Loss of sarcolemma nNOS in sarcoglycan-deficient muscle

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ABSTRACT **nNOS, anchored to the sarcolemma through its interactions with the dystrophin–glycoprotein complex, is dramatically reduced in dystrophin-deficient *mdx* mice and Duchenne muscular dystrophy patients. Recent evidence suggests that loss of nNOS in dystrophin-deficient muscle may contribute significantly to the progression of muscle pathology through a variety of mechanisms. To investigate whether nNOS plays a role in other forms of muscular dystrophy, we analyzed protein expression of nNOS in several sarcoglycan-deficient animal models of muscular dystrophy as well as patients with primary mutations in the sarcoglycan genes. Primary mutations in α-, β-, δ-, and γ-sarcoglycan result in autosomal recessive limb girdle muscular dystrophy (AR-LGMD). We report that loss of the sarcoglycan–sarcospan complex in muscle causes a dramatic reduction in the levels of nNOS expression at the membrane, even in the presence of normal dystrophin and syntrophin expression. Furthermore, we show that expression of three out of four sarcoglycans is not sufficient to maintain nNOS at the sarcolemma. Our data suggest that loss of nNOS may contribute to muscle pathology in AR-LGMD with primary mutations in the sarcoglycans.—Crosbie, R. H., Barresi, R., Campbell, K. P. Loss of sarcolemma nNOS in sarcoglycan-deficient muscle. *FASEB J.* 16, 1786–1791 (2002)**

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In skeletal and smooth muscle fibers, the dystrophin–glycoprotein complex (DGC) is located at the sarcolemma, where it provides a mechanical linkage between the extracellular matrix and the intracellular actin network (1). It is thought that this connection serves to stabilize the muscle membrane during contraction (2, 3). Primary mutations in the dystrophin gene cause Duchenne muscular dystrophy (DMD), which is a devastating disease characterized by progressive muscle weakness and respiratory or cardiac failure. In DMD patients, loss of dystrophin results in loss of the entire DGC complex (4–7). Likewise, a nonsense mutation in the murine dystrophin gene (*mdx*) eliminates expression of dystrophin; consequently, the DGC proteins are reduced at the sarcolemma (for a review, see ref 8). The key to developing therapies and treatments for muscular dystrophies lies in understanding the functions of the DGC and other molecules associated with the DGC.

The core components of the DGC are organized into three distinct subcomplexes (7, 9–12): cytoskeletal proteins, dystrophin, and syntrophins; the dystroglycans (α and β subunits); and the sarcoglycan–sarcospan subcomplex. The four sarcoglycans are single-pass transmembrane glycoproteins referred to as α-, β-, γ-, and δ-sarcoglycan (13). Sarcospan is a tetraspanin-like molecule tightly associated with the sarcoglycans (14–16). Many other proteins have recently been shown to associate with the DGC, including dystrobrevin (17, 18), ε-sarcoglycan (19, 20), caveolin-3 (21–24), and neuronal nitric oxide synthase or nNOS (25). nNOS is particularly interesting given the well-characterized biological role of nitric oxide, produced by NOS, as a second messenger. nNOS is absent from the sarcolemma of dystrophin-deficient muscle (25, 26). Biochemical studies have shown that nNOS interacts with the PDZ domain of syntrophin, thereby anchoring nNOS to the sarcolemma (25, 26).

Nitric oxide mediates diverse functions in skeletal muscle and regulates muscle development (27, 28), metabolism (29), contraction (30), and muscle blood flow (31, 32). Emerging data suggest that loss of nNOS in DMD muscle may result in aberrant regulation of adrenergic vasoconstriction (31–34). Sander and colleagues have measured the responsiveness of blood flow in normal and DMD muscle during exercise and found that dystrophin loss impairs regulation of the vasoconstrictor response (33). These results are supported by previous work showing that dystrophin-deficient *mdx* mice as well as nNOS null mice are unable to control blood flow to muscle during exercise (31). A model is forming whereby loss of dystrophin in DMD patients causes a secondary reduction of nNOS from the sarcolemma (26, 35). Without membrane-localized nNOS, dystrophin-deficient muscle maintains abnormal vasoconstriction, even during periods of active exercise (33, 35). Without proper vascular dilation and

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subsequent blood flow, skeletal and cardiac muscles suffer focal necrosis (for a review, see ref 36).

It is now clear that aberrant production of nitric oxide by mislocalized, cytosolic nNOS does not significantly contribute to DMD pathology, as evidenced by persistent dystrophic pathology in mice lacking both dystrophin and nNOS (37, 38) and by studies with *mdx* extraocular muscles (39). Recently, the important role of nitric oxide as an anti-inflammatory and cytoprotective molecule has gained attention. Studies from Tidball and colleagues have shown that overexpression of nNOS greatly reduces muscle pathology in *mdx* mice, with a concomitant decrease in cytotoxic macrophages (40). Loss of normal levels of nitric oxide has been shown to exacerbate inflammation and membrane damage in dystrophinopathy (40, 41). The potential of nNOS to improve *mdx* muscle pathology raises the possibility that nitric oxide-related therapies may be beneficial for treatment of dystrophinopathy (40). To determine whether nNOS is absent in other forms of muscular dystrophy, we analyzed the expression of nNOS in autosomal-recessive muscular dystrophy with normal dystrophin expression. We show that nNOS is reduced from the sarcolemma in sarcoglycan-deficient limb girdle muscular dystrophy (LGMD).

MATERIALS AND METHODS

Animal models

Wild-type (C57BL/10) and *mdx* (C57BL/10ScSn) mice, obtained from Jackson Laboratories (Bar Harbor, ME), were maintained at the University of Iowa Animal Care Unit in accordance with animal usage guidelines. Male F1B and BIO14.6 cardiomyopathic hamsters were obtained from Bio-Breeders (Fitchburg, MA). We have previously reported the generation and initial characterization of the α-SG-deficient (Sgca null) mice (42), δ-SG-deficient mice (Sgcd null) (43), and β-SG-deficient mice (Sgcb null) (44).

Patient samples

A clinical description and mutational analysis of patients have been described (16). Analysis of all four sarcoglycans and sarcospan has also been reported for all of the muscle biopsies analyzed (16). Pathological controls included myotonic dystrophy, congenital muscular dystrophy, and LGMD 2A linked to chromosome 15 (calpain deficiency), which had normal levels of nNOS at the sarcolemma. All tissues were obtained and tested in agreement with the Human Subjects Institutional Review Board of the University of Iowa.

Antibodies

nNOS antibodies (rabbits 200 and 201) were generated against recombinant nNOS (rat amino acids 1–200)-glutathione-S-transferase (GST) fusion protein, as described (37). Affinity-purified antibodies were used in all experiments, as described (37).

Immunofluorescence

Transverse muscle cryosections (7 μm) were fixed with 4% freshly depolymerized paraformaldehyde for 10 min at room temperature (RT). Muscle sections were washed with TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% triton X-100, pH 7.4) and incubated with affinity-purified nNOS antibody (1:10) overnight at RT (37). After washing with Tris-buffered saline, sections were incubated with either Cy3- or FITC-conjugated secondary antibodies at a dilution of 1:250 (Jackson Immunoresearch, West Grove, PA) for 1 h at room temp. After washing with TBS, the slides were mounted with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA) and observed under a Bio-Rad MRC-600 laser scanning confocal microscope (Hercules, CA). Digitized images were captured under identical conditions.

Western blot

KCl-washed microsomal membranes were purified from mouse skeletal muscle as described (14). Membranes (50 μg) were resolved under reducing conditions by 3–15% SDS-PAGE and transferred to nitrocellulose (Immobilon-N) membranes (Millipore Corp., Bedford, MA). Membranes were probed with anti-nNOS antibodies, as described (37) and developed using enhanced chemiluminescence (Supersignal; Pierce Chemical Co., Rockford, IL).

RESULTS AND DISCUSSION

Mutations in α-, β-, γ-, and δ- sarcoglycan genes are responsible for limb girdle muscular dystrophy type 2D (45–47), 2E (48, 49), 2C (50, 51), and 2F (52, 53), respectively. Sarcoglycanopathies primarily involve wasting of the shoulder and girdle muscles along with calf hypertrophy (for a review, see refs 8, 13). Although the precise function of the sarcoglycans is unknown, the complex is clearly important for normal muscle physiology since their absence results in muscular dystrophy and cardiomyopathy (45, 48, 49, 53, 54).

We sought to determine whether loss of the sarcoglycan–sarcospan subcomplex would perturb sarcolemmal expression of nNOS. We first examined a naturally occurring BIO 14.6 hamster as a model for δ-SG-deficient LGMD (55, 56). A large deletion in the δ-SG gene (57, 58) causes selective loss of the entire sarcoglycan–sarcospan subcomplex from the BIO 14.6 skeletal muscle (15, 59, 60). We demonstrate that nNOS is reduced dramatically from the sarcolemma of quadriceps muscle from adult BIO 14.6 hamsters (**Fig. 1**). Skeletal muscle from tongue and esophagus also lacked nNOS expression as a consequence of δ-SG deficiency (data not shown).

In addition to this naturally occurring hamster model for AR-LGMD, our laboratory has created several sarcoglycan-deficient mice by targeted disruption of the α-, β-, and δ-sarcoglycan genes (42–44). Targeted deletions of the sarcoglycan genes in mice have resulted in animal models with severe muscular dystrophy (42–44, 61, 62). β-, δ-, and γ-sarcoglycan null mice also have an associated cardiomyopathy (43, 44, 61) and δ- and β-sarcoglycan-deficient mice show severe micro-

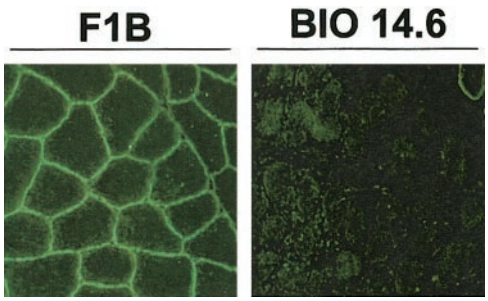


Figure 1. Analysis of nNOS in δ-SG-deficient BIO 14.6. Skeletal muscle from quadriceps muscle was analyzed for nNOS expression by indirect immunofluorescence with affinity-purified polyclonal antibodies to nNOS. Staining was visualized with FITC-conjugated secondary antibodies. Transverse cryosections from wild-type hamster (F1B) and the δ-SG-deficient BIO 14.6 hamster (BIO 14.6) are shown.

constrictions of the vasculature (43, 44). These microconstrictions reduce normal blood flow and thereby contribute significantly to the severity of the muscular and cardiac phenotypes (63).

To extend our studies to other models of sarcoglycan deficiency, we analyzed skeletal muscle from mice lacking α-, β-, and δ-sarcoglycan (Sgca, Sgcb, and Sgcd, respectively). In all mice, the sarcoglycan–sarcospan subcomplex is completely missing from the skeletal muscle sarcolemma whereas dystroglycan and dystrophin levels are nearly normal (15, 42–44). Indirect immunofluorescence of quadriceps muscle demonstrates that nNOS is severely reduced from the sarcolemma of Sgca, Sgcb, and Sgcd mice (**Fig. 2*A***). nNOS staining in wild-type (wt) and *mdx* muscle is shown for comparison (Fig. 2*A*).

To examine nNOS protein levels in sarcoglycan null muscle, KCl-washed skeletal muscle microsomes were prepared from wild-type, *mdx*, Sgca, Sgcb, and Sgcd mice. Protein samples were analyzed by immunoblotting with anti-nNOS antibodies. nNOS levels are substantially reduced in Sgcd and Sgcb muscle vs. wild-type levels (Fig. 2*B*). nNOS protein is nearly absent in skeletal muscle membranes from adult *mdx* and Sgca mice (Fig. 2*B*).

To determine whether these findings pertained to human sarcoglycan-deficient LGMD, we analyzed patients with mutations in α-, β-, or γ-sarcoglycan genes (16). In most cases of sarcoglycan-deficient LGMD, mutations in one sarcoglycan gene lead to the absence or a significant reduction of the tetrameric sarcoglycan complex within the skeletal muscle sarcolemma. We have investigated the membrane localization of nNOS in > 30 autosomal recessive LGMD biopsies (some with defined mutations and others with as-yet-uncharacterized primary mutations). Skeletal muscle cryosections from these patients had been stained with antibodies to each sarcoglycan and sarcospan (15). In **Fig. 3** we show a representative case of autosomal recessive LGMD with complete deficiency of the sarcoglycan–sarcospan subcomplex (case 20). This patient has a primary mutation in the β-sarcoglycan gene (Fig. 3*B*) and a reduction of nNOS staining at the sarcolemma (Fig. 3*A*, case 20).

We extended our studies to patients with partial loss of the sarcoglycan–sarcospan subcomplex. Normal expression of β-sarcoglycan (case 17) or δ-sarcoglycan (case 18), despite the absence of the other sarcoglycans and sarcospan, was observed in two patients with primary mutations in the α-sarcoglycan gene (16). In Fig. 3 we show that these LGMD-2E patients also have lower levels of nNOS at the sarcolemma. Clearly, expression of a single sarcoglycan protein is not sufficient to maintain nNOS localization to the sarcolemma. In our survey of LGMD patients, we identified two interesting γ-sarcoglycanopathy cases. Muscle from patient 24 had near-normal expression of α-, β-, and δ-sarcoglycan and patient 23 had normal levels of β- and δ-sarcoglycan. We found that nNOS immunostaining was reduced in both of these patient samples (Fig. 3*A, B*).

Our report clearly demonstrates that nNOS is reduced in sarcoglycan-deficient muscle. We show that the presence of two or three sarcoglycans at the sarco-

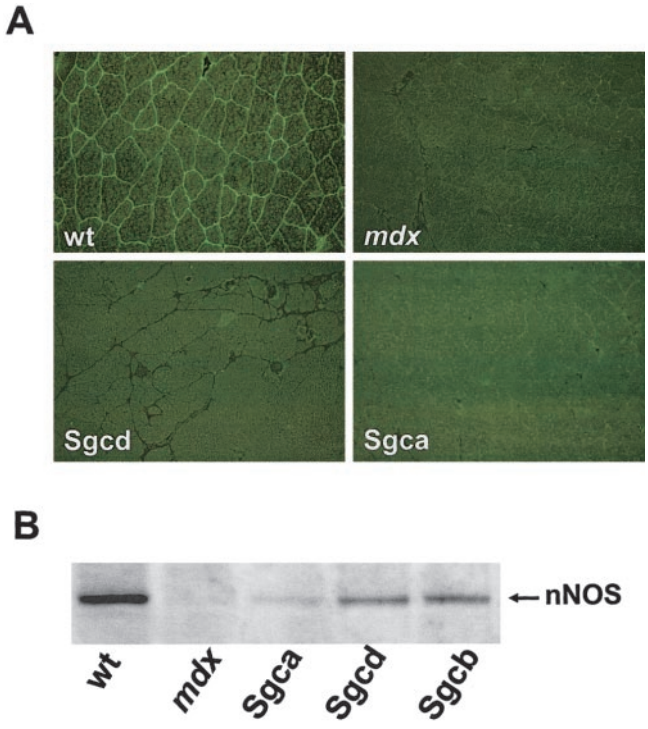
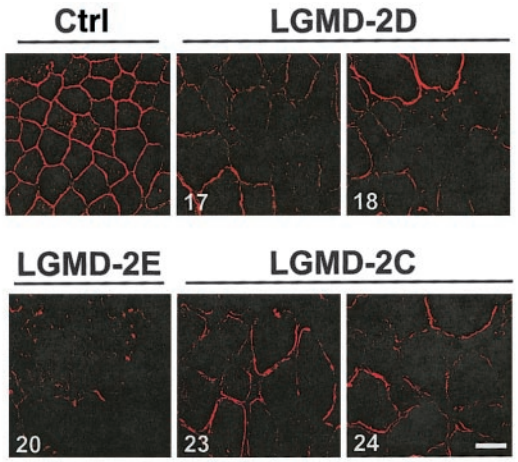


Figure 2. nNOS expression is reduced in sarcoglycan-deficient muscle. *A)* Immunohistochemical analysis of nNOS in sarcoglycan-deficient muscle. Transverse skeletal muscle cryosections from wild-type (wt), dystrophin-deficient (*mdx*), α-SG null (Sgca), and δ-SG null (Sgcd) mice were labeled with polyclonal antibodies against nNOS. nNOS staining was visualized by indirect immunofluorescence with FITC-conjugated secondary antibodies. *B)* Biochemical analysis of nNOS protein levels in sarcoglycan-deficient muscle. KCl-washed microsomes were prepared from skeletal muscle of wt, dystrophin-deficient (*mdx*), α-SG null (Sgca), δ-SG null (Sgcd), and β-SG null (Sgcb) mice. Microsome protein samples (50 μg per lane) were electrophoretically separated on a 3–15% SDS-polyacrylamide gel and transferred to nitrocellulose. Nitrocellulose transfers were stained with affinity-purified antibodies against nNOS. Lanes are marked accordingly.

A



B

Case	Mutation in Primary Gene	
17	α-SG	Arg(77)Cys/FS(118) to X(212)
18	α-SG	Arg(77)Cys/Val(247)Met
20	β-SG	Ser(114)Phe/Ser(114)Phe
23	γ-SG	Δ bp423-630/Δ bp702-825
24	γ-SG	Glu(176)X/Glu(176)X

Figure 3. Reduction of nNOS in AR-LGMD patients with sarcoglycan mutations. *A)* Skeletal muscle biopsies from patients with known mutations in the sarcoglycan genes were analyzed for expression of nNOS using indirect immunofluorescence. Muscle from a normal human control is shown for comparison (ctrl). Patients with primary mutations in α-SG (LGMD-2D, cases 17 and 18), β-SG (LGMD-2E, case 20), and γ-SG (LGMD-2C, cases 23 and 24) are shown. Bar, 100 μm. *B)* Sarcoglycan mutations were determined by direct sequence analysis of patient genomic DNA (16). The sarcoglycan–sarcospan subcomplex is partially lost in these cases of AR-LGMD (16). X, premature stop; FS, frameshift; Δ bp, deletion of DNA bases.

lemma (Fig. 3, cases 23 and 24) is insufficient for membrane localization of nNOS. Several possible scenarios could account for the reduction of nNOS in sarcoglycanopathies. First, loss of the sarcoglycan–sarcospan subcomplex may cause conformational changes in nNOS binding sites within syntrophin. Dystrophin and syntrophin expression at the sarcolemma is not affected by absence of the sarcoglycan–sarcospan subcomplex. However, perturbation in the structural integrity of the DGC may alter syntrophin's PDZ domains, which have been shown to interact directly with nNOS (25). Evidence of the altered conformational structure of the DGC in sarcoglycan null muscle is best illustrated by α-and β-dystroglycan. Although the dystroglycans are properly localized to the sarcolemma, interaction between the α and β subunits is severely weakened by the absence of sarcoglycans and sarcospan (64). Second, several studies have suggested that expression and proper localization of nNOS to the sarcolemma require not only α1-syntrophin, but also other molecules (for a review, see ref 65). This possibility is strengthened by studies with dystrophin transgenic *mdx* mice. Truncated dystrophin without syntrophin binding domains was able to anchor syntrophin and nNOS to the sarcolemma. This suggests that besides direct binding to dystrophin, α1-syntrophin and nNOS may associate with the sarcolemma via unknown mechanisms. One candidate is the voltage-gated sodium channel, which has been shown to bind to α1-syntrophin (66). Last, loss of nNOS may be a secondary consequence of muscle pathology. Increased levels of Ca^{+2}-activated proteases in dystrophic muscle may be responsible for premature degradation of nNOS protein. This is consistent with data from Gossrau and colleagues suggesting that nNOS is not directly associated with the sarcoglycans (67). FJ

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