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# Neuronal nitric oxide synthase and dystrophin-deficient muscular dystrophy

**(Duchenne muscular dystrophy/*mdx* mice/syntrophin/skeletal muscle/myotendinous junctions)**

WEN-JINN CHANG*, SUSAN T. IANNACCONE†, KIM S. LAU*, BETTIE SUE S. MASTERS‡, TIMOTHY J. MCCABE‡, KIRK MCMILLAN*§, ROANNA C. PADRE*, MELISSA J. SPENCER¶, JAMES G. TIDBALL¶, AND JAMES T. STULL*‖

*Department of Physiology, University of Texas Southwestern Medical Center, Dallas, TX 75235; †Department of Neurology, Texas Scottish Rite Hospital and University of Texas Southwestern Medical Center, Dallas, TX 75235; ‡Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78284; and ¶Department of Physiological Science, University of California, Los Angeles, CA 90095

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ABSTRACT **Neuronal nitric oxide synthase (nNOS) in fast-twitch skeletal muscle fibers is primarily particulate in contrast to its greater solubility in brain. Immunohistochemistry shows nNOS localized to the sarcolemma, with enrichment at force transmitting sites, the myotendinous junctions, and costameres. Because this distribution is similar to dystrophin, we determined if nNOS expression was affected by the loss of dystrophin. Significant nNOS immunoreactivity and enzyme activity was absent in skeletal muscle tissues from patients with Duchenne muscular dystrophy. Similarly, in dystrophin-deficient skeletal muscles from *mdx* mice both soluble and particulate nNOS was greatly reduced compared with C57 control mice. nNOS mRNA was also reduced in *mdx* muscle in contrast to mRNA levels for a dystrophin binding protein, α1-syntrophin. nNOS levels increased dramatically from 2 to 52 weeks of age in C57 skeletal muscle, which may indicate a physiological role for NO in aging-related processes. Biochemical purification readily dissociates nNOS from the dystrophin–glycoprotein complex. Thus, nNOS is not an integral component of the dystrophin–glycoprotein complex and is not simply another dystrophin-associated protein since the expression of both nNOS mRNA and protein is affected by dystrophin expression.**

Duchenne muscular dystrophy (DMD) is characterized by progressive muscle wasting and inflammation that begins at about 3 years of age (1). Although the deficient gene product in DMD has been identified as dystrophin, a membrane-associated protein (2, 3), the relationship between the protein's absence and muscle pathology is not understood. Current views support the proposal that dystrophin is a structural protein, so that its absence results in a weakened cell membrane that can be more easily damaged (4, 5). This putative structural role for dystrophin is supported by several observations including similarities between the primary structure of the molecule and other cytoskeletal proteins (6), changes in membrane stiffness in dystrophin-deficient muscle (4, 5), and disruptions of structural protein associations with the cell membrane in dystrophin deficient muscle (7).

More recently, analyses of the primary structure of dystrophin-associated proteins (DAPs) have indicated that dystrophin may interact with regulatory proteins as well as structural proteins at the cell surface. Syntrophin, a dystrophin-binding protein, contains a GLGF motif similar to neuronal nitric oxide synthase (nNOS) (8). This motif may mediate protein–protein associations and regulate enzymatic activities in a diverse population of proteins (9). Additionally, dystrophin appears to bind to a 299-aa nNOS peptide containing the GLGF motif (10). It was also reported that skeletal muscle nNOS is diffusely distributed in the cytoplasm rather than concentrated at the cell membrane in the absence of dystrophin (10). The cytosolic distribution of nNOS in dystrophic muscle also corresponds to a decrease in nNOS activity in the cell membrane fraction. These changes in intracellular distribution and reduced membrane activity of nNOS in dystrophic muscle have stimulated speculation that nNOS-related defects may contribute to the pathophysiology of DMD.

We examine two questions concerning nNOS in dystrophin-deficient muscle. First, we test whether total nNOS content in dystrophic muscle changes at the protein or mRNA levels. Previous investigations have shown that other DAPs are present at substantially lower concentrations in dystrophin-deficient muscle. The mRNA of at least two of these proteins, dystroglycan and syntrophin, are present at normal concentrations in dystrophic muscle (11, 12). These findings show that DAPs may be normally synthesized in dystrophin-deficient muscle, but not stabilized at the cell membrane, leading to their loss (13–15).

Second, we test the hypothesis that reduced activity of nNOS at the cell membrane contributes substantially to the pathophysiology of dystrophin-deficient muscle. Our approach is to analyze the change in nNOS distribution, concentration, and activity over the course of muscular dystrophy in *mdx* mouse muscle. *mdx* mice, like DMD humans, are dystrophin mutants (16, 17). However, they differ importantly from DMD humans in that *mdx* muscle successfully regenerates after a period of necrosis, whereas DMD pathology is progressive (18, 19). If the course of the disease reflects the loss of nNOS from the cell membrane, regeneration may correspond to recovery of normal nNOS distribution and activity. Utrophin, which is also a syntrophin binding protein, is capable of partially compensating for the absence of dystrophin by stabilizing DAPs at the cell surface. If nNOS stabilization at the cell membrane via compensatory association with utrophin is a component of *mdx* muscle regeneration, we anticipate an increase in membrane association of the enzyme during regeneration.

## MATERIALS AND METHODS

**Tissue Extraction and Western Blot Analysis.** Skeletal muscle tissue was homogenized in 40 volumes (wt/vol) of buffer A (50 mM Tris·HCl, pH 7.5/1 μg of aprotinin per ml/2 μg of leupeptin per ml/2 μM tetrahydrobiopterin/1 mM DTT/1 μg of pepstatin A per ml/10 μg of soybean trypsin inhibitor per ml/1 mM benzamidine/1 mM EDTA/0.5 mM

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Abbreviations: nNOS, neuronal nitric oxide synthase; DMD, Duchenne muscular dystrophy; DGC, dystrophin–glycoprotein complex; DAP, dystrophin-associated protein.

§Present address: Pharmacopeia, Cranbury, NJ 08512.
‖To whom reprint requests should be addressed.

phenylmethylsulfonyl fluoride). The homogenate was centrifuged for 30 min at 100,000 × *g* to separate the membrane pellet fraction from the cytosol fraction. The pellet fraction was resuspended in 40 volumes of buffer A.

Tissue extracts were resolved by SDS/PAGE, and proteins were transferred to nitrocellulose paper. The transferred blot was incubated with antisera [raised to recombinant nNOS (20, 21) or dystrophin antibody, Dys-1 (NovoCastra, Newcastle, U.K.)] followed by the horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies. Immunoreactivity was detected by ECL chemiluminescence (Amersham) (16, 17).

**NOS Activity Assays.** NOS activity was measured by the conversion of [$^3$H]L-arginine to [$^3$H]citrulline (22).

**Immunolocalization.** Tibialis anterior muscles were dissected from *mdx* and C57 mice at 8 week and 1 year of age and rapidly frozen in isopentane cooled in liquid nitrogen. Longitudinal, frozen sections through the myotendinous junction region and cross sections through the mid-belly region of the muscles were cut at 10 μm and stored at −20°C until used for immunohistochemistry. Immunolabeled sections were double-labeled for nNOS and dystrophin to test for antigen colocalization using a mouse monoclonal antibody (clone DYS2) generated against a polypeptide consisting of the 17 carboxy-terminal amino acids of human dystrophin (generously donated by Louise Anderson, Newcastle General Hospital, Newcastle, U.K.) and a rabbit polyclonal antibody to nNOS (20). Before antibody incubations, sections were treated with 50 mM Tris (pH 7.5) containing 150 mM NaCl, 0.2% gelatin, 3.0% bovine serum albumin, and 0.05% Tween-20 for 20 min to block nonspecific binding. The sections were also pretreated for 30 min with 40 μg of goat anti-mouse IgG Fab fragments per ml to block endogenous immunoglobins in the tissue samples so that they would not bind the secondary antibody directed against mouse IgG. The sections were then incubated for 3 h at room temperature in 8.8 μg of anti-NOS per ml and 20 μg of anti-dystrophin per ml in 50 mM Tris (pH 7.5) and 150 mM NaCl. Following buffer rinses, the sections were incubated with tetramethylrhodamine B isothiocyanate-conjugated goat anti-rabbit IgG and fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 3 h. The sections were then rinsed, mounted, and viewed.

**RNA Isolation and Northern Blot Analysis.** RNA was isolated according to the method of Chomczynski and Sacchi (23). Total RNA (30 μg) was separated on a 0.7% agarose gel followed by transfer to a nylon membrane. The transferred blot was incubated at 80°C for 2 h followed by prehybridization for 30 min at 65°C in Rapid-hyb buffer (Amersham). The membrane was then incubated for 1.5 h at 65°C with [α-$^{32}$P]-labeled ATP probe for rat brain C-terminal nNOS, mouse α1-syntrophin (a gift from Stanley Froehner, University of North Carolina), and mouse β-actin. After extensive washing, autoradiography was performed.

**Ribonuclease Protection Assay.** Antisense riboprobes were synthesized with linearized plasmids containing the cDNAs encoding mouse α1-syntrophin, mouse nNOS (prepared by reverse transcriptase–PCR of total RNA from mouse skeletal muscle) and mouse β-actin (Ambion, Austin, TX). Specifically, α1-syntrophin cDNA cloned into Bluescript SK vector was linearized with *Bam*HI. This produced a 291-nt C-terminal transcript with T7 RNA polymerase of which 227 nt (nt 1902–2128) would hybridize with α1-syntrophin mRNA. Mouse nNOS cDNA was prepared from total mouse skeletal muscle RNA by reverse transcriptase–PCR using primers that amplified a 552-bp region (3573–4114) (24). The 552 bp PCR product was subcloned into a pCRII vector (Invitrogen) and linearized with Acc-1 to yield an antisense transcript of 232 nt with T7 RNA polymerase of which 164 nt were homologous to the mouse nNOS mRNA. A mouse 309-nt β-actin riboprobe was synthesized from a linearized plasmid from Ambion, of which 250 nt were homologous to the mouse β-actin mRNA. All three probes were radiolabeled with [α-$^{32}$P]UTP to a final radiospecific radioactivity of ≈2 μCi (1 Ci = 37 GBq) per pmol of probe. Radiolabeled probes (≈40,000 cpm each) were hybridized with 25–30 μg of total RNA at 45°C for 18 h in a final volume of 20 μl hybridization buffer consisting of 80% deionized formamide, 100 mM sodium citrate, 300 mM sodium acetate, and 1 mM EDTA at pH 6.4. Nonhybridized RNA and probe were digested with 0.5 units of RNase A and 20 units of RNase T1 in a final volume of 200 μl RNase digestion buffer (Ambion). RNA digestion was performed at 37°C for 30 min and terminated by addition of 300 μl RNase inactivation/precipitation mixture (Ambion). Precipitation of fragments protected by the RNA probe was performed by centrifugation after freezing at −20°C for 30 min. Protected fragments were dissolved in formamide sample buffer and resolved on a 5% polyacrylamide/8 M urea gel. Known amounts of the three riboprobes were used as standards on the gel. Resolved bands were quantified with a Molecular Dynamics PhosphorImager.

**Isolation of Dystrophin–Glycoprotein Complex (DGC).** The procedure for purifying dystrophin in the DGC was performed according to the method of Senter *et al.* (25). Rabbit skeletal muscle was homogenized in buffer B [0.3 M KCl/20 mM sodium phosphate/20 mM sodium pyrophosphate/1 mM $MgCl_2$/0.5 mM EGTA/1 mM EDTA, pH 7.0/protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride/1 mM benzamidine/2 μg of leupeptin per ml/1 μg of aprotinin per ml/1 mM pepstatin)]. The homogenate was centrifuged at 14,000 × *g* for 20 min. The pellet was resuspended in buffer B and centrifuged as above. The supernatant S2 fraction was centrifuged at 142,000 × *g* for 30 min generating the S3 supernatant and P3 pellet fractions, respectively. The pellet (P3) was resuspended in 0.6 M KCl/0.303 M sucrose in 50 mM Tris (pH 7.4) containing protease inhibitors.

## RESULTS

**nNOS Is Enriched in Myotendinous Junctions and Costameres.** Indirect immunofluorescence of double-labeled C57 mouse muscle shows a close correspondence between the distribution of nNOS and dystrophin at the muscle fiber surface (Fig. 1). Both nNOS and dystrophin were observed to be enriched at sites of close association between muscle fibers and the extracellular matrix. In particular, the myotendinous junction displayed high concentrations of both proteins. nNOS was also shown to be enriched in periodic bands, costameres, that are distributed along the lateral surfaces of the muscle fibers and coincide with the distribution of Z-discs in the muscle. Cross-sections in the mid-belly region of the muscle showed that nNOS and dystrophin are not always homogeneously distributed at the membrane in a single transverse plane of an individual cell. Some cells show both proteins enriched at the surface of fibers adjacent to perimysium or epimysium (Fig. 1). nNOS was not observed at the muscle cell surface of *mdx* mice examined at 8 weeks or 1 year of age (data not shown).

**nNOS Is Absent in Skeletal Muscle from DMD Patients.** Enzyme assays of skeletal muscle homogenates of biopsies from humans without DMD showed NOS activity present in both supernatant and pellet fractions with the majority (80%) of the NOS activity in the pellet fraction (Fig. 2*A*). However, there was little NOS activity in the supernatant fraction and no measurable activity in the pellet fraction in the biopsy samples from DMD patients. A typical Western blot showed no measurable nNOS immunoreactivity in the pellet or supernatant fractions of the DMD muscle samples (Fig. 2*B*). Careful examination of overexposed Western blots revealed the possible presence of some nNOS immunoreactivity in the pellet fractions, but still no detectable immunoreactivity in the supernatant fractions of DMD samples. Densitometry of these

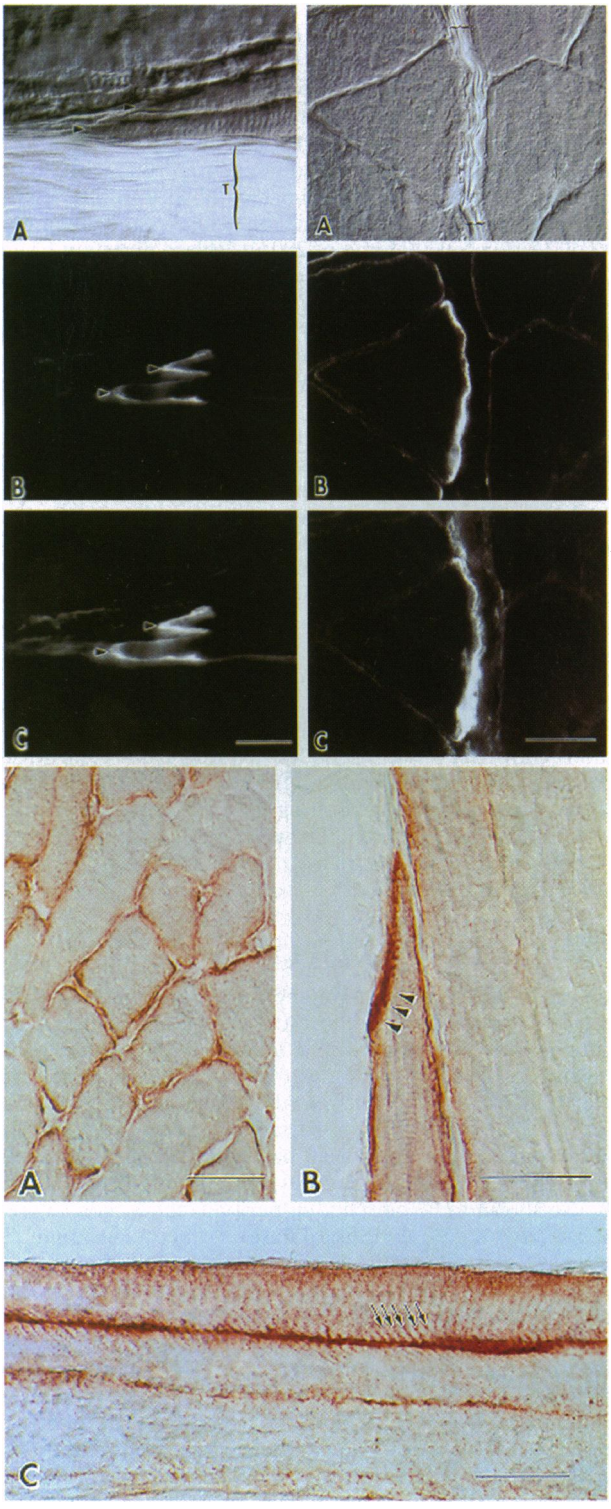


FIG. 1. Immunolocalization of nNOS in skeletal muscle. (*Upper Left*) nNOS and dystrophin are enriched at myotendinous junctions. Shown is a longitudinal section through the myotendinous junction (MTJ) region of adult C57 mouse tibialis anterior with tendon indicated by the bracket (T). The MTJs of two fibers are indicated by arrowheads. (*A*) Nomarski optics image. (*B*) Indirect immunofluorescence of the same field showing the site of anti-nNOS binding at the MTJ. (*C*) Indirect immunofluorescence of the same field showing anti-dystrophin binding at the MTJ. (Bar = 40 μm.) (*Lower*) nNOS is enriched at costameres. Indirect immunofluorescence shows the distribution of nNOS on adult C57 muscle fibers. (*A*) Cross-sectioned muscle fibers. (*B*) Longitudinal section of muscle fibers showing nNOS concentrated at the MTJ (arrowheads). (*C*) Longitudinal section of three muscle fibers. The plane of section of the upper fiber passes along the fiber surface so that many of the costameres located at the fiber surface, and labeled with anti-nNOS, are apparent. (Bars = 40 μm.) (*Upper Right*) nNOS and dystrophin are enriched at the sarcolemmal region opposing the perimysium. Cross-section of 8-week-old C57 mouse quadriceps muscle was double-immunolabeled for nNOS and dystrophin. (*A*) Nomarski optics image showing a region of muscle containing a band of perimysial connective tissue separating groups of fibers (bracket). (*B*) Indirect immunofluorescence of the same field showing anti-nNOS binding at the muscle cell surfaces. nNOS and dystrophin are much more highly concentrated at the surface of the fiber apposing the perimysium (shown in *C*). (Bar = 20 μm.)

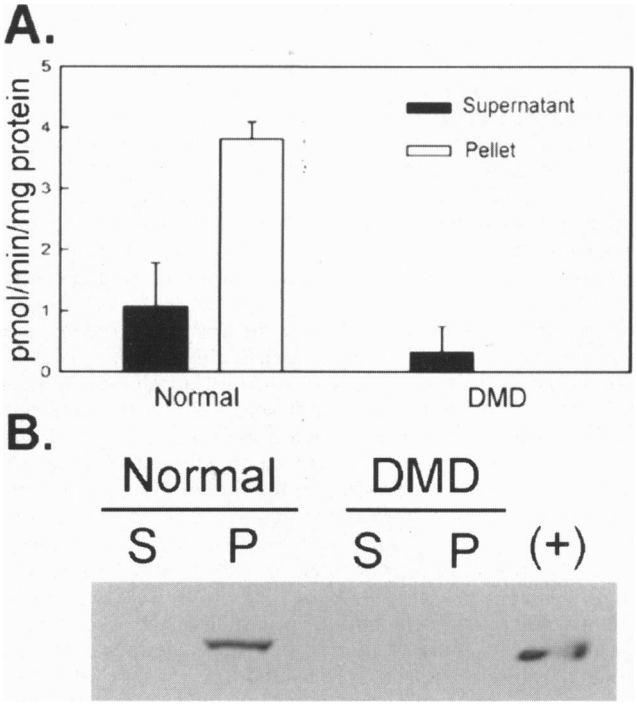


FIG. 2. nNOS expression is decreased in skeletal muscle of DMD patients. Skeletal muscle samples were obtained from patients with and without DMD by needle biopsy (1). (*A*) NOS activity. Results represent mean values and SEM for at least three separate experiments with duplicate measurements. Closed bars, supernatant fractions; open bars, pellet fractions. (*B*) Western blot analysis of nNOS in supernatant (S) and pellet (P) fractions; lane +, 2 ng recombinant nNOS. Control biopsies included the following muscles: quadriceps (age, 5 years), quadriceps (age, 10 years), deltoid (age, 8 years), and deltoid (age, 15 years); DMD biopsies include gastrocnemius (age, 4 years), quadriceps (age, 8 years), quadriceps (age, 8 years), and quadriceps (age, 2.5 years). All of the DMD biopsies showed no dystrophin staining using three antibodies against the rod domain (DYS-1), C terminus (DYS-2), and N terminus (DYS-3) of dystrophin (data not shown).

Western blots showed at least a 25-fold decrease in nNOS immunoreactivity in the pellet fraction compared with biopsies from humans without DMD. Since the Western blots showed no significant nNOS immunoreactivity in the supernatant fraction of normal human muscle, this NOS activity is probably endothelial NOS. As expected, there was no difference in the amount of myosin in normal and DMD biopsies (data not shown). Thus, the lack of dystrophin in human skeletal muscle results in a marked loss of nNOS protein, similar to the loss of other DGC proteins that bind dystrophin (14).

**nNOS Is also Attenuated in *mdx* Skeletal Muscle.** C57 mice have higher NOS activity and nNOS immunoreactivity in both supernatant and pellet fractions relative to *mdx* mice (Fig. 3). In C57 mouse skeletal muscle 48% of nNOS is in the supernatant fraction (Fig. 3) in contrast to 20% in human muscle (Fig. 2).

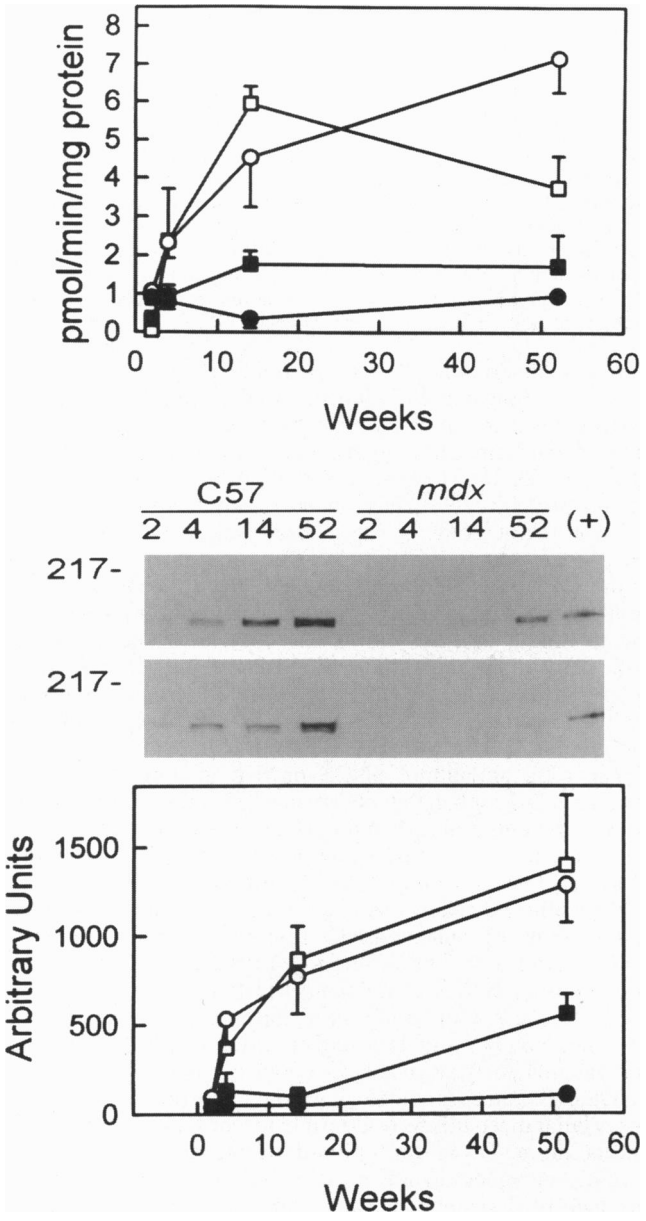


FIG. 3. nNOS expression is decreased in skeletal muscle of *mdx* mice. (*Top*) NOS activity. Open squares, supernatant fractions from control C57 mice; open circles, pellet fractions from control mice; solid squares, supernatant fractions from *mdx* mice; solid circles, pellet fractions from *mdx* mice. The symbols show mean values and SEM for at least four determinations. (*Middle*) Western blots of supernatant (*Upper*) and pellet (*Lower*) fractions, respectively. The abscissa indicates ages in weeks. (*Bottom*) Quantitative densitometry of Western blots shown in *Middle* panel. The symbols are the same as for samples described in *Top* panel for control versus *mdx* samples compared on the same blot. Results represent mean and SEM values for each age group.

The amount of both nNOS immunoreactivity and enzyme activity were attenuated in muscles obtained from *mdx* at 2 weeks (prenecrotic), 4 weeks (necrotic), and 14 weeks or older (postnecrotic). In *mdx* mice, the supernatant fraction had higher nNOS immunoreactivity relative to the pellet fraction, especially in muscles from 1-year-old mice (Fig. 3). Measurement of the amount of immunoreactivity by densitometry showed that the difference in the pellet fractions between control and *mdx* was 16- and 12-fold at 14 weeks and 52 weeks, respectively (Fig. 3). In contrast, the difference in the supernatant fractions between 14-week- and 52-week-old animals was 9- and 3-fold, respectively. There was no apparent difference in nNOS amounts in brains of C57 and *mdx* mice where 85% of the enzyme was in the soluble fraction (data not shown).

Interestingly, there was an age-dependent increase in nNOS levels in C57 mouse muscle with a 17- and 14-fold increase in nNOS in the supernatant and pellet fractions, respectively, in muscles from 1-year-old animals compared with 2-week-old mice (Fig. 3). Attention to the ages of mice is needed to assess physiological and pathophysiological functions of skeletal muscle nNOS.

**mRNA for nNOS, but Not α1-Syntrophin, Is Decreased in Dystrophin-Deficient Muscle.** The loss of dystrophin leads to the loss of dystroglycans and syntrophin proteins in the DGC (26), but mRNA levels are similar in control and dystrophin-deficient muscles (14). However, results from Northern blots showed that nNOS mRNA is decreased in *mdx* muscle compared with C57 muscle (Fig. 4). There were no differences in mRNA levels of α1-syntrophin and β-actin between C57 and *mdx* at 12 weeks or 12 months of age. Relative to β-actin mRNA levels, nNOS mRNA in C57 mouse muscle is 5-fold higher than in *mdx* muscle at 12 weeks, and 12-fold higher at 52 weeks (Fig. 4). mRNA levels for α1-syntrophin are similar for *mdx* muscles compared with C57 at both 12 weeks and 12 months (Fig. 4).

A ribonuclease protection assay was used to confirm the Northern blot analysis. The same levels of α1-syntrophin and β-actin mRNA are present in muscles of both C57 and *mdx* mice (Fig. 4). mRNA levels of α1-syntrophin, but not β-actin, increased between 12 and 52 weeks and did not differ between control or *mdx* mice. However, there was less nNOS mRNA from *mdx* muscles compared with control muscles (Fig. 4). These two methods of analysis show nNOS mRNA levels in skeletal muscle from *mdx* mice are significantly less than dystrophin-containing muscles.

**nNOS Is Not an Integral Component of the DGC.** The loss of nNOS in DMD patients and *mdx* mice prompted us to analyze by quantitative Western blots the association of nNOS with the DGC. The dystrophin–glycoprotein membrane fraction was isolated from rabbit skeletal muscle according to the method of Senter *et al.* (25) with the subsequent purification of the DGC by wheat-germ agglutinin agarose column chromatography (25, 27). After homogenization in an extraction buffer containing 0.3 M KCl, 20 mM phosphate, and 20 mM pyrophosphate at pH 7.0, 37% of nNOS protein was present in the supernatant S1 fraction after centrifugation at 14,000 × *g* compared with only 10% of dystrophin (data not shown). After resuspending the pellet and centrifugation, the supernatant S2 fraction contained 40% and 42% of the total nNOS and dystrophin, respectively. When the S2 fraction was further fractionated using high speed centrifugation, most of the nNOS remained in the S3 supernatant fraction (Table 1). In contrast, the supernatant S3 fraction contained little dystrophin (<10%) while the membrane pellet P3 fraction contained 76%.

The DGC membrane fraction (P3) was also subjected to digitonin solubilization followed by wheat-germ agglutinin–agarose column chromatography (27). The small amount of nNOS present in the P3 fraction was not adsorbed by the column, whereas the *N*-acetylglucosamine eluted fractions containing dystrophin did not have nNOS immunoreactivity (data not shown). Although nNOS may associate with some protein in the DGC, nNOS is not an integral component of the complex.

## DISCUSSION

In the present investigation, we have shown a close correspondence between the distribution of nNOS and dystrophin at the muscle fiber surface, which supports the view that there may be interdependence in their distribution (10). In particular, both proteins are greatly enriched at myotendinous junctions and costameres, which are sites of force transmission across the

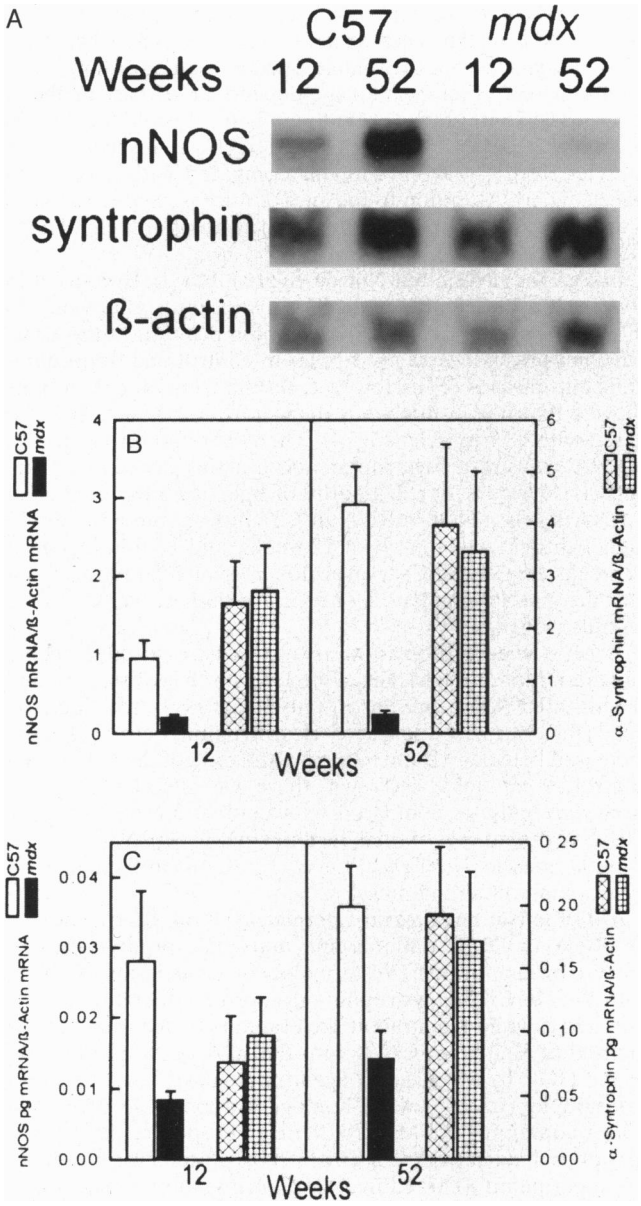


FIG. 4. nNOS levels, but not α1-syntrophin mRNA levels, are decreased in *mdx* muscle. (*A*) Northern blot analysis of nNOS, α1-syntrophin, and β-actin in C57 and *mdx* mice. The abscissa indicates the age of the mice in weeks. The autoradiogram is representative of samples analyzed from C57 and *mdx* mice, respectively. (*B*) Relative amounts of mRNA measured from Northern blots. The data were normalized to β-actin mRNA in the same samples. (*C*) Amount of mRNA obtained from the ribonuclease protection assay. Data represent the mean and SEM values normalized to β-actin mRNA.

muscle cell membrane. Although nNOS and dystrophin are codistributed, our biochemical results show that nNOS does not have the high affinity for dystrophin as DAPs.

The recent findings that nNOS concentration and distribution are modified in dystrophic muscle (10) provided an example of a dystrophin-associated regulatory molecule affected by the disease. There is a potential role of NO in protecting cells from damage by the reactive oxygen intermediate, superoxide, when there is an imbalance in the production of either nitric oxide or superoxide because of the altered chemistry for the production of highly reactive free radical, peroxynitrite (28). A mechanism by which recovery could occur would be if nNOS were able to bind and be stabilized by a dystrophin homologue, utrophin (29, 30), that is upregulated

Table 1. nNOS does not copurify with dystrophin

| Fraction | nNOS, % | Dystrophin, % |
|---|---|---|
| Supernatant S2 | 100 | 100 |
| Supernatant S3 | 63 | 7 |
| Pellet P3 | 10 | 76 |

Rabbit skeletal muscle was homogenized and centrifuged as described under *Materials and Methods*. After centrifugation at 14,000 × *g* for 20 min, the relative amounts of dystrophin and nNOS were measured by quantitative densitometry of Western blots in the supernatant fraction (S2). This fraction was centrifuged at 142,000 × *g* for 30 min, generating the soluble supernatant S3 fraction and the membrane pellet P3 fraction, respectively.

during regeneration (31–33). Utrophin is normally present only at the neuromuscular junction (34), but in the absence of dystrophin, it is expressed more broadly on the muscle fiber surface and is present at high concentrations in myotendinous junctions (35, 36). However, nNOS is never observed enriched at the muscle cell surface in *mdx* muscle examined from prenecrotic, necrotic, or regenerated stages. Thus, regeneration of *mdx* muscle is not attributable to localization of nNOS to the muscle cell membrane, and nNOS is not able to interact stably with utrophin, which becomes highly concentrated at the myotendinous junction during *mdx* muscle regeneration (35, 36). The immunohistochemical observations reported here support our findings that there is no difference in nNOS activity or concentration in the particulate fraction of *mdx* mouse muscle at 4, 14, or 52 weeks of age.

The total amount of nNOS protein in *mdx* muscle was substantially less than that observed in C57 muscle. This differs from a previous publication reporting 80% total nNOS activity and protein in *mdx* muscle compared with C57 muscle (10). However, they also showed that the total NOS activity in both supernatant and pellet fractions of *mdx* muscles was 20% of the total activity present in similar fractions from C57 muscles. One possibility for the differences in Western blot results is overexposure in their experiments which would underestimate the differences. Our results show that the lack of dystrophin does not simply result in a redistribution of nNOS from the sarcolemma to the cytosol. Considering that almost half of nNOS in C57 mice is soluble, it will be important to determine the cellular mechanisms responsible for the loss of both soluble and bound nNOS in dystrophin-deficient muscles.

nNOS deficiency in *mdx* muscle is not simply attributable to the lack of dystrophin in stabilizing the protein at the cell membrane. Analysis of the change in the amount nNOS activity and protein during development and aging of C57 and *mdx* muscle show that regulatory mechanisms other than the presence of dystrophin are important in determining nNOS levels. In 2-week-old muscle, which is before the onset of necrosis in *mdx* muscle, nNOS activity and amount of protein are at similar low levels in both *mdx* and C57 muscle. Thus, low nNOS levels alone are not adequate to explain the dystrophic pathology. Furthermore, dystrophin expression alone does not explain the higher amount of NOS in healthy muscle, since 2-week-old C57 muscle contains membrane-associated dystrophin. In evaluating the time course of dystrophin expression in *mdx* and C57 muscle, the most striking difference is the steep increase in the amount of nNOS between weeks 2 and 4 in C57 muscle that does not occur in *mdx* muscle. Because nNOS and dystrophin expression are not coincident in normal muscle, it will be important to determine the regulatory influences for this dramatic increase in nNOS.

The reduced concentration of nNOS mRNA in dystrophic muscle may also reflect an influence of dystrophin on regulatory mechanisms that control nNOS expression, rather than merely serving to stabilize nNOS protein at the cell surface. The absence of dystrophin resulted in greatly reduced dystroglycan and syntrophin concentrations, although no significant

change in dystroglycan or syntrophin mRNA concentrations were observed (11–13, 15). These DAPs may be expressed at normal rates in dystrophin-deficient muscle, but their amounts are reduced because they are not properly assembled or associated with the cell membrane, leading to degradation. While this manuscript was under review, it was reported that nNOS interacts with α1-syntrophin in muscle and PSD-95 in brain (37). Thus, the loss of this DAP along with dystrophin could contribute to nNOS degradation. However, nNOS differs from this general scheme in that its low mRNA concentration in dystrophin-deficient muscle indicates the existence of a regulatory defect in addition to, or other than, reduced nNOS stability. Additional studies are needed to identify the involved cellular mechanisms.

We conclude nNOS does not redistribute from the sarcolemma to the cytosol in dystrophic muscle. Furthermore, low levels of nNOS are not simply due to the failure of nNOS to associate with the DGC at the cell membrane with an increase in its degradation. There is a defect in nNOS expression as a component of the disease, indicating that nNOS is not just another DAP. Further, our findings show that defective nNOS expression alone is inadequate to explain the pathophysiology of dystrophin deficiency, because there is no substantial recovery of nNOS in regenerating *mdx* muscle. However, we cannot conclude from these findings that deficiencies in NO production in dystrophin-deficient muscle do not exacerbate the disease.

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