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*Invited Review*

# Nitric oxide synthase in skeletal muscle fibers: a signaling component of the dystrophin-glycoprotein complex

**Z. Grozdanovic and H.G. Baumgarten**
Institute of Anatomy, Freie Universität Berlin, Berlin, Germany

**Summary.** The present review deals with the anatomical distribution, physiological importance, and pathological implications of the neuronal-type nitric oxide synthase (nNOS) in skeletal muscle. Throughout the body, nNOS is located beneath the sarcolemma of skeletal muscle fibers. In rodents, nNOS is enriched in type IIb muscle fibers, but is more homogenously distributed among type II and type I fibers in humans and subhuman primates. It is accumulated on the postsynaptic membrane at the neuromuscular junction. An increased concentration of nNOS is noted at the sarcolemma of muscle spindle fibers, in particular nuclear bag fibers, which belong to type I fibers. The association of nNOS with the sarcolemma is mediated by the dystrophin-glycoprotein complex. Specifically, nNOS is linked to α1-syntrophin through PDZ domain interactions. Possibly, it also directly binds to dystrophin. The activity and expression of nNOS are regulated by both myogenic and neurogenic factors. Besides acetylcholine, glutamate has also been shown to stimulate nNOS, probably acting through N-methyl-D-aspartate receptors, which are colocalized with nNOS at the junctional sarcolemma. Functional studies have implicated nitric oxide as a modulator of skeletal muscle contractility, mitochondrial respiration, carbohydrate metabolism, and neuromuscular transmission. A clinically relevant aspect of nNOS is its absence from the skeletal muscle sarcolemma of patients with Duchenne muscular dystrophy (DMD). A concept is presented which suggests that, as a consequence of the disruption of the dystrophin-glyoprotein complex in DMD, nNOS fails to become attached to the sarcolemma and is subject to downregulation in the cytosol.

**Key words:** Nitric oxide synthase, Nitric oxide, Skeletal muscle, Dystrophin, Muscular dystrophy

*Offprint requests to:* Dr. Zarko Grozdanovic, Institute of Anatomy, Department of Neurobiology and Tumor Biology, Universitätsklinikum Benjamin Franklin, Königin-Luise-Strasse 15, D-14195 Berlin, Germany

## Introduction

Since the discovery that the biological activity of the endothelium-derived relaxing factor is due to the synthesis and release of the free radical nitric oxide (NO) (Ignarro et al., 1987; Palmer et al., 1987), an impressive body of evidence has been produced unravelling NO as a multifunctional messenger which is involved in a wide range of physiological processes, such as as the control of vascular resistance, the regulation of blood fluidity, immune response to invading pathogens and tumor cells, the synaptic transmission and modulation as well as the relaxation of smooth muscle in the wall of visceral organs. NO is formed intracellularly by the action of NO synthase (NOS; EC 1.14.13.39), an iron protoporphyrin IX (heme)- and flavin-containing oxidoreductase, which catalyzes a five electron oxidation of a guanidino-nitrogen of L-arginine to yield NO and L-citrulline. Three isoforms of NOS have been identified which share common catalytic properties, but otherwise differ with respect to their chromosomal localization, cellular expression, and molecular regulation. The neuronal (nNOS) and endothelial (eNOS) isoforms are constitutive and dependent on $Ca^{2+}$/calmodulin. The inducible NOS (iNOS) is regulated at the transcriptional level by numerous agents generated during an immune or inflammatory reaction. As NO is a readily diffusible, short-lived agent which, on the basis of its physico-chemical properties, is able to interact with an array of molecular targets in- and outside the cells, advances in our knowledge of the biological actions of NO are derived to a large extent from studies using inhibitors of NOS or, more recently, targeted disruption of the genes encoding for the NOS isoenzymes (Huang and Fishman, 1996). Comprehensive review articles and multi-authored books are available containing in-depth discussions of the various aspects of NO function, and the reader is referred to these sources for detailed information (e.g., Moncada et al., 1991; Nathan, 1992; Moncada and Higgs, 1993; Bredt and Snyder, 1994;

Schmidt and Walter, 1994; Vincent, 1995; Feelisch and Stamler, 1996; Lincoln et al., 1997). Most recently, it has become increasingly evident that skeletal muscle fibers are quantitatively the most important source of nNOS, which is attached to the muscle fiber membrane *via* the dystrophin-associated protein complex. Thus, it is strategically positioned to transduct information originating from outside the muscle fibers to the fiber interior and *vice versa*. The signals leading to an enhanced NO synthesis in muscle fibers are beginning to be elucidated, and a number of potential targets have already been identified. The present account is intended to present an up-to-date summary of this rapidly expanding field of NO research.

## Mechanisms of nNOS targeting to the skeletal muscle sarcolemma

### *Distribution of nNOS within skeletal muscle*

That nNOS may be expressed in the skeletal muscle under normal conditions was first suggested by Nakane et al. (1993). In a series of biochemical and molecular biology studies, Nakane and colleagues have shown that cloned human nNOS is expressed at high levels in human skeletal muscle. Indeed, nNOS mRNA levels and enzyme activity were found to be higher in human skeletal muscle than human brain. Furthermore, most of the NOS activity, determined by the conversion of [$^3$H]arginine to [$^3$H]citrulline, was detected in membrane fractions of skeletal muscle.

Subsequent histochemical investigations have provided strong support for the notion that nNOS is specifically associated with the skeletal muscle plasma membrane (i.e., sarcolemma). By immunohistochemistry, Kobzik et al. (1994) showed nNOS to be preferentially localized to the periphery of rat skeletal muscle fibers. Grozdanovic et al. (1995c) used a combination of immunochemical and enzyme histochemical methods to systematically examine the molecular distribution of nNOS in a wide range of skeletal muscles in mice, rats, gerbils, hamsters, guinea-pigs, and marmoset monkeys. Both nNOS immunoreactivity and NADPH diaphorase staining, a histochemical reaction during which a water-soluble tetrazolium salt is reduced to an insoluble formazan as a result of electron flow down the reductase domain of nNOS (see e.g., Weinberg et al., 1996), were regularly concentrated at the myofiber surface (Fig. 1). In some muscle fibers, however, a punctate NADPH diaphorase staining pattern was also observed in the fiber interior, being more or less diffusely distributed throughout the sarcoplasm. Similar findings were obtained in human skeletal muscles (Grozdanovic et al., 1996). There is circumstantial evidence from histochemical experiments that the cytoplasmic NADPH diaphorase labeling does not indicate the presence of nNOS (Nakos and Gossrau, 1994; Grozdanovic et al., 1995a,b; Grozdanovic, 1998). This conclusion is supported by the results of Kusner and Kaminski (1996) showing that all NADPH diaphorase activity in muscle is due to a single protein. These findings are at odds with those of Frandsen et al. (1996), who maintain that nNOS immunostaining and NADPH diaphorase activity occur both in the sarcolemma region and the cytoplasm of the human skeletal muscle. The basis for this discrepancy is elusive.

These data are confirmed by ultrastructural studies. Using immunogold-labeling electron microscopy, Langer et al. (1996) and Wakayama et al. (1997) noted an accumulation of gold particles in the subsarcolemmal region of rat and human skeletal muscles, respectively. In addition, Wakayama et al. (1997) have found that gold

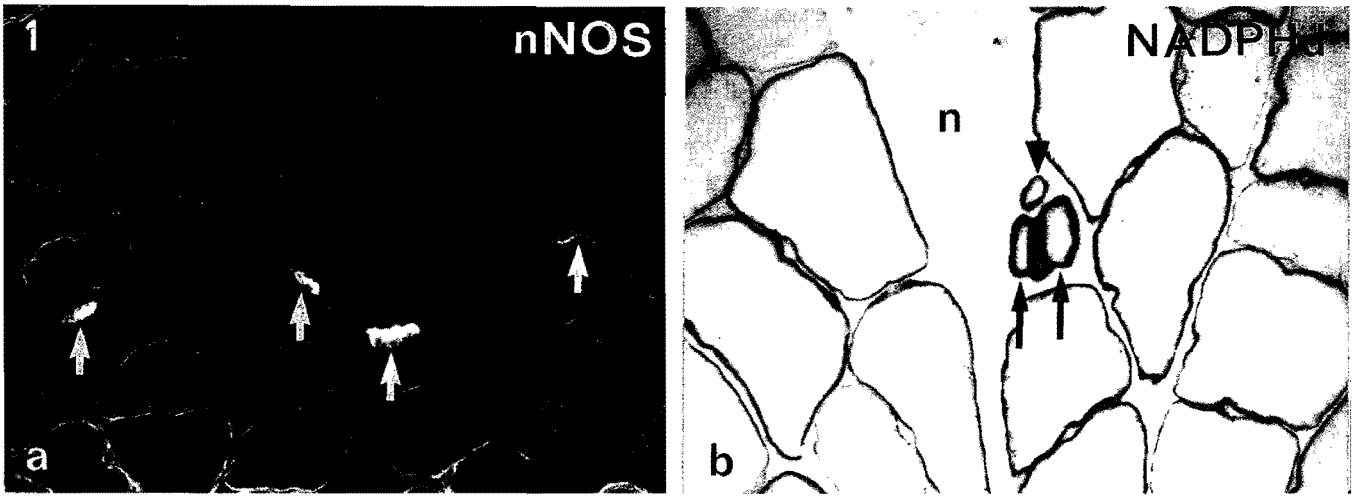


**Fig. 1.** Demonstration of nNOS by immunofluorescence labeling **(a)** and NADPH diaphorase (NADPHd) histochemistry **(b)** at the sarcolemma of striated muscle fibers in the rat. **a.** nNOS is concentrated at muscle endplates (arrows, tongue). **b.** In addition to extrafusal fibers, nNOS is also present at the sarcolemma of intrafusal nuclear bag (arrows) and nuclear chain fibers (arrowhead, gastrocnemius muscle). n: Nerve. x 100

particles tend to occur in clusters and to be more numerous in the vicinity of mitochondria. This latter finding is particularly intriguing in view of the role of NO in the regulation of mitochondrial respiration, as detailed below.

In general, skeletal muscles are composed of a heterogenous population of fibers that differ with respect to their cytochemical, physiological, and biochemical properties, providing the basis for the classification of muscle fibers into type I, the slow fibers, and several subtypes of the fast fibers, or type II fibers, the exact number (and designation) of which varies according to the methods used (Kelly and Rubinstein, 1996; Zhang et al., 1998). As originally suggested by Kobzik et al. (1994), nNOS is differentially distributed among muscle fibers. Histochemically, fiber type correlations were assessed by double staining for myofibrillar ATPase activity (Kobzik et al., 1994; Grozdanovic et al., 1995c) or immunoreactivity for myosin heavy chain isoforms (Grozdanovic and Gossrau, 1998). Both approaches have yielded comparable results indicating that nNOS is selectively enriched in type II fibers. In type I fibers, the sarcolemmal nNOS labeling is very faint, but obviously not completely negative. Furthermore, type II fibers that are particularly well labeled are found to exhibit low staining levels for oxidative enzymes, such as NADH tetrazolium reductase, succinate dehydrogenase, and cytochrome *c* oxidase (Grozdanovic et al., 1995c). Taken together, these data suggest that, at least in rodents, nNOS is selectively expressed in type IIb fibers, the fast glycolytic fibers. This interpretation is supported by biochemical data. First, NOS activity is shown to vary among skeletal muscles according to the proportion of type II fibers (Kobzik et al., 1994), and, second, total nNOS protein is found to occur at higher levels in the extensor digitorum longus muscle, which is composed of predominantly type II fibers, than the soleus muscle, which consists primarily of type I fibers (Chao et al., 1997). In humans, however, nNOS is more uniformly distributed among type II and type I fibers, or may even predominate in type I fibers (Frandsen et al., 1996).

Several authors have noted a concentration of nNOS at the neuromuscular junction, at least in skeletal muscles of mammals (Brenman et al., 1996; Gath et al., 1996; Kusner and Kaminski, 1996; Oliver et al., 1996; Chao et al., 1997; Grozdanovic et al., 1997a; Grozdanovic and Gossrau, 1997, 1998) (Fig. 1a). It may be of interest that no such accumulation is found in skeletal muscles of turtles (Gossrau and Grozdanovic, 1998a) or pigeons (Gossrau and Grozdanovic, 1998b). Although it has been proposed that nNOS resides within the presynaptic motor nerve terminal (Oliver et al., 1996), the bulk of evidence now favors a postsynaptic localization of nNOS. Thus, nNOS protein expression at the neuromuscular junction was little affected up to two weeks following peripheral nerve sectioning and degeneration (Kusner and Kaminski, 1996; Chao et al., 1997). Furthermore, as a consequence of a deficiency of dystrophin at the muscle fiber sarcolemma, nNOS immunolabeling and, to a lesser extent, NADPH diaphorase staining were found to be diminished at the neuromuscular junction of *mdx* mice skeletal muscles (Grozdanovic et al., 1997a; Grozdanovic and Gossrau, 1998).

Grozdanovic et al. (1995c) observed a conspicuous nNOS activity at the sarcolemma of the specialized muscle fibers in muscle spindles, the so-called intrafusal fibers, which are encircled by sensory endings responding to changes in muscle length (Fig. 1b). Often, the intensity of nNOS staining on the intrafusal fibers was stronger than on the surrounding extrafusal fibers (Grozdanovic et al., 1995c; Chao et al., 1997). This was particularly true in the case of nuclear bag fibers, especially bag 2 fibers, which were more heavily labeled than bag 1 fibers and nuclear chain fibers (Gossrau and Grozdanovic, 1997). Moreover, Gossrau and Grozdanovic (1997) have shown that bag 2 fibers and segments of bag 1 fibers are immunoreactive for the slow myosin heavy chain isoform, whereas nuclear chain fibers contain the fast isoform. It should, however, be emphasized that nNOS is almost exclusively found in the polar regions of intrafusal fibers, which stain strongly for dystrophin and other components of the dystrophin-glycoprotein complex (see below), and contain a concentration of mitochondria and myofibrils. In constrast, either weak or no nNOS staining is present in the central regions of intrafusal fibers, which are endowed with sensory axons (Gossrau and Grozdanovic, 1997).

Chang et al. (1996) reported an elevated accumulation of nNOS immunostaining at the myotendinous junction, which is the region of close apposition between the ends of muscle fibers and the tendinous attachments, acting to transmit contractile forces to the surrounding connective tissue matrix. It is well known that dystrophin expression is accentuated at the myotendinous junction. We, however, could not reproduce the former finding by using both nNOS immunofluorescence and NADPH diaphorase histochemistry (Grozdanovic and Gossrau, unpublished observations).

### *Association of nNOS with the dystrophin-glycoprotein complex*

The mechanisms which are responsible for the attachment of nNOS to the sarcolemma have begun to be unravelled following the demonstration by Brenman et al. (1995) that nNOS is complexed with dystrophin, a large rod-like cytoskeletal protein anchored to the sarcolemma *via* a glycoprotein complex (see below). They have shown that nNOS, which is primarily particulate, copurifies with the dystrophin-glycoprotein complex in skeletal muscle extracts from wild-type mice, but not from dystrophin-deficient *mdx* mice. In an analogous set of experiments, Brenman et al. (1995) have found that the dystrophin complex copurifies with nNOS in muscle extracts from normal mice, but not from nNOS knockout mice. However, as demonstrated

by Chang et al. (1996), nNOS is probably not directly bound to dystrophin, but is instead linked to some protein in the dystrophin-glycoprotein complex.

Further evidence is based on the histochemical analysis of nNOS localization in various neuromuscular disorders of humans and related animal models. Both nNOS immunoreactivity and NADPH diaphorase staining were found to be drastically reduced or completely absent from the sarcolemma of patients afflicted with the most severe type of the dystrophinopathies, Duchenne muscular dystrophy (DMD) (Brenman et al., 1995; Grozdanovic et al., 1996), and of *mdx* mice (Brenman et al., 1995; Grozdanovic et al., 1997a; Grozdanovic and Gossrau, 1998), but showed no obvious alterations in non-dystrophin-associated muscular dystrophies as well as certain inflammatory and metabolic muscle diseases (Grozdanovic et al., 1997c). Dystrophinopathies are a group of muscle disoders which are due to abnormalities of the dystrophin gene located on chromosome Xp21 (Hoffman et al., 1987; Koenig et al., 1988; for a recent review on the differential diagnosis of dystrophinopathies, see Kakulas, 1996). In DMD skeletal muscle, dystrophin is markedly diminished (Arahata et al., 1988; Bonilla et al., 1988; Hoffman et al., 1988; Nicholson et al., 1990; Bulman et al., 1991; Voit et al., 1991), whereas in Becker muscular dystrophy (BMD), a milder allelic variant of DMD, it is present but abnormal in molecular weight, abundance, or both (Hoffman et al., 1988). Understanding of the molecular pathogenesis of muscle damage in DMD has been facilitated by the identification of an oligomeric complex of integral and peripheral membrane proteins which, together with dystrophin, create a linkage between the cytoskeleton and the extracellular matrix. As a consequence of dystrophin deficiency in DMD, all of the dystrophin-associated proteins are severely reduced at the sarcolemma (Ervasti and Campbell, 1993; Matsumura and Campbell, 1993, 1994; Campbell, 1995).

The dystrophin-associated proteins are organized into dystroglycan, sarcoglycan, and syntrophin complexes. The dystroglycan complex consists of two subunits, α-dystroglycan and ß-dystroglycan, which are derived from a common precursor protein (Henry and Campbell, 1996; Matsumura et al., 1997). ß-Dystroglycan is a membrane-spanning glycoprotein which binds to the cysteine-rich region of the C-terminal domain of dystrophin inside the muscle fiber and to α-dystroglycan, a peripheral proteoglycan. α-Dystroglycan itself interacts with merosin (laminin α2-chain). The sarcoglycan complex consists of four transmembrane proteins, designated as α-sarcoglycan (originally named adhalin), ß-sarcoglycan, γ-sarcoglycan, and δ-sarcoglycan, which are linked to ß-dystroglycan (Sunada and Campbell, 1995; Ohlendieck, 1996; Nigro et al., 1996; Jung et al., 1996). It is suggested that the sarcoglycan proteins interact with dystrophin at the inner surface of the muscle membrane. Attached to the distal C-terminal domain of dystrophin are components of the syntrophin triplet, namely α1-syntrophin, ß1-syntrophin, and ß2-syntrophin (Ahn and Kunkel, 1995; Yang et al., 1995). The syntrophins contain two pleckstrin homology domains (Gibson et al., 1994) and one PDZ domain (Adams et al., 1995). PDZ domains, which are also known as the GLGF domains or the DHR domains, function as protein-recognition modules in a diverse group of cytoskeletal proteins and enzymes at sites of cell-cell communication, such as synaptic junctions (Cho et al., 1992; Gomperts, 1996).

This raises the question as to which component of the dystrophin-glycoprotein complex serves to link nNOS to the sarcolemma. As already mentioned, nNOS does not interact directly with dystrophin *in vitro* (Chang et al., 1996). These data are somewhat challenged by the *in vivo* work. Thus, analysis of nNOS localization in transgenic *mdx* mice expressing truncated portions of the dystrophin gene suggests that association of nNOS with the sarcolemma requires full-length dystrophin (Chao et al., 1996). Immunoreactivity for nNOS in transgenic *mdx* mice expressing full-length dystrophin or truncated dystrophin lacking the 330 nucleotides of exons 71–74 near the C-terminus (Δ330) was indistinguishable from wild-type mice. It was, however, absent at the sarcolemma of *mdx* mice expressing truncated dystrophin lacking exons 17–48 (ΔE17–48) of the spectrin-like motif or the C-terminal 71 kDa of dystrophin (Dp 71). Moreover, nNOS immunostaining was absent from the sarcolemma of patients with mild and intermediate BMD caused by deletions in the rodlike and N-terminal domain of dystrophin, respectively (Chao et al., 1996). It may, therefore, be supposed that nNOS binds directly to the central region of dystrophin (Chao et al., 1996).

From the histochemical work it may further be inferred that sarcoglycans are not required for anchoring nNOS to the sarcolemma. In a comparative study, Gossrau et al. (1996) have shown that nNOS is normally present at the sarcolemma of turtle and chicken skeletal muscles, which are, however, devoid of α-sarcoglycan. In addition, examination of muscle biopsies of patients suffering from neurogenic muscle diseases has revealed that nNOS is reduced or abolished at the sarcolemma of atrophic muscle fibers, whereas α-sarcoglycan is not changed (Grozdanovic et al., 1997b). This is supported by findings of Chao et al. (1996). They have found intact nNOS immunolabeling at the sarcolemma of two patients with a primary α-sarcoglycan deficiency. It is well established that a loss of any single sarcoglycan protein leads to a secondary deficiency in the immunostaining for other components of the complex, whereas the immunostaining for dystrophin and dystroglycan are little affected (Bönnemann et al., 1996; Brown, 1997).

In their 1995 paper, Brenman and colleagues have disclosed within the N-terminal region of nNOS the presence of a PDZ domain, which has been suspected to mediate the interaction of nNOS with the dystrophin complex (Brenman et al., 1995). In a follow-up paper, Brenman et al. (1996) have provided conclusive

evidence for this idea through a series of elegant *in vitro* and *in vivo* studies. Their findings indicate that nNOS is targeted to the syntrophin complex on the cytoplasmic face of the sarcolemma due to binding of its PDZ domain to the PDZ domain of α1-syntrophin. By immunostaining, nNOS and α1-syntrophin are found to colocalize underneath the sarcolemma of normal mice muscles.

In a further study from Bredt's laboratory, it has been shown that nNOS mRNA is alternatively spliced in skeletal muscle, yielding a muscle-specific isoform, nNOSμ, which contains an additional 102 base pair segment between exons 16 and 17 (Silvagno et al., 1996). This segment encodes for a 34 amino acid insert between the calmodulin- and FMN-binding domains. The expression of nNOSμ occurs coincidently with the myotube fusion.

The patterns of distribution of nNOS and α1-syntrophin in skeletal muscle are not always identical. In wild-type mice, nNOS is concentrated in fast fibers, whereas α1-syntrophin occurs in both fast and slow fibers (Brenman et al., 1996). Furthermore, in *mdx* mice, α1-syntrophin is lost from the extrajunctional sarcolemma, but it is intact at the junctional sarcolemma (Peters et al., 1994; Yang et al., 1994; Chao et al., 1996). By contrast, nNOS is deficient at both these sites, supporting the assumption that the sarcolemmal attachment of nNOS cannot fully be explained by its interaction with α1-syntrophin, at least in the junctional region. The discrepancy between nNOS and α1-syntrophin localization has also been noted during postnatal muscle development (Chao et al., 1996). At P3

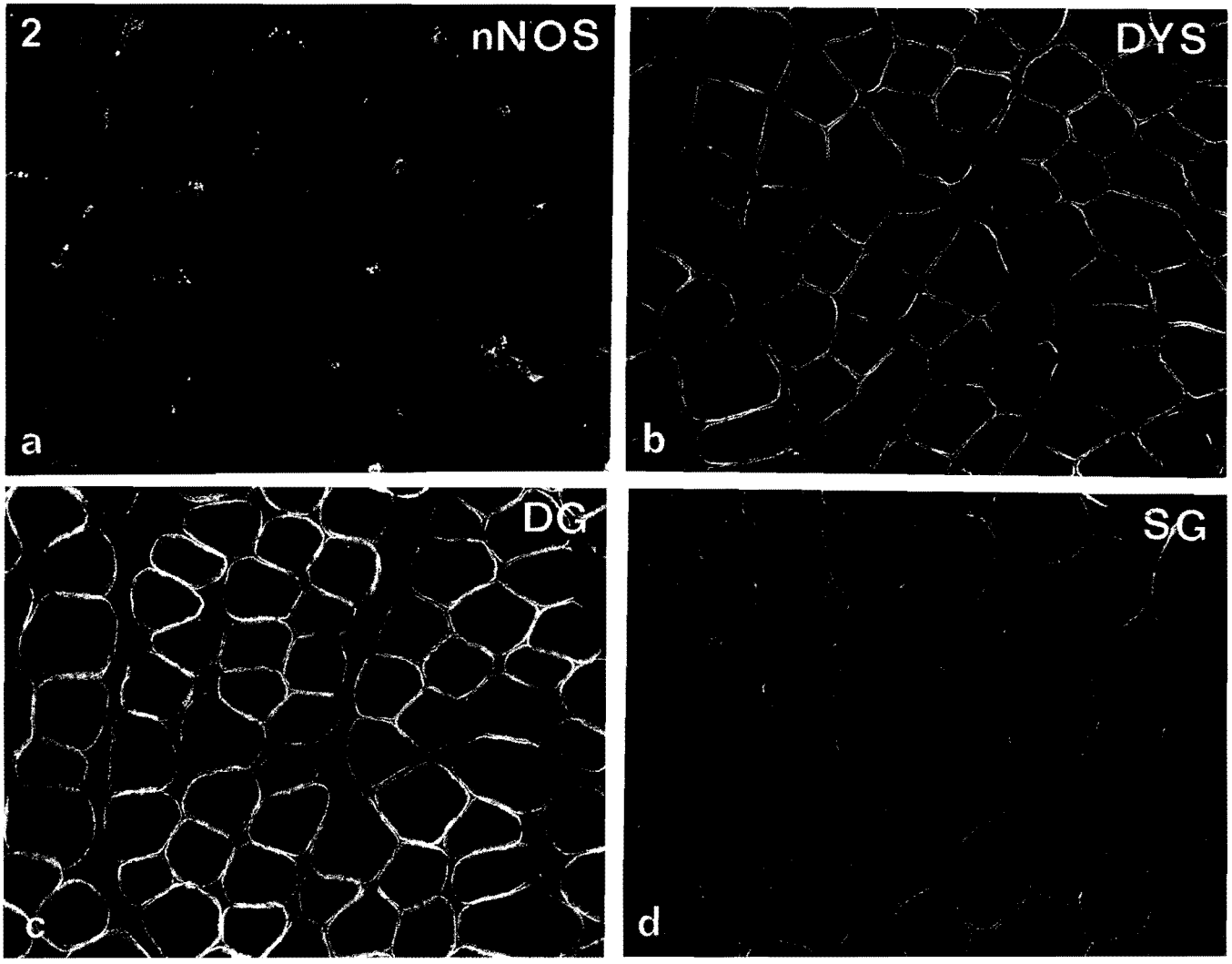


**Fig. 2.** Immunohistochemical analysis of nNOS **(a)**, dystrophin (DYS; **b**), ß-dystroglycan (DG; **c**), and α-sarcoglycan (SG; **d**) in a skeletal muscle biopsy specimen from a patient aged 3 months afflicted with idiopathic clubfoot. No specific nNOS labeling is detectable at the sarcolemma, although other components of the dystrophin-glycoprotein complex are expressed and localized in a regular fashion. x 100

and P7 in rats, dystrophin and nNOS were observed only at the extrajunctional sarcolemma, but both became enriched at the junctional sarcolemma at P12 and P60. In contrast, α1-syntrophin was localized at the extrajunctional sarcolemma and was concentrated at the junctional sarcolemma in all stages evaluated. The close relationship between nNOS and dystrophin was also obvious in the study of Christova et al. (1997). These authors have, however, shown that nNOS and dystrophin are coincident at both the junctional and extrajunctional sarcolemma as early as P1. In human fetal muscle, the localization of syntrophin parallels that of dystrophin, and both subsarcolemmal proteins become localized at an earlier stage than extracellular (α-dystroglycan) and transmembrane (ß-dystroglycan and α-sarcoglycan) components of the dystrophin-glycoprotein complex (Tomé et al., 1994). However, although both dystrophin and syntrophin are fully expressed and orderly localized at the sarcolemma in the fetal skeletal muscle, nNOS does not attach to the sarcolemma until an age of about 5 years (Grozdanovic et al., 1998) (Figs. 2, 3). Most probably, the expression of the nNOS gene is delayed compared to that of dystrophin and syntrophin. There is, however, accentuated accumulation of nNOS staining inside some of the developing muscle fibers, which is detected as early as about 3 years of age, suggesting that nNOS may exist in a soluble form in the sarcosol prior to becoming attached to the sarcolemma (Fig. 3a).

These investigations may also help to understand the mechanisms for the regulation of nNOS at the sarco-

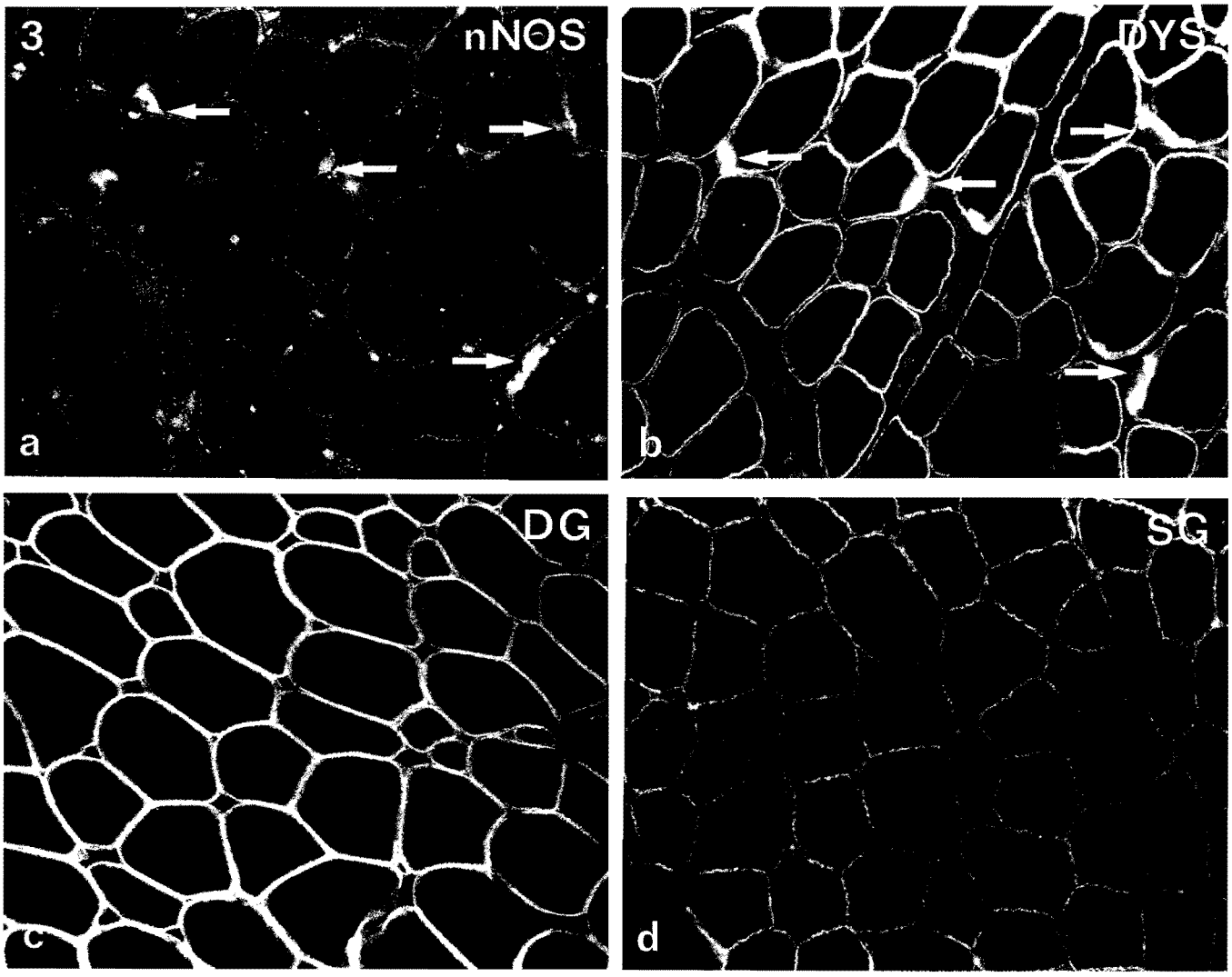


**Fig. 3.** Immunohisochemical analysis of nNOS **(a)**, DYS **(b)**, DG **(c)**, and SG **(d)** in a skeletal muscle biopsy specimen from a patient aged 5 years suffering from a mild myopathy. The intensity of nNOS staining at the sarcolemma is very light compared to the situation in adults. Nevertheless, a selective enrichment of nNOS is discernible at neuromuscular junctions, where it is colocalized with DYS (arrows). In addition, nNOS labeling is diffusely distributed inside muscle fibers. x 100

lemma. As already stated, dystrophin and α1-syntrophin are diminished at the extrajunctional sarcolemma of *mdx* mice, resulting in a secondary deficiency of nNOS. However, at the junctional sarcolemma of *mdx* mice, α1-syntrophin is localized in a regular pattern, whereas nNOS is severely reduced and dystrophin is completely lacking. It is tempting to speculate that a portion of nNOS that is directly bound to α1-syntrophin and does not require dystrophin is preserved at the junctional sarcolemma of *mdx* mice. Previous studies have shown that α1-syntrophin is present in the depths and on the crests of the junctional folds, while dystrophin is restricted to the depths (Byers et al., 1991; Sealock et al., 1991). It has, therefore, to be asked which molecule serves to anchor α1-syntrophin to the junctional sarcolemma in the absence of dystrophin. Utrophin, which is localized at the crests of the junctional folds (Bewick et al., 1992), is a likely candidate, since it can bind to syntrophin *in vitro* (Kramarcy et al., 1994). However, utrophin immunostaining is attenuated at the junctional sarcolemma of *mdx* mice (Grozdanovic and Gossrau, 1998). Another potential candidate is the N-methyl-D-aspartate (NMDA) receptor. This receptor belongs to a family of ionotropic glutamate receptors and shows significant permeability to $Ca^{2+}$ ions. Molecular cloning has revealed a diversity of NMDA receptor complexes, which are composed of various combinations of the principal subunit NR1 and different modulatory NR2 subunits. The subunit composition determines the pharmacological and/or electrophysiological properties of the NMDA receptor subtypes (Nakanishi, 1992; Sucher et al., 1996).

### Mechanisms of nNOS regulation

In brain, NO production is tightly and selectively coupled to NMDA receptor channel activation leading to a rise in the cytosolic $Ca^{2+}$ concentration (Garthwaite and Boulton, 1995). This is facilitated by the targeting of nNOS to macromolecular signal transduction complexes in the postsynaptic density (Brenman and Bredt, 1997). Using the yeast two-hybrid system, Brenman et al. (1996) have identified two postsynaptic density proteins, PSD-95 and PSD-93, which interact with nNOS via the PDZ domains. Previously, Kornau et al. (1995) have raised evidence for an association of PSD-95 with the intracellular tail of NMDA receptors. In the latter study, the second PDZ domain of PSD-95 has been found to bind to the tSXV motif in the C-termimal domain of NR2. In central neurons, therefore, the functional coupling of nNOS to $Ca^{2+}$ inflow through NMDA receptors may be accomplished by a ternary complex which includes PSD-95.

Evidence is accumulating to suggest that NMDA receptors may play a role in nNOS regulation at the myoneural junction. Berger et al. (1995) were the first to demonstrate the presence of NR1 on postsynaptic muscle membranes. This finding was confirmed by Grozdanovic and Gossrau (1998), who also found that nNOS colocalizes with NR1 at the junctional sarcolemma (Fig. 4). Moreover, in *mdx* mice muscles, NR1 is preserved at the postjunctional membrane, together with a portion of nNOS (Grozdanovic and Gossrau, 1998) and α1-syntrophin (see above). In analogy to the situation in the central nervous system, the existence at the myoneural junction of an oligomeric complex that is made up of NMDA receptor, syntrophin/dystrophin, and nNOS may be postulated (Grozdanovic and Gossrau, 1997) (Fig. 5). With regard to this, it would be interesting to know whether a component of the syntrophin complex interacts with NMDA receptors at the endplate membrane. There is, however, circumstantial support from electrophysiological studies that the production of NO in skeletal muscle is dependent upon

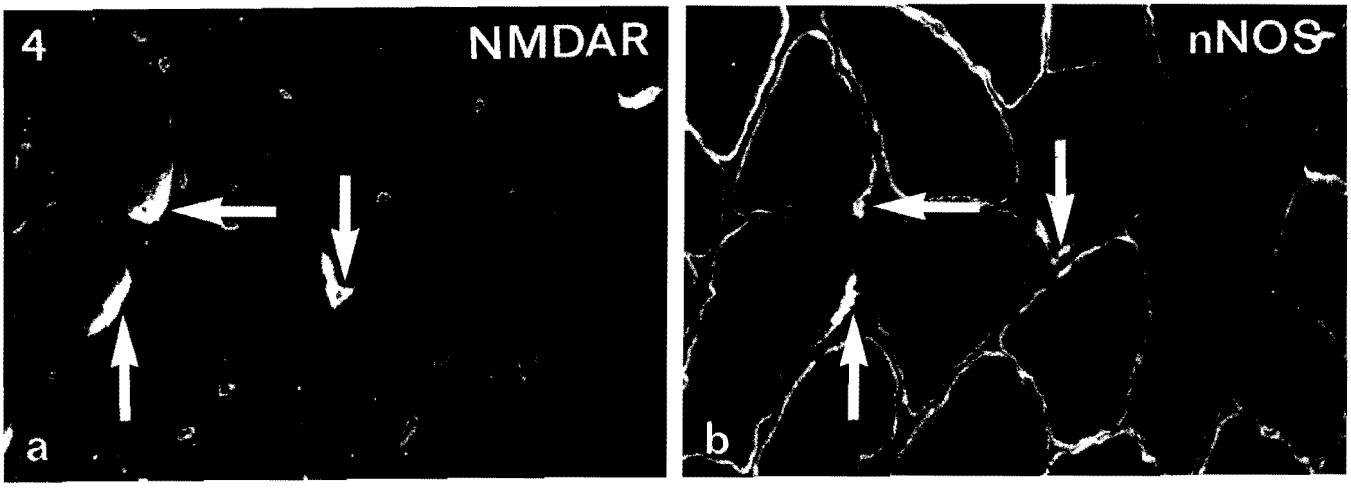


**Fig. 4.** Serial sections through the rat gastrocnemius muscle stained for NMDA receptor (NMDAR) **(a)** and nNOS **(b)** immunoreactivity. NMDAR and nNOS are colocalized at the endplate membrane (arrows). Outside the endplate region, nNOS but no NMDAR is present at the muscle membrane. Note that the nuclei of muscle fibers react positively with the antibody against NMDAR. x 100

NMDA receptor stimulation (see below).

Nevertheless, it has to be admitted that our picture of the regulation of nNOS activity in skeletal muscle is rather fragmentary. In contrast, a concept is emerging that implicates motor nerve activity as a crucial factor in the control of nNOS expression. As shown by Grozdanovic et al. (1997b,c) in muscle biopsy specimens of patients suffering from neurogenic diseases, both nNOS immunolabeling and NADPH diaphorase staining are reduced or totally abolished at the sarcolemma of muscle fibers that display varying degrees of atrophy. Comparable, but somewhat conflicting findings, have been obtained in studies dealing with the consequences of nerve sectioning on the expression of nNOS protein in experimental animals. Oliver et al. (1996) noted a diminution of nNOS immunostaining in the extrasynaptic portion of the sarcolemma and a complete absence in the synaptic portion two weeks following denervation. Using the same experimental design, Kusner and Kaminski (1996) reported a mere reduction in the intensity of nNOS immunoreactivity and NADPH diaphorase staining in the endplate region. In contrast, Chao et al. (1997) did not observe any significant change in the level of nNOS protein or nNOS immunoreactivity in denervated muscle fibers. In the latter study, however, denervation was accompanied by an upregulation of nNOS mRNA levels in skeletal muscle.

A recent study by Reiser et al. (1997) has drawn attention to the importance of myogenic factors in the regulation of nNOS expression in skeletal muscle. They have reported an increased level of nNOS expression as a result of enhanced muscle contractile activity elicited by chronic electrical stimulation *in vivo*. This was accompanied by fast-to-slow fiber type transformation, indicating that augmented nNOS activity and fiber phenotype are not functionally related.

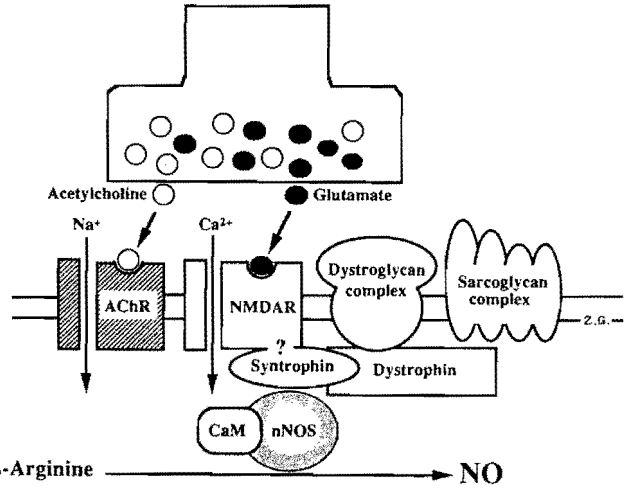


**Fig. 5.** Schematic representation of the putative relationship between the NMDA receptor (NMDAR) and nNOS at the neuromuscular junction. AChR: Acetylcholine receptor; CaM: Calmodulin.

## Functional implications and molecular targets of NO

NO exerts several distinct effects on various aspects of skeletal muscle function, such as contractility, mitochondrial respiratory chain, glucose metabolism, and neuromuscular transmission. Notwithstanding, the underlying mechanisms of action are not perfectly understood.

The effects of NO on contractile function are complex. Kobzik et al. (1994) found that in rat diaphragm NOS inhibitors increased isometric force production at submaximal stimulation frequencies, whereas NO donors depressed force production. However, Morrison et al. (1996) showed that inhibition of NO synthesis by $N^{w}$-nitro-L-arginine (L-NA) did not change maximal isometric force and unloaded shortening velocity, but attenuated shortening velocity and power production of muscle bundles contracting against an external load. These findings suggest that NO is necessary for optimal myofilament function. Important in this regard is the observation that L-NA differentially affected the two parameters of unloaded shortening velocity, $V_{max}$ and $V_0$ (Morrison et al., 1996). Thus, $V_{max}$, which is a composite estimate of whole muscle shortening velocity, was reduced by L-NA, whereas $V_0$, which is a measure of the maximal velocity of the fastest fibers in the muscle, was not altered. The hypothesis has been put forward that NO may selectively promote the detachment rate of a slower cross-bridge population in the muscle (Morrison et al., 1996). Taken together, it may be assumed that NO decreases the efficiency of excitation-contraction coupling by acting at a site proximal to the myofilaments. This assumption is supported by the study of Mészáros et al. (1996), who produced evidence that NO acts to diminish the rate of $Ca^{2+}$ release from the sarcoplasmic reticulum by inhibiting the ryanodine receptor $Ca^{2+}$ release channel.

The mechanisms by which NO modulates skeletal muscle contractile function are beginning to be elucidated. Clearly, several mechanisms of NO action are proposed. In general, many of the physiological effects of NO are mediated by the stimulation of the soluble guanylate cyclase, which catalyzes the conversion of GTP to guanosine 3':5'-cyclic monophosphate (cGMP) (e.g., Schmidt et al., 1993). This seems also to be the case in skeletal muscle. The increase in the contractile force caused by NOS inhibition is in part prevented by 8-bromo-cGMP, a cGMP analogue, as well as dipyridamole, a phosphodiesterase inhibitor (Kobzik et al., 1994). By immunohistochemistry, cGMP has been localized to the sarcolemma region of muscle fibers (Kobzik et al., 1994). How then might cGMP act to promote skeletal muscle relaxation? Available data suggest that elevated cGMP levels may lower intracellular $Ca^{2+}$ concentration by activating the $Ca^{2+}$-ATPase in the membrane of the

sarcoplasmic reticulum (Reid, 1996). Whether this effect is mediated by a cGMP-dependent protein kinase (cGMP kinase) is not known. It has to be pointed out that, according to Chao et al. (1997), immunoreactivity for type I cGMP kinase is restricted to the junctional sarcolemma. Not all effects of NO can be ascribed to cGMP-related mechanisms. For instance, Mészáros et al. (1996) have found that the inhibition of the ryanodine receptor by NO is independent of cGMP formation. Therefore, the existence of further target molecules has to be postulated. A potential candidate are reactive oxygen intermediates (ROI). In actively contracting muscle, ROI are produced at an increased rate (Davies et al., 1982; Reid et al., 1992a,b; Diaz et al., 1993), resulting in an augmented excitation-contraction coupling (Reid et al., 1993) by enhancing $Ca^{2+}$ release from the sarcoplasmic reticulum due to oxidation of the sulfhydryl groups on the $Ca^{2+}$ release channels (Trimm et al., 1986; Liu et al., 1994) and the $Ca^{2+}$ pumps (Scherer and Deamer, 1986). NO has been postulated to reverse sulfhydryl oxidation, thereby opposing the effects of ROI and, consequently, to depress contractile function (Reid, 1996). Certainly, further studies are needed, which, hopefully, will shed more light on the mechanisms of NO action on skeletal muscle contraction. On the other hand, contraction is considered to be a major stimulus for NO formation in skeletal muscle fibers. Balon and Nadler (1994) demonstrated NO release from resting incubated skeletal muscle preparations *in vitro*, which was enhanced by prior electrical stimulation (Balon and Nadler, 1994; see also Reiser et al., 1997).

The ability of NO to react with heme or Fe–S groups of proteins, such as enzymes in the mitochondrial respiratory chain, accounts for its involvement in the regulation of oxidative metabolism. As stated above, nNOS is intimately related to mitochondria in the subsarcolemmal cytoplasm in extrafusal (Wakayama et al., 1997) as well as intrafusal muscle fibers (Gossrau and Grozdanovic, 1997). Moreover, the endothelial isoform of NOS is presumed to occur within skeletal muscle mitochondria. However, the pieces of evidence are not entirely consistent. By light microscopy, immunoreactivity to eNOS has been reported in the cytoplasm of rat skeletal muscle fibers rich in mitochondria, irrespective of the fiber myosin ATPase type (Kobzik et al., 1995). In contrast, guinea-pig (Gath et al., 1996; Young et al., 1997a) and human (Frandsen et al., 1996) skeletal muscle fibers fail to stain positively following immunohistochemistry using antisera against eNOS. On the other hand, Bates et al. (1996) have detected eNOS antigen in isolated rat skeletal muscle mitochondria using electron microscopic immunogold-labeling. Despite these inconsistencies, evidence from biochemical studies clearly supports a role for NO in the regulation of mitochondrial respiration. Kobzik et al. (1995) have demonstrated $Ca^{2+}$-dependent NO production in isolated diaphragm mitochondria. These same authors have also shown L-arginine to inhibit $O_2$ consumption of mitochondria (Kobzik et al., 1995). In fact, as reviewed by Lancaster (1997), NO has the capacity to modulate the activity of various components of the electron-transport chain. The finding that cytochrome oxidase is reversibly inhibited by NO (Cleeter et al., 1994; Brown, 1995) may provide deeper insight into the actions of NO on tissue oxygenation, since "...in addition to inducing increased vascular oxygen delivery by vasodilation, increased NO may also reversibly inhibit oxygen consumption in tissue surrounding the vessel thus extending the diffusion distance of $O_2$ into tissue" (Lancaster, 1997).

So far, few reports have dealt with the involvement of NO in carbohydrate metabolism of skeletal muscle. As early as 1994, Balon and Nadler have claimed that $N^W$-monomethyl-L-arginine (L-NMMA), another inhibitor of NOS, causes a decrease in basal 2-deoxyglucose transport. This observation was later confirmed and extended by Young et al. (1997b). In an *in vitro* preparation of rat soleus muscle, sodium nitroprusside (SNP), an NO donor, increased the rate of 2-deoxyglucose transport as well as the rates of net lactate and [$^{14}$C]lactate release in the presence of insulin. These findings indicate that NO increases the rate of glucose transport by acting independently of insulin. Furthermore, SNP reduced the insulin-stimulated rate of incorporation of [U-$^{14}$C]glucose into glycogen, supporting the notion that NO restrains insulin-mediated glycogen synthesis. In addition, the insulin-stimulated rate of conversion of [U-$^{14}$C]glucose into $^{14}CO_2$ was augmented by SNP, suggesting that NO promotes glucose oxidation. The effects of SNP on glucose transport and metabolism were blocked by LY-83583 (6-analino-5,8-quinolinedione), an inhibitor of soluble guanylate cyclase, and/or methylene blue, which, however, inhibits both soluble guanylate cyclase and NOS. On the other hand, the rate of net lactate release was augmented by 8-bromo-cGMP. These data converge to indicate that NO stimulates muscle metabolism by promoting glucose transport and utilization.

In view of the increased concentration of nNOS at the myoneural junction, it is not surprising that NO has been ascribed functions related to nerve-muscle communication. Wang et al. (1995) evaluated the effects of NO on synaptic events in nerve-muscle co-cultures prepared from *Xenopus* embryos. They found a reduction of spontaneous and induced synaptic currents in response to NO donors. Moreover, the synaptic suppression produced by repetitive, depolarizing stimulation of the postsynaptic myocyte was reversed by hemoglobin and NOS inhibitors. These results have been interpreted to indicate that NO serves as a retrograde messenger which is liberated from a postsynaptic myocyte whose activity is asynchronous to that of the presynaptic terminal, leading to feedback inhibition of synaptic activity. This action of NO, which is probably mediated by cGMP, may contribute to the elimination of polyneuronal innervation of muscle fibers during development.

In a series of studies, Urazaev et al. (1995, 1996, 1997) have produced convincing evidence for a participation of NO in the regulation of the muscle fiber membrane potential. They measured the membrane potential of denervated rat diaphragm muscle fibers kept in a tissue culture medium. After 3 h, a decrease in membrane potential was noted, which amounted to about 8 mV. It was reduced to about 3 mV by addition of glutamate or NMDA to the medium. This effect was blocked by the simultaneous application of glutamate and an NOS inhibitor, $N^{w}$-nitro-L-arginine methyl ester (L-NAME) (Urazaev et al., 1995). The involvement of the NO/cGMP system in the protection of the membrane potential following denervation was substantiated in a follow-up study (Urazaev et al., 1996). The reduction in the early post-denervation depolarization evoked by SNP was antagonized by hemoglobin, 1*H*-[1,2,4]oxadiazolo [4,3-*a*]quinoxalin-1-one, a selective inhibitor of the soluble guanylate cyclase, and methylene blue. Possibly, NO interacts with acetylcholine to prevent the post-denervation depolarization. The protective action of acetylcholine and carbachol on the membrane potential was blocked by $Ca^{2+}$ channel antagonists, L-NAME, and hemoglobin, but not by tubocurarine or ouabain (Urazaev et al., 1997). It has, therefore, been concluded that acetylcholine activates NOS in a $Ca^{2+}$-dependent manner, leading to enhanced NO generation, which provides feedback information about the functional state of the neuromuscular junction to presynaptic nerve terminals (Urazaev et al., 1997).

### Pathophysiology of nNOS in the dystrophin-deficient skeletal muscle

As discussed in detail above, all authors agree that both nNOS protein and its associated histochemical activity are no longer demonstrable at the skeletal muscle sarcolemma of patients suffering from DMD and of *mdx* mice. Controversies, however, exist regarding the fate of nNOS as a result of the lack of dystrophin. According to Brenman et al. (1995), total nNOS protein is reduced by about 75% in DMD muscle compared with normal muscle. In contrast, nNOS protein and activity are moderately decreased in *mdx* muscle, amounting to about 80% of the control levels (Brenman et al., 1995). Interestingly, NOS activity is abolished in the particulate fraction from *mdx* muscle, but is actually accumulated (at levels about 75% higher than in wild-type) in the soluble fraction (Brenman et al., 1995). The findings of this study and their interpretation are questioned by Chang et al. (1996). These latter authors have found that both particulate and soluble nNOS are greatly reduced in skeletal muscles from *mdx* mice compared with wild-type mice. In addition, they have noted a decrease in nNOS mRNA levels in *mdx* mice muscles (Chang et al., 1996).

Consequently, there are opposing views on the significance of NO in the molecular pathogenesis of DMD. Brenman et al. (1995) have hypothesized that, due to the absence of dystrophin, nNOS dissociates from the sarcolemma and accumulates in the cytosol. They have further speculated that an altered regulation of nNOS may promote the interaction of NO with superoxide and contribute to muscle fiber necrosis. Since type II fibers of rats are selectively enriched in nNOS, this fact has been suggested to explain their preferential necrosis in DMD. A number of lines of evidence are not consistent with this model. First, we have disclosed a temporal dissociation between nNOS and dystrophin localization in postnatal human muscle (Grozdanovic et al., 1998). By histochemistry, nNOS has been detected in the area of the sarcolemma in muscle biopsy samples from children aged about 5 years. However, dystrophin is present from as early as nine weeks of gestation in developing human muscle (Clerk et al., 1992). Therefore, a likely conclusion is that, because of the dystrophin gene defect, nNOS is not properly assembled into the dystrophin-glycoprotein-complex, i.e., it *fails to attach* to the sarcolemma, rather than becoming detached. Second, in human skeletal muscle, nNOS is expressed at the sarcolemma of both type I and type II fibers, being probably more abundant in type I fibers (Frandsen et al., 1996). This more uniform pattern of nNOS distribution among human muscle fibers cannot account for the differential fiber type involvement in DMD. Third, the dramatic reduction of nNOS in DMD muscle can hardly result in the production of abnormally high NO levels. As referred to above, Chang et al. (1996) have found a reduction of nNOS mRNA in *mdx* muscle, suggesting that the absence of dystrophin may affect the transcription, processing and/or stability of nNOS mRNA. In contrast, levels of mRNA for dystroglycan and syntrophin are not significantly different in dystrophic vs. normal muscle (Ervasti and Campbell, 1993; Matsumura and Campbell, 1994). It has, therefore, been proposed that these dystrophin-associated proteins may be normally synthesized and correctly assembled in dystrophin-deficient muscle, but not stabilized at the sarcolemma, leading to their accelerated degradation. The attenuation of nNOS in muscles of DMD patients can probably be best explained by the failure of nNOS to become incorporated into the sarcolemma, with subsequent downregulation.

It is well accepted that excessive or unregulated NO production may play a contributing role in various forms of cellular injury associated with vascular, inflammatory, and neurodegenerative disorders (Gross and Wolin, 1995). However, NO *per se* seems to be neither highly reactive nor toxic (Varner and Beckman, 1995). Indeed, it has been suggested that low concentrations of NO may exert protective effects against peroxide-mediated cytotoxicity (Wink et al., 1995). Conversely, it may be hypothesized that a reduction in NO generation may promote tissue injury by nullifying this protective action. A recent study has demonstrated that total cellular proteins in DMD skeletal muscle are quantitatively more oxidized than in normal muscle (Haycock et al., 1996). Speculatively, NO may normally serve to prevent the

oxidative damage of protein constituents in skeletal muscle during states of increased production, such as contraction, by opposing the action of ROI. Certainly, the absence of dystrophin is the primary biochemical abnormality in DMD muscle. However, it does not cause any obvious handicap until the afflicted patients are 4-5 years old (Gorospe et al., 1997). Curiously, nNOS has been shown to be inserted into the sarcolemma at about this age. Of course, this may be a pure coincidence, or yet another piece of evidence in support of the role of NO as a protective agent against the deleterious consequences of unrestrained action of ROI in skeletal muscle.

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