Targeting Nitric Oxide to Its Targets (43950F)

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Abstract. **Nitric oxide (NO) is now recognized as a major messenger molecule in the nervous system. In specific neuronal pathways, NO functions alternatively as a neurotransmitter and as a second messenger. Neuronal-type nitric oxide synthase is a widely distributed (nNOS or Type I) calmodulin-regulated enzyme and is coupled to a variety of neurotransmitter systems in brain and peripheral tissues. NO formation is linked in cerebellum to NMDA receptor activity, in myenteric neurons to neuronal nicotinic receptor activity, and in skeletal muscle to sarcolemmal depolarization. Coupling of nNOS activity to alternative calcium sources appears to be mediated, in part, by tethering nNOS to specific membrane proteins. In skeletal muscle, nNOS physically associates with the sarcolemmal dystrophin complex through a GLGF protein-association motif present near the N terminus of nNOS. This GLGF motif is likely also involved in anchoring nNOS to synaptic membrane complexes in brain. Subcellular targeting of nNOS represents a crucial mechanism for regulation of NO actions in the nervous system.** [P.S.E.B.M. 1996, Vol 211]

The discovery of nitric oxide (NO) as the endothelial-derived relaxation factor (EDRF) that regulates cyclic guanosine-3′-5′-monophosphate (cGMP) levels in the vascular system (1, 2) prompted investigations of the possible role for NO in controlling cGMP levels in nervous tissue. Within the brain, highest levels of cGMP are found in cerebellum, where excitatory transmitters rapidly elevate cGMP levels both *in vitro* (3) and *in vivo* (4). In 1988, Garthwaite and co-workers found that cerebellar cells stimulated with the excitatory amino acid glutamate, release a diffusible factor with physical and biological properties of EDRF (5). Definitive evidence for a role for NO in controlling cGMP levels in brain derived from studies showing that NO synthase (NOS) inhibitors such as mono-methyl arginine block glutamate-linked enhancements of cGMP levels (6, 7).

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0037-9727/96/2111-0041$10.50/0

Nitric Oxide in Neuronal Function

Intensive investigation over the past several years has implicated neuron-derived NO in diverse processes associated with neuronal signaling. Synaptic functions for NO were first demonstrated in the peripheral nervous system. These studies resolved pharmacologic observations which had puzzled physiologists for over 20 years. Following the development of adrenergic blocking agents such as guanethidine in the early 1960s, it became clear that certain actions of the autonomic nervous system were subserved by non-adrenergic, noncholinergic (NANC) nerves (8). The further development of additional adrenergic and cholinergic blockers has allowed the characterization of NANC nerves throughout the body. These pathways play a particularly important role in producing relaxation of smooth muscle in the gastrointestinal, urogenital and respiratory tracts and in the cerebral circulation (9). Studies by numerous investigators in the mid-1980s indicated similarities between the NANC transmitter and the endothelial-derived relaxing factor described by Furchgott and Zawadzki (1). Both agents were found to mediate smooth muscle relaxation by activating guanylyl cyclase (10). In addition, hemoglobin, which binds NO, was found to block both endothelium-dependent relaxation and NANC-mediated dilation of the bovine retractor penis (11, 12).

In each of these regions, immunohistochemical studies have established that NOS is present in the

expected NANC nerves (13, 14). At the electron microscopic level, NOS occurs on specialized intraneuronal membrane structures and is selectively concentrated in neuronal axons (15, 16). Smooth muscle cells contain the "receptor" for NO in this pathway, the soluble guanylyl cyclase. Thus, in specific pathways of the peripheral nervous system, NO functions as a NANC neurotransmitter and fulfills all the classical criteria of a neurotransmitter. A unique aspect of this pathway is that NO is not packaged in lipid lined vesicles like other transmitters but instead is synthesized in nerve terminals and rapidly diffuses across cell membranes to influence transcellular targets. The immediate blockage of NO-mediated transmission by NOS inhibitors supports this model and suggests that neurons do not store inactive NO adducts.

Physiological roles for NO in the central nervous system (CNS) are suggested by the discrete and highly conserved distribution of NOS in subpopulations of central neurons of all mammalian brains so far examined (17, 18). The unique capacity of NO to function as a rapidly diffusible messenger suggests possible functions for NO in mediating certain actions in the nervous system which require a fast-acting retrograde messenger. On theoretical grounds, NO has been suggested to function as a retrograde messenger subserving use-dependent modification of synaptic efficacy during the establishment of ordered cortical connections (19). Experimental support for this suggestion was provided by several laboratories that independently found that NOS inhibitors block hippocampal long-term potentiation (LTP), a model of learning and memory, in the CA-1 region of the hippocampus (20–23). Application of nitroarginine, a NOS inhibitor, to hippocampal slices blocks LTP formation. Injection of nitroarginine into pyramidal cells of the hippocampus also inhibits LTP, suggesting that NO might act as a retrograde messenger for LTP passing from pyramidal cells to Schaffer collateral terminals. Simultaneous intercellular recording from electrically isolated nearby CA1 pyramidal cells suggests that NO mediates a locally distributed synaptic potentiation in hippocampus (24). Analogous pharmacologic studies suggest a role for NO in long-term depression, a model of motor learning, in the cerebellum (25, 26). However, several studies find no role for NO in mediating LTP. Controversy over the role of NO in LTP appears to be explained by studies which show that the effectiveness of NOS inhibitors in blocking LTP depends on the precise stimulation protocol used (27–29). Thus both NO-dependent and NO-independent forms of LTP appear to operate in forms of hippocampal LTP.

Nitric Oxide Actions in Skeletal Muscle

Though endogenous NO was originally discovered as a major mediator of smooth muscle relaxation recent studies demonstrate a role for NO in skeletal muscle (30). nNOS mRNA is highly abundant in human skeletal muscle and exists at levels that exceed those in human brain (31). Interestingly, biochemical assays of skeletal muscle homogenates indicate that >90% of NOS activity is membrane associated and is not released by washing microsomal membranes with 0.5 *M* NaCl (31). Western blot analysis indicates strong expression of neuronal-type NOS in skeletal muscles. NOS activity in a variety of individual muscles correlates closely with fast twitch (type II) muscle fiber composition (30). Immunohistochemical localization of nNOS shows prominent labeling of sarcolemma in a subset of muscle fibers in diaphragm. ATPase histochemical typing identifies nNOS positive fibers as fast twitch consistent with observations that NOS activity in isolated muscles increases as the proportion of fast twitch fibers increases (30).

Pharmacological modulators of NOS signaling have been used to determine the role of NO in skeletal muscle contraction. Incubation of rat diaphragm muscle with NOS inhibitors causes a dramatic shift to the left of the force-frequency relationship indicating an increase in contractile force (30). These increases in contractile force by nNOS inhibition are reversed by the exogenous NO donors S-nitroso-N-acetylcysteine (SNAC) and sodium nitroprusside (30). Relaxation of smooth muscle by NO is mediated by cGMP. Similarly agents which increase intracellular cGMP, such as 8-bromo-cGMP and dipyridamole, significantly reverse the contractile increases caused by inhibition of nNOS (30). In addition, the guanylyl cyclase inhibitor LY 83583 increases relative force production during submaximal tetanic contraction. Finally, immunohistochemistry with a monoclonal antibody to cGMP reveals that cGMP is enriched at the sarcolemma in the immediate vicinity of nNOS. Taken together, this body of evidence suggests that endogenous NO produced in active muscle near the sarcolemma opposes contractile force.

nNOS Associates with the Dystrophin Complex in Muscle

A striking aspect of this work is that nNOS is selectively present at the sarcolemmal membrane. Subcellular fractionation of mouse quadriceps skeletal muscle indicates that nNOS is anchored both to integral membrane proteins of microsomal membranes and to proteins in cytoskeleton (32). Thus, approximately 75% of nNOS protein and enzyme activity remain membrane associated following extensive washing of muscle heavy microsomes with 0.5 *M* NaCl. Solubilization of washed membranes with 0.5% Triton X-100 releases about ½ of this particulate nNOS with the remainder in the cytoskeletal pellet. By contrast, eNOS, which is N-terminally myristolated, is quanti-

tatively solubilized from skeletal muscle extracts by Triton X-100.

This differential fractionation suggests that unique determinants in nNOS anchor this isoform to the skeletal muscle cytoskeleton. The amino acid sequence of nNOS contains a 230 amino acid N-terminal domain which is not present in eNOS (Fig. 1A). Beyond this extended N-terminus of nNOS the two proteins share >60% sequence identity. Similar enzymatic activities of eNOS and nNOS suggest that the unique N terminus of nNOS may not be required for catalytic activity. Indeed, a deletion mutant, nNOSΔ1-226, lacking the first 226 amino acids exhibits essentially normal NOS catalytic activity. Thus, both the wild-type and nNOSΔ1-226 mutant display similar V_{max}, and K_m for arginine as well as regulation by calcium/calmodulin.

Rather than regulating catalytic activity the N-terminal domain instead targets nNOS to skeletal muscle sarcolemma (32). Within this domain, nNOS contains a 66 amino acid motif which bears homology to a heterogeneous family of signaling enzymes that share the property of being localized to specialized cell-cell junctions (Fig. 1B). Proteins containing this motif, which is named "GLGF" for a conserved tetrapeptid (33), include: *dlg*-1, the product of the lethal discs-large tumor suppressor gene that localizes to the undercoat of the septate junction in Drosophila (34); disheveled (*dsh*), a gene required for planar cell polarity in Drosophila; PSD-95, a brain-specific protein that localizes to the post-synaptic density in mammals (33); ZO-1 and -2, peripheral membrane proteins that localize to tight junctions (zona occludens) of epithelial and endothelial cells (35, 36); and certain protein tyrosine phosphatases, such as PTP1E, which are thought to localize at the junction between the plasma membrane and the cytoskeleton. Interestingly, this domain shares homology to syntrophins (syn), a family of recently cloned dystrophin-binding proteins, which co-localize with NOS beneath the sarcolemmal membrane of skeletal muscle (37, 38). In fact, syntrophins are more closely related to nNOS in this domain than any other known gene (Fig. 1B), suggesting a possible role for dystrophin in regulating the sarcolemmal localization of nNOS.

Association of nNOS with dystrophin was demonstrated by means of wheat germ agglutinin (WGA)–Sepharose affinity chromatography (32). nNOS, which lacks glycosylation sites, is not expected to adhere to a wheat germ column while dystrophin, which is bound to a glycoprotein complex, is routinely purified by this technique (39).To ensure specificity of this association parallel experiments were conducted with tissue from *mdx* mice which specifically lack dystrophin due to a non-sense mutation(40), but express nNOS at near normal levels. Quadriceps from wild-type and *mdx* mice were homogenized and solubilized in buffer containing 0.5 *M* NaCl and 1% digitonin. Solubilized homogenates were applied to succinylated (WGA)–Sepharose columns which were extensively washed with buffer containing 0.5 *M* NaCl and 0.5% Triton X-100. Tightly bound proteins were affinity eluted with 0.5 *M* N-acetyl-D-glucosamine (NAG). Western blot analysis reveals elution of nNOS in NAG eluates from wild-type but not *mdx* tissue (Fig. 2A).

In order to quantitate enrichment of nNOS by sWGA chromatography larger scale purifications were conducted from rat skeletal muscle tissue. Western blot analysis for nNOS in equally loaded (5 μg/lane) fractions from sWGA chromatography revealed a striking enrichment in the NAG elute (Fig. 2B). nNOS was typically purified approximately 60-fold by sWGA chromatography (32). To evaluate the percent retention of nNOS by sWGA, fractions were reloaded onto SDS gels with 6-fold more total protein (21 μg) in "load" and "flow through" lanes (21 μg) than in wash and eluate lanes (3.5 μg) (Fig. 2C). Semi quantitative

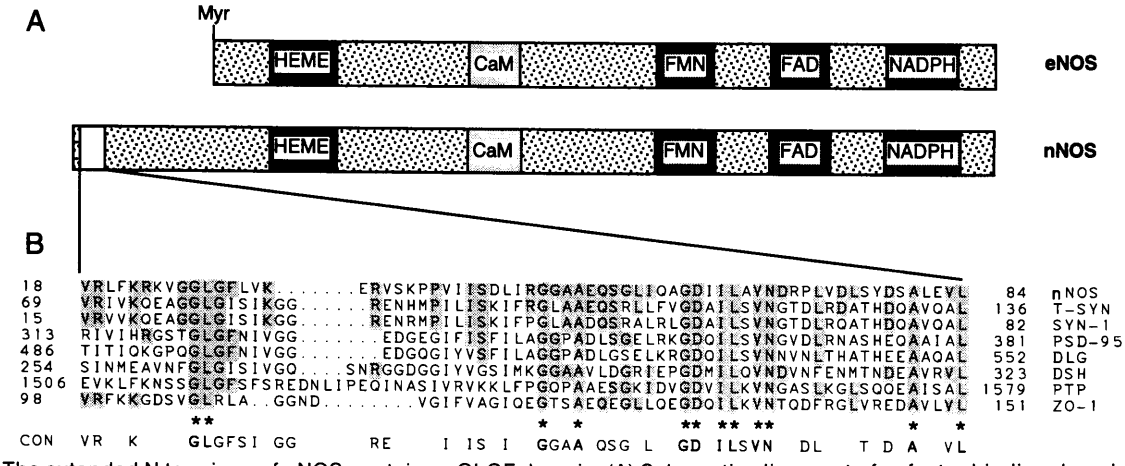

Figure 1. The extended N terminus of nNOS contains a GLGF domain. (A) Schematic alignment of cofactor binding domains of eNOS and nNOS indicating N-terminal myristoylation of eNOS and extended N terminus of nNOS. (B) Alignment of the GLGF/dystrophin binding domain of nNOS with syntrophins and a family of other cytoskeletal associated proteins. (Adapted from Ref. 32.)

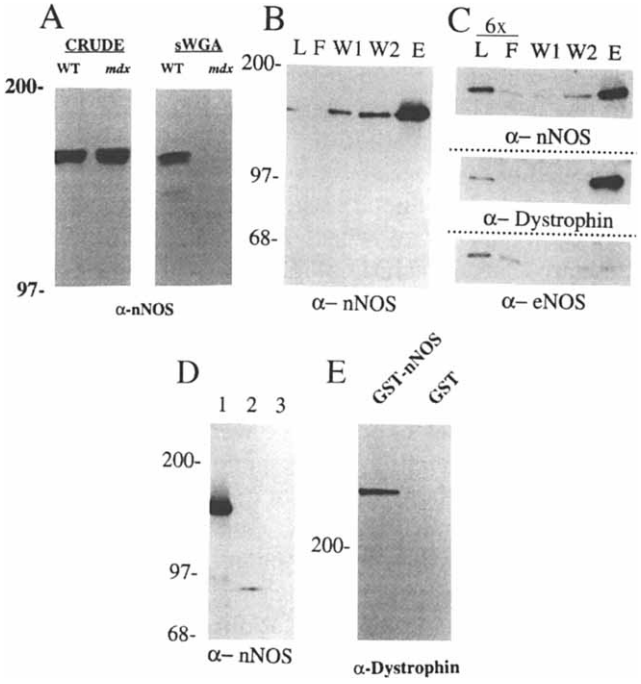

Figure 2. Association of nNOS and dystrophin in skeletal muscle. (A–C) Dystrophin-associated glycoprotein complex was purified by succinylated wheat germ agglutinin (sWGA) chromatography. (A) Western blotting indicates that total nNOS levels in crude extracts are similar in wild-type and *mdx* muscle. nNOS coelutes with dystrophin on sWGA affinity column in muscle homogenates from wild-type (WT) mouse. nNOS does not adhere to a sWGA column in extracts from *mdx* mouse. (B) Western blotting for nNOS from equally loaded (5 μg/lane) fractions from sWGA chromatography shows large enrichment of nNOS in NAG eluate fractions. L, load; F, flow through; W1, 500 m*M* NaCl wash; W2, 500 m*M* NaCl and 0.5% Triton X-100 wash; E, 0.3 *M* NAG eluate. (C) Reloading samples from sWGA column with 6-fold higher protein in load and flow through (21 μg/lane) than wash and eluate fractions (3.5 μg/lane) shows purification of nNOS and dystrophin but not eNOS by sWGA. (D) Immunoprecipitation from NAG eluate fractions indicates that a monoclonal antibody to dystrophin (12 n*M*) potently precipitates nNOS (Lane 1). Control experiments containing a monoclonal anti-*myc* antibody (12 n*M*) or lacking any primary antibody (Lane 2 and 3, respectively) fail to precipitate nNOS. (E) Glutathione sepharose beads bound to GST or GST nNOS (1–299) were incubated with solubilized skeletal muscle membranes. After extensive washing, the beads were eluted with 0.2% SDS, proteins were separated by SDS-PAGE and retention of dystrophin protein was analyzed by Western blotting. (Adapted from Ref. 32.)

analysis indicated that approximately 80% of particulate nNOS adhered to the sWGA column. This same blot was probed with an antibody to eNOS which is similar to nNOS but lacks a GLGF domain. eNOS did not specifically adhere to the sWGA column and only residual amounts of eNOS were found in NAG eluate fractions.

Immunoprecipitation experiments were performed to determine whether nNOS associates with dystrophin complexes eluting from sWGA with NAG. Samples were incubated for 1 hr with a monoclonal antibody to α-dystrophin or α-*myc* (9E10) as control and immunocomplexes pelleted with anti-mouse IgG beads. Western blot analysis revealed potent immunoprecipitation of nNOS with 2.0 μg/ml (12 n*M*) α-dystrophin and no precipitation of nNOS by α-*myc* (12 n*M*) or in incubations lacking primary antibody (Fig. 2D).

To directly evaluate binding of dystrophin associated complexes to the N-terminal domain of nNOS a protein containing glutathione-S-transferase (GST) fused to the first 299 amino acids of nNOS was coupled to glutathione beads. The GST-nNOS (1–299) beads and control GST beads were incubated with solubilized homogenates of mouse skeletal muscle. After extensive washing of the beads, bound proteins were eluted with sample buffer. Western blotting indicated that GST-nNOS (1–299) beads but not control beads retained dystrophin protein (Fig. 2E).

Absence of Sarcolemmal NOS in Dystrophic Muscle

Dystrophin contains binding sites for actin (41) and β-dystroglycan (42), such that dystrophin partitions with both the cytoskeleton and the sarcolemma of skeletal muscle. A similar distribution for skeletal muscle nNOS raises the possibility that this subcellular localization of nNOS is mediated by dystrophin binding. The absence of dystrophin leads to a dramatic reduction in dystrophin-associated proteins, including α- and β-dystroglycans and syntrophin, in the sarcolemma of patients with Duchenne muscular dystrophy and *mdx* mice (43). nNOS expression is also disrupted in dystrophic muscle. Overall nNOS levels and enzyme activity are modestly decreased (~80% of control levels) in skeletal muscle from *mdx* mice; however, nNOS is entirely absent from the sarcolemma in these animals. Thus, in skeletal muscle from *mdx* mice, nNOS is quantitatively removed from membrane fractions following a 0.5 *M* NaCl wash; no nNOS protein or enzyme activity were detected in the detergent extract or cytoskeletal fractions (Fig. 3, A and B).

Immunohistochemical studies further identifies an absence of sarcolemmal nNOS in *mdx* skeletal muscle. nNOS immunofluorescence is found restricted to the sarcolemma of a subset of rat extensor digitorum longus skeletal muscle fibers (Fig. 3C). However, nNOS immunoreactivity is absent from the sarcolemma of *mdx* muscle (Fig. 3C) (32). This derangement of nNOS is specific for dystrophin abnormalities. Thus, the sarcolemmal distribution of nNOS is not altered in *dy* mice, which have severe muscular dystrophy, but have a normal distribution of dystrophin at the sarcolemma (44). nNOS-like immunoreactivity in skeletal muscle is a product of the nNOS locus as staining is absent in $nNOS^{-/-}$ mice.

The localization of nNOS in human muscle tissues is similar to that in rodent. In normal human biopsies nNOS, dystrophin and spectrin are colocalized be-

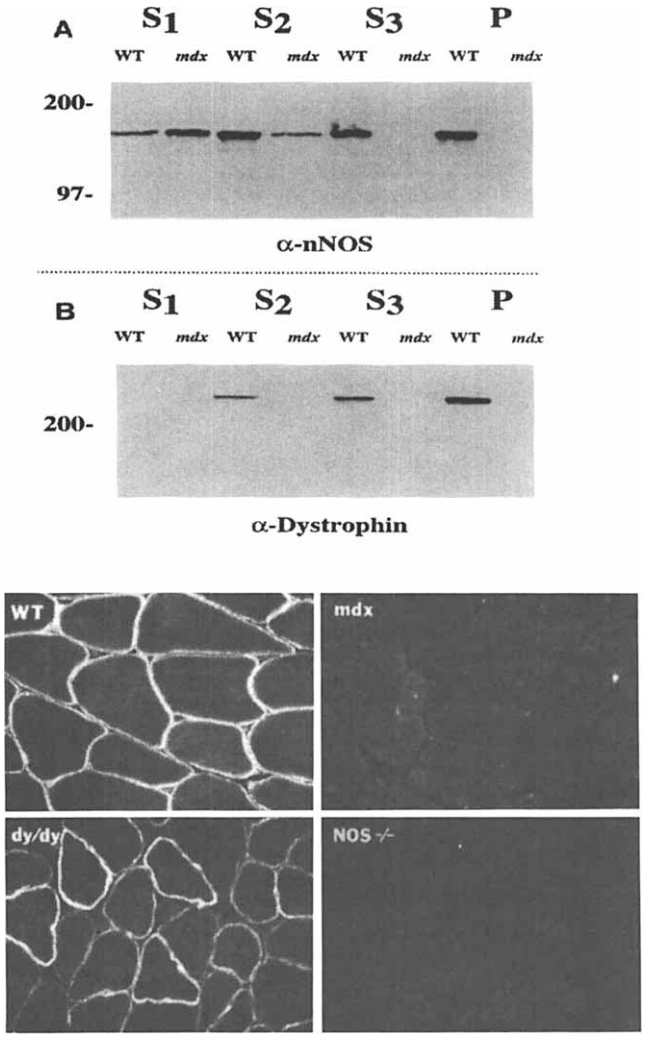

Figure 3. nNOS is selectively absent from sarcolemma of *mdx* skeletal muscle. Subcellular fractions (S_1, S_2, S_3, and P) of mouse quadriceps from wild-type (WT) and *mdx* mice (age matched at 7–8 weeks) were prepared. (A) Western blotting indicates that significant nNOS remains in an insoluble pellet (P) following sequential extraction of WT mouse quadricep homogenates with 100 m*M* NaCl (S_1), 500 m*M* NaCl (S_2), and 0.5% Triton (S_3). By contrast, nNOS in skeletal muscle of *mdx* mice is largely extracted from membranes with 100 m*M* NaCl (S_1) and is completely removed with 500 m*M* NaCl (S_2). (B) Dystrophin is enriched in detergent extract (S_3) and cytoskeletal pellet fractions (P) in wild-type mice and is absent from *mdx* muscle. (C) Immunofluorescent staining for nNOS in quadriceps of wild-type, *mdx*, *dy*, and nNOS knockout mouse was performed using an affinity-purified polyclonal antibody. nNOS immunostaining is present at the surface membranes of skeletal muscle fibers from wild-type (WT) mouse, but is absent from *mdx* mouse skeletal muscle sarcolemma. Homozygous *dy* dystrophic mice display normal sarcolemmal nNOS labeling of intact fibers. Skeletal muscle from NOS knockout mouse is entirely devoid of immunostaining. Cryostat muscle sections from wild-type, *mdx*, *dy*, and nNOS knockout mouse were stained under identical condition using an affinity-purified nNOS antiserum and a FITC linked secondary antibody. Magnification: ×400. (Adapted from Ref. 32.)

neath the sarcolemma of muscle fibers (Fig. 4B). In biopsies from patients with Duchenne muscular dystrophy, the disruption of dystrophin results in absence of nNOS staining of sarcolemma. Normal sarcolemmal labeling for spectrin confirmed that this structural cytoskeleton was not disrupted in the Duchenne muscular dystrophy tissues (Fig. 4B).

Absence of dystrophin in Duchenne muscular dystrophy results in disruption of the dystrophin associated glycoprotein complex and a dramatic reduction in overall levels of certain dystrophin associated proteins in muscle. Similarly, nNOS levels are dramatically reduced in human dystrophic muscle (Fig. 4A). Densitometric scanning of nNOS immunoreactive bands in equally loaded Western blots revealed approximately 75% decrease of nNOS in Duchenne muscular dystrophy tissues. Immunoblotting for spectrin confirmed that similar amounts of protein were loaded in all cases and that the structural cytoskeleton was intact (Fig. 4A).

The physical association of nNOS with dystrophin provides a valuable clue to understanding mechanisms for NO and dystrophin mediated signaling in skeletal muscle. Recently, the dystrophin associated glycoprotein complex (DAGC) has been proposed to mediate neurite-induced clustering of acetylcholine receptors via tight binding of α-dystroglycan to agrin (45, 46), a neuron-derived component of the extracellular matrix. Binding of nNOS to dystrophin beneath skeletal muscle sarcolemma completes the link between the extracellular matrix and signal transduction and may help to explain signaling mediated through the DAGC.

Translocation of nNOS from sarcolemma to myocyte cytosol in dystrophic muscle may also have implications for the pathogenesis of muscular dystrophy. A toxic role for endogenous NO is well characterized in the immune system (47). NO mediates cytotoxicity of induced macrophages by inactivating of enzymes containing critical iron-sulfur enzymes, including ribonucleotide reductase, mitochondrial aconitase, and succinate:ubiquinone reductase (48). In addition to killing tumor cells and pathogens, NO can be neurotoxic. The primary stimulus for NO synthesis in central neurons is activation of NMDA-type glutamate receptors (49). Overactivity of NMDA receptors is implicated in numerous neurodegenerative processes including stroke, Alzheimer's disease, Huntington's chorea, and amyotrophic lateral sclerosis (for recent review see Ref. 50). A causal role for NO in several models of NMDA-mediated degeneration is implied by neuroprotective effects of NOS antagonists (51). More recent studies with nNOS "knockout" mice confirm that endogenous NO contributes to neurodegeneration (51).

Because endogenous NO contributes to cytotoxicity of macrophages in the immune system (47) and neurodegeneration in brain (51), it seems possible that the inappropriate cytosolic nNOS activity in dystrophic muscle is toxic to myocytes. Free radical oxygen intermediates, which occur at high levels in skeletal

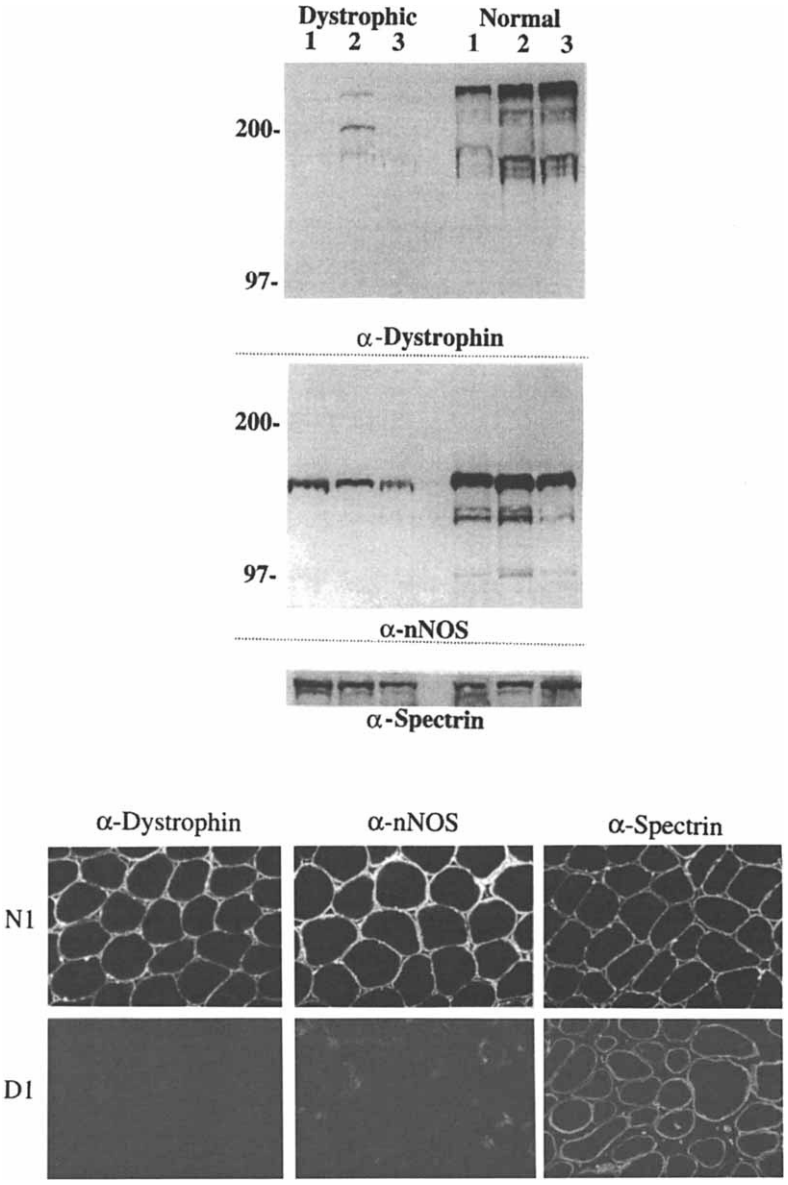

Figure 4. nNOS is absent from sarcolemma of Duchenne muscular dystrophy muscle fibers. (A) Skeletal muscle tissue homogenates from three cases of Duchenne muscular dystrophy and three normal muscle biopsies were resolved by SDS/PAGE. Immunoblot analysis confirms that dystrophin is essentially absent from Duchenne muscular dystrophy muscle. nNOS levels are significantly decreased in all three cases of Duchenne muscular dystrophy. Spectrin levels are nearly equal in normal and Duchenne muscular dystrophy biopsies. (B) Skeletal muscle cryosections of normal (N1) or Duchenne muscular dystrophy (D1) skeletal muscle were immunostained with antibodies to dystrophin, nNOS and spectrin. Magnification: ×200. (Adapted from Ref. 32.)

muscle (52, 53), are known to contribute to cytotoxic damage in various muscle diseases including Duchenne muscular dystrophy (54). Altered regulation of nNOS in dystrophic muscle may augment the toxic interaction of NO and superoxide and contribute to myofiber necrosis. Fast-twitch muscle fibers are preferentially affected in Duchenne muscular dystrophy. However, dystrophin and previously identified components of the dystrophin-associated complex are equally distributed between fast- and slow-twitch fibers (55). The selective enrichment of nNOS in fast-twitch muscle fibers (30) could help explain the preferential degeneration of this fiber type seen in Duchenne muscular dystrophy (56).

Targeting of nNOS to Neuronal Membranes

Cellular studies of nNOS in skeletal muscle provide a model for understanding similar synaptic targeting of nNOS in neurons. In early studies, nNOS was characterized from cytosolic fraction of brain which facilitated biochemical isolation of the protein (57). Cloning of the nNOS cDNA reveals a lack of a hydrophobic signal sequence or predicted membrane spanning domains (58). Subsequent studies indicate that the majority of nNOS immunoreactivity in neurons is associated with rough endoplasmic reticulum and specialized electron dense synaptic membrane structures. Thus, over 60% of NOS activity in associated with the particulate fraction of cortical extracts (59), and over 85% of NOS immunoreactivity in monkey visual cortex is identified as axonal or dendritic profiles (60).

Mechanisms for anchoring nNOS to neuronal membranes are not yet certain. The subcellular distribution of nNOS in brain of *mdx* mouse is normal, suggesting that the dystrophin complex does not play a

major role in regulation of nNOS disposition in neurons. Molecular studies, however, have identified a family of dystrophin-related proteins in brain. It is possible that these protein complexes may play a role in anchoring nNOS to synaptic membranes. Alternatively, the GLGF motif of nNOS may associate with an entirely distinct class of proteins in brain. One attractive candidate is neuro-fibromatosis 2 (NF-2), a recently cloned tumor suppressor protein. NF2 is enriched in brain shares sequence homology to band 4.1 and p55, proteins that have been shown to bind GLGF motifs *in vitro*. Another possibility is that PSD-95 serves as a synaptic anchor for nNOS in brain. PSD-95 itself contains three GLGF domains and is co-localized with nNOS at synaptic densities in numerous neuronal populations (Brenman J, Bredt DS, in preparation).

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