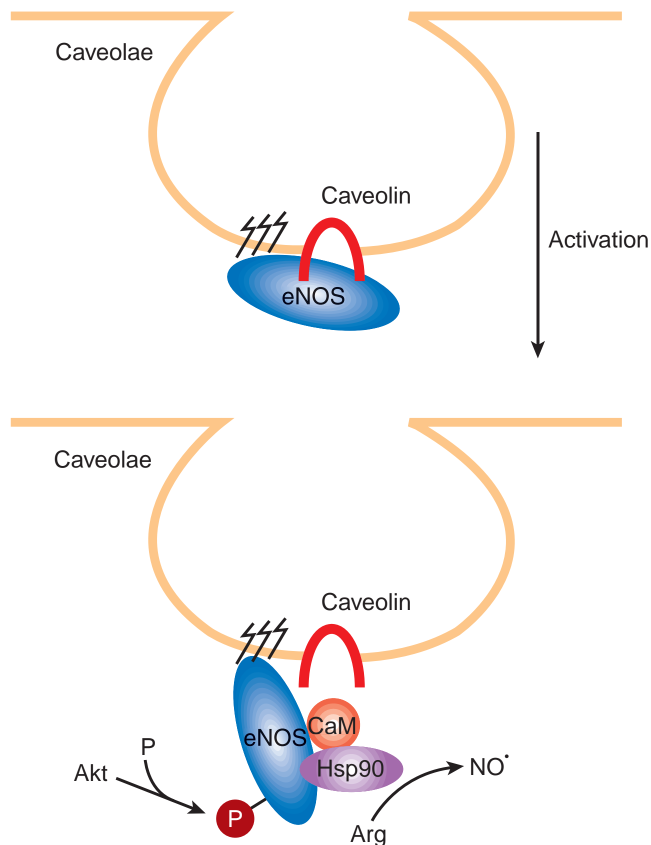

Fig. 3. Regulation of eNOS in caveolae. Myristoylation and palmitoylation (jagged lines) target eNOS to plasma membrane caveolae. Interaction with caveolin inhibits eNOS activity. Endothelial cell stimuli such as shear stress, recruit Hsp90 and activate eNOS by recruiting calcium and calmodulin (CaM). Phosphorylation of eNOS by Akt also increases activity.

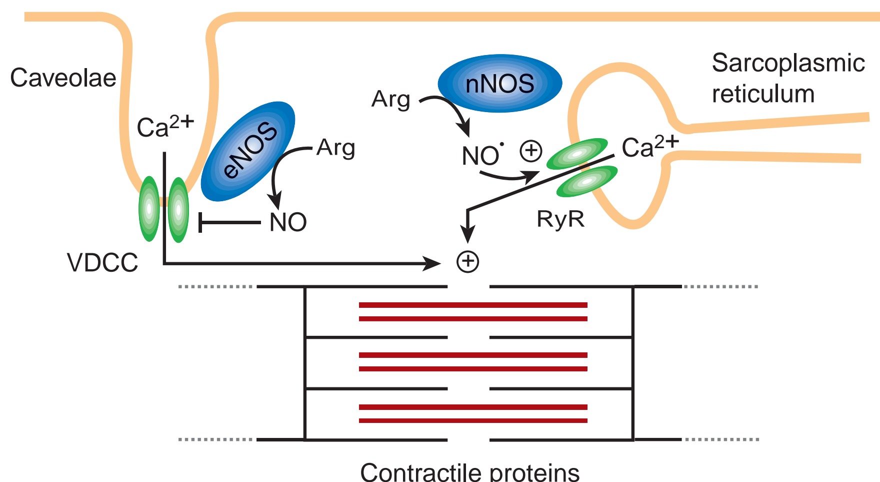

Fig. 4. Opposing roles for eNOS and nNOS in regulating heart contraction. NO formed by eNOS inhibits L-type voltage-dependent calcium channels (VDCC) to decrease muscle contraction. By contrast, NO produced by nNOS on the sarcoplasmic reticulum stimulates calcium influx through the ryanodine receptor (RyR) to augment cardiac contractility.

adenylyl cyclase, formation of cyclic AMP, and activation of protein kinase A (PKA) (Schultz et al., 1990). Formation of cyclic GMP in association with eNOS activity in caveolae inhibits L-type calcium channels by two mechanisms. First, cyclic-GMP-dependent protein kinase directly phosphorylates L-type calcium channels to inhibit their function. Also, cyclic GMP activates a cyclic AMP phosphodiesterase-2. Together, these pathways allow eNOS in caveolae to blunt β-adrenergic-induced contractility (Bloch et al., 1999). By contrast, nNOSμ activity in cardiomyocytes increases contractility. nNOSμ in cardiac muscles localizes to the sarcoplasmic reticulum and associates with ryanodine receptors, cardiac calcium release channels (Xu et al., 1999). Whereas eNOS activity inhibits L-channel function, nNOSμ-derived NO stimulates calcium release from the ryanodine receptor (Xu et al., 1998). This activation of the ryanodine receptor does not involve cyclic GMP but rather direct S-nitrosylation of free thiols on the channel protein (Xu et al., 1998).

Targeting of nNOSμ to the sarcoplasmic reticulum versus caveolar targeting of eNOS can therefore explain the opposing influences of these NOS isoforms on the heart (Fig. 4). As shown in the recent study by Hare and collaborators, mutant mice lacking specific NOS isoforms have opposite changes in cardiac function (Barouch et al., 2002). At baseline, nNOS-knockout mice (which also lack nNOSμ) have relatively normal cardiovascular function whereas eNOS-knockout mice have high blood pressure, an elevated heart rate and enlarged left ventricular chambers. Importantly, nNOS-knockout mice exhibit a suppressed ionotropic response to β-adrenergic stimulation, whereas eNOS mice show an enhanced response to isoproterenol. These effects on cardiac function reflect changes in myocyte calcium cycling such that myocytes from nNOS knockout mice show attenuated responses to isoproterenol whereas cells from eNOS mutants show enhanced responses. Interestingly, mice lacking both nNOS and eNOS show defects in calcium cycling that resembles that of nNOS, which suggests the nNOS effects in heart predominate.

Abnormalities in sarcoplasmic reticulum calcium cycling often cause cardiac hypertrophy. Indeed nNOS knockout mice show age-related left ventricular hypertrophy. eNOS knockouts also show left ventricular hypertrophy though it is associated with hypertension. Mutant mice lacking both nNOS and eNOS show greater cardiac hypertrophy than either single mutant (Barouch et al., 2002). This additivity in the cardiac phenotype emphasizes the independent roles of nNOS and eNOS in cardiac function and suggests a lack of cross-talk in the signaling in cardiomyocytes. Since cardiac hypertrophy is often associated with heart failure and myocardial infarction, modulation of NO level in heart seems a valuable therapeutic target.

Perspectives

The diversity of NO actions in the body is paralleled by numerous cellular mechanisms for regulating the family of NOS enzymes. Interactions of NOS enzymes with cytoskeletal scaffolding proteins, specialized lipid domains and protein phosphorylation cascades allow NO to integrate into a variety of signaling pathways. Molecular dissection of these complex NOS regulatory mechanisms will further our understanding of NO signal transduction. Because abnormalities of NO signaling are linked to numerous pathological conditions, these studies have important implications for diseases of skeletal muscle, brain and heart.

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