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Nitrosative stress elicited by nNOS μ delocalization inhibits muscle force in dystrophin-null mice

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Abstract

The mechanism of force reduction is not completely understood in Duchenne muscular dystrophy (DMD), a dystrophin-deficient lethal disease. Nitric oxide regulates muscle force. Interestingly, neuronal nitric oxide synthase μ (nNOS μ), a major source of muscle nitric oxide, is lost from the sarcolemma in DMD muscle. We hypothesize that nNOS μ delocalization contributes to force reduction in DMD. To test this hypothesis, we generated dystrophin/nNOS μ double knockout mice. Genetic elimination of nNOS μ significantly enhanced force in dystrophin-null mice. Pharmacological inhibition of nNOS yielded similar results. To further test our hypothesis, we studied δ -sarcoglycan-null mice, a model of limb-girdle muscular dystrophy. These mice had minimal sarcolemmal nNOS μ delocalization and muscle force was less compromised. Annihilation of nNOS μ did not improve their force either. To determine whether nNOS μ delocalization itself inhibited force, we corrected muscle disease in dystrophin-null mice with micro-dystrophins that either restored or did not restore sarcolemmal nNOS μ . Similar muscle force was obtained irrespective of nNOS μ localization. Additional studies suggest that nNOS μ delocalization selectively inhibits muscle force in dystrophin-null mice via nitrosative stress. In summary, we have demonstrated for the first time that nitrosative stress elicited by nNOS μ delocalization is an important mechanism underlying force loss in DMD.

Keywords

dystrophin; Duchenne muscular dystrophy; nitrosative stress; nitrotyrosination; nNOS; δ -sarcoglycan; mdx; mdx4cv; ryanodine receptor 1; S-nitrosylation

Introduction

Duchenne muscular dystrophy (DMD) is a lethal muscle disease caused by dystrophin gene mutation [1]. Dystrophin is a sub-sarcolemmal protein that connects the extracellular matrix to the cytoskeleton. The absence of dystrophin weakens the sarcolemmal integrity. This mechanical defect has been suggested as the primary pathogenic underpinning for DMD [2], yet the full-spectrum of DMD pathophysiology remains elusive [3].

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Author contribution statement

DD and LD designed the study. CH, DD, LD, YL, and YY carried out experiments. DD, LD, YL, and YY analysed data. DD wrote the paper.

Besides providing a mechanical support to the sarcolemma, dystrophin helps recruit muscle-specific neuronal nitric oxide synthase μ (nNOS μ) to the membrane [4]. In DMD, nNOS μ delocalizes from the sarcolemma and the relative cytosolic NOS activity is elevated [5–12]. When activated by calcium, nNOS generates nitric oxide (NO), a major muscle oxidant and a critical muscle force regulator [13–15]. Currently, it is not clear whether the relative increase of cytosolic NOS activity affects force generation in DMD. Herein, we hypothesize that nNOS μ delocalization contributes to muscle weakening in DMD.

To test this hypothesis, we crossed nNOS μ knockout mice with dystrophin-deficient mdx4cv mice and generated nNOS μ /dystrophin double-deficient (n-dko) mice [9,16–18]. In support of our hypothesis, the specific muscle forces were significantly improved when nNOS μ was eliminated. Administration of the nNOS inhibitor 7-nitroindazole (7-NI) to dystrophin-deficient mdx mice also increased specific muscle force. To further understand the relationship between nNOS μ delocalization and force loss in dystrophic muscle, we eliminated nNOS μ from δ -sarcoglycan-null mice, a model for limb girdle muscular dystrophy 2F. In contrast to dystrophin-deficient mice, sarcolemmal nNOS μ was largely preserved and muscle strength was less compromised in δ -sarcoglycan-null mice [19]. Importantly, muscle force was not improved in δ -sarcoglycan/nNOS μ double-deficient mice (δ /n-dko). To determine whether delocalized nNOS μ inhibited force in a non-dystrophic muscle, we corrected muscle disease in mdx mice with transgenic micro-dystrophin expression. Sarcolemmal nNOS μ expression was restored in the R16–17/ Δ C, but not the Δ R4–23/ Δ C, strain. However, both strains yielded identical muscle forces. Collectively, our data suggest that nNOS μ delocalization selectively weakened the force of dystrophic muscle. To elucidate the underlying mechanism(s), we compared muscle pathology, utrophin expression, and markers for nitrosative stress in mdx4cv and n-dko mice. There was a nominal difference in muscle pathology [9,18]. Utrophin levels were not up-regulated in n-dko mice either. However, protein nitrotyrosination and ryanodine receptor 1 (RyR1) S-nitrosylation were greatly mitigated in n-dko mice. In summary, we have revealed nNOS μ delocalization as a previously unappreciated mechanism underlying force loss in DMD. In this model, delocalized nNOS μ is activated by high cytosolic free calcium in dystrophic muscle. This leads to supra-physiological levels of reactive nitrogen species (RNS) production. Muscle force is weakened by RNS-mediated nitrosative modification of RyR1 and other yet to be defined contractile proteins.

Materials and methods

Animals

All animal experiments were approved by the institutional animal care and use committee and were in accordance with NIH guidelines. The breeding pairs for C57Bl/6 (BL6), C57Bl/10 (BL10), mdx4cv, mdx, δ -SG KO, and nNOS μ KO mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The experimental n-dko and δ /n-dko mice were generated by crossing nNOS μ KO mice with mdx4cv and δ -SG KO mice, respectively. The founder lines of human micro-dystrophin transgenic mice were generated at the University of Missouri transgenic core. The micro-gene expression was controlled by the skeletal specific human skeletal α -actin promoter and the bovine growth hormone pA [20]. The experimental microgene transgenic mice were obtained after backcrossing with mdx mice for at least five generations. Only male mice were used in the study. Mouse age and sample size for each experimental group are detailed in Table 1.

To inhibit nNOS activity, 7-NI (5 mg/ml, dissolved in peanut oil; Sigma, St Louis, MO, USA) was injected into 7-month-old mdx mice intraperitoneally at a dose of 50 mg/kg daily. Control mdx mice were injected with the same volume of peanut oil. After 1 month of treatment, mice were euthanized for functional assay.

Western blot

Whole muscle lysate and membrane enriched microsomal preparations were prepared according to published protocols [4,19,21,22]. Full-length wild-type dystrophin was detected with a mouse monoclonal antibody against the dystrophin C-terminal domain (Dys-2, 1: 100; Novocastra, Newcastle, UK). Human micro-dystrophin was detected with a human dystrophin N-terminal specific monoclonal antibody (Dys-3, 1: 100; Novocastra). Utrophin was detected with a mouse monoclonal antibody against utrophin amino acid residues 768–874 (1: 200; BD Biosciences, San Diego, CA, USA). nNOS μ was detected with a rabbit polyclonal antibody against the N-terminal end of nNOS (1: 1000; Upstate, Lake Placid, NY, USA). The size of the band suggests that it is nNOS μ rather than nNOS β . α -Sarcoglycan was detected with a mouse monoclonal antibody (1: 1000; Vector Laboratories, Burlingame, CA, USA). 3-Nitrotyrosine was detected with a mouse monoclonal antibody (1: 3000; Caymen Chemicals, Ann Arbor, MI, USA).

To detect total cellular RyR1, the sarcoplasmic reticulum membrane fraction was prepared as described by Saito *et al* in the presence of 1% protease inhibitor (Roche, Indianapolis, IN, USA) [23]. To detect S-nitrosylated RyR1, the sarcoplasmic reticulum membrane fraction was further purified using a resin-assisted capture method as reported by Forrester *et al* [24]. RyR1 was detected with a mouse monoclonal antibody (1: 1000; Affinity Bioreagents, Golden, CO, USA). The loading controls included a monoclonal antibody against α -tubulin (1: 3000; Sigma), a monoclonal antibody against α -actin (1: 3000; Sigma), and rapid blue staining of duplicated gels (Geno Technology, St Louis, MO, USA).

Immunofluorescence staining and *in situ* NOS activity staining

Immunofluorescence staining was performed essentially as described previously [19,22]. Full-length and micro-dystrophin were revealed with Dys-2 (1: 30) and Dys-3 (1: 20), respectively. Utrophin was revealed with a mouse monoclonal antibody against the utrophin N-terminal domain (1: 20; Vector Laboratories). nNOS was revealed with a rabbit polyclonal antibody against an epitope near the C-terminal end of nNOS (1: 2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). NOS activity staining was performed on cryosections according to our published protocol [4]. The histochemical staining revealed the NADPH diaphorase activity of NOS.

Histopathology and serum creatine kinase (CK) assay

Central nucleation quantification was performed as described previously [25]. Serum CK level was determined using a CK liqui-UV test kit from Stanbio Laboratory (Boerne, TX, USA) as described before [22].

Muscle force measurement

Twitch and tetanic forces of the extensor digitorum longus (EDL) muscle were measured *ex vivo* using a 305B dual-mode servomotor transducer (Aurora Scientific Inc, Aurora, Ontario, Canada) according to our published protocol [19,22,25]. Twitch and tetanic forces of the tibialis anterior (TA) muscle were measured *in situ* with a 305C-LR dual-mode servomotor transducer (Aurora Scientific Inc) according to a published protocol [26]. The specific muscle force was calculated by dividing the absolute muscle force by the muscle cross-sectional area (CSA) (kN/m²). Muscle CSA was calculated according to the following equation: CSA = (muscle mass, in g)/[(muscle density, in g/cm³) $\times$ (length ratio) $\times$ (optimal muscle length, in cm)]. 1.06 g/cm³ is used as the muscle density [27]. The length ratio refers to the ratio of optimal fiber length to optimal muscle length. The length ratios for the EDL and TA muscles are 0.44 and 0.6, respectively [28,29].

Statistical analysis

Data are presented as mean $\pm$ standard error of the mean. Statistical analysis was performed with SPSS software (SPSS, Chicago, IL, USA). Statistical significance among multiple groups was determined by one-way ANOVA followed by Bonferroni *post-hoc* analysis. For two-group comparison, statistical significance was determined by the Student *t* -test. Difference was considered significant when $p < 0.05$.

Results

Specific forces of dystrophin-deficient mice are enhanced by nNOS μ elimination or nNOS inhibition

In dystrophin-deficient mice, nNOS μ was lost from the sarcolemma. However, it was not completely eliminated from muscle cells (Figures 1A–1C) [5–7]. To determine whether mislocalized nNOS μ affected muscle contractility, we generated n-dko mice by crossing nNOS μ -null mice with mdx4cv mice [16,17] (Figures 1A–1C). Both parental strains were on the BL6 background.

We first examined force in young adult male mice (Figures 1D, 1E, and Table 1). The EDL muscle of n-dko mice yielded significantly greater specific twitch and tetanic forces than that of mdx4cv mice (Figure 1D). Similar results were obtained from the TA muscle, although the improvement in specific twitch force appeared marginal in the TA muscle (Figure 1E). At 18 months of age, the EDL muscle of n-dko mice also showed significantly higher specific tetanic forces than that of mdx4cv mice (Figure 1F). However, genetic elimination of nNOS μ did not change the eccentric contraction profile (Supporting information, Supplementary Figures 1A–1C).

To validate the results of n-dko mice, we treated adult mdx mice with daily administration of 7-NI. Mdx mice are on the BL10 background and they carry a different mutation. One month after treatment, specific forces were significantly enhanced (Figure 1G). Interestingly, 7-NI-treated mdx mice appeared to be more resistant to eccentric contraction injury during the first two rounds of lengthening contraction (Supporting information, Supplementary Methods, Supplementary Figure 1D).

Genetic elimination of nNOS μ does not increase specific muscle force in δ -sarcoglycan knockout mice

To further decipher the relationship between nNOS μ delocalization and force loss in dystrophic mice, we created δ /n-dko mice by crossing nNOS μ -null mice with BL6 background δ -sarcoglycan-null mice (Figure 2). We have previously shown that age- and gender-matched δ -sarcoglycan-null mice yielded significantly higher specific twitch and tetanic forces than those of mdx4cv mice [19]. Interestingly, nNOS μ was minimally delocalized from the sarcolemma in δ -sarcoglycan-null mice (Figures 2A and 2B). Genetic elimination of nNOS μ did not increase specific force of δ -sarcoglycan-null mice either (Figure 2C).

nNOS μ delocalization alone is not sufficient to inhibit force in non-dystrophic muscle

To determine whether nNOS μ mislocalization per se impeded force production, we generated two strains of micro-dystrophin transgenic mdx mice. One strain expressed the R16–17/ Δ C microgene which contains the nNOS-binding domain. The other strain expressed the Δ R4–23/ Δ C microgene that does not carry the nNOS-binding domain (Figure 3A). Similar levels of micro-dystrophin expression were detected (Figures 3B–3D). Muscle histopathology was also effectively ameliorated in both strains (data not shown) [4]. However, sarcolemmal nNOS μ expression was restored only in the R16–17/ Δ C strain

(Figure 3C; data not shown for nNOS immunostaining). NOS activity was detected at the sarcolemma in the R16–17/ Δ C, but not the Δ R4–23/ Δ C, strain (Figure 3D). Interestingly, despite a considerably high cytosolic nNOS μ level, the Δ R4–23/ Δ C transgenic mice yielded a contractile profile identical to that of the R16–17/ Δ C strain (Figure 3E and Supporting information, Supplementary Methods, Supplementary Figure 1E).

Delocalized nNOS μ induces nitrosative stress in dystrophin-deficient mice

Our results so far suggest that nNOS μ delocalization selectively inhibits force production in dystrophin-deficient muscle. To understand why nNOS μ delocalization reduced force in dystrophic muscle, we compared muscle pathology in mdx4cv and n-dko mice. Consistent with previous reports [9,18], we observed similar levels of myofiber size variation, central nucleation, inflammation, regeneration, and fibrosis (Figure 4A and Supporting information, Supplementary Methods, Supplementary Figure 2). We also measured the severity of sarcolemmal leakage by immunoglobulin infiltration and the serum CK level. There was also no significant difference between mdx4cv and n-dko mice (Figure 4A and Supporting information, Supplementary Methods, Supplementary Figure 2). Utrophin is a known compensatory protein for dystrophin. Its up-regulation may explain force gain in n-dko mice. To our surprise, utrophin appeared slightly reduced in n-dko mice compared with that of mdx4cv mice (Figure 1A).

NO is one of the primary free radicals in muscle [14,15]. It is also a well-established regulator of muscle contractility [13,30]. To determine whether disruption of NO redox homeostasis contributed to our observation, we examined several nitrosative stress markers in mdx4cv and n-dko mice. 3-Nitrotyrosine is generated when the tyrosine residues are modified by reactive nitrogen species (RNS) [13,14]. On western blot, we observed prominent 3-nitrotyrosine bands in mdx4cv mice (Figure 4B) [31,32]. Total cellular RyR1 levels were quite comparable among the different strains. However, S-nitrosylated RyR1 was greatly increased in mdx4cv mice (Figure 4C) [24,33]. Importantly, protein nitrotyrosination and RyR1 S-nitrosylation were minimized in n-dko mice (Figures 4B and 4C).

In the Δ R4–23/ Δ C strain of micro-dystrophin transgenic mdx mice, nNOS μ delocalization did not reduce force (Figure 3E). One likely explanation was that delocalized nNOS μ did not induce nitrosative stress in this context. We evaluated protein nitrotyrosination (Figure 4D). Indeed, minimal 3-nitrotyrosine bands were detected in Δ R4–23/ Δ C microgene transgenic mdx mice (Figure 4D). Collectively, these results suggest that nNOS μ delocalization inhibits force via elevated RNS and subsequent nitrosative modification (Figure 5). In the absence of nitrosative stress, muscle force is not compromised.

Discussion

nNOS is a well-established signalling molecule [13,34]. It plays an important role in many aspects of skeletal muscle activities [13]. Several different nNOS isoforms have been identified in skeletal muscle. These include 135 kD nNOS β and 165 kD nNOS μ . nNOS β does not contain the PDZ domain and it resides within the Golgi complex [35]. nNOS μ is the predominant nNOS isoform in muscle. It carries a unique N-terminal PDZ domain. The β -finger of the nNOS μ PDZ domain interacts with the PDZ domain of α -syntrophin [36,37]. The groove of the nNOS μ PDZ domain interacts with dystrophin spectrin-like repeats 16/17 [4]. Through these interactions, nNOS μ is anchored to the sarcolemma. Victor and co-workers examined the haemodynamic response during muscle contraction in mdx mice and DMD patients [10,38]. They demonstrated that NO generated from nNOS μ counteracts α -adrenergic vaso-constriction and hence prevents functional ischaemia in contracting muscle. The loss of dystrophin leads to nNOS μ delocalization from the sarcolemma. In the absence

of NO, muscle vessels cannot fully relax during contraction. Eventually, it leads to focal ischaemic injury in myofibres that are already weakened by the loss of dystrophin. Recent studies have further provided the physiological relevance of membrane-associated nNOS μ in muscle fatigue and muscular dystrophy [4,39,40].

In addition to facilitating blood perfusion, NO also facilitates muscle repair by promoting satellite cell activation and fusion, increasing trophic differentiating factors (follistatin and hepatocyte growth factor) and modifying chromatin [41–44]. As a matter of fact, several studies have revealed a beneficial effect of NO donors or NO-releasing agents (such as arginine) in mdx mice [45–48]. Wehling *et al* overexpressed nNOS in the cytoplasm and the sarcolemma via the transgenic approach. They found that supra-physiological levels of nNOS mitigated histological lesions in mdx mice [49]. Collectively, these studies support a NO and/or nNOS-based therapy for DMD.

While increasing NO/nNOS levels appear beneficial, there is also evidence suggesting possible disadvantages. Stamler and co-workers have provided convincing evidence that NO inhibits muscle force [50]. Furthermore, they demonstrated that NO-mediated force inhibition occurs under high oxygen tension (a situation mimicking oxidative/nitrosative stress) [51]. Recent studies have begun to elucidate the underlying molecular mechanism [52,53]. Specifically, it was found that NO impairs skeletal muscle contractility via nitrosylative modification of RyR1 [33,52–54]. In addition to a negative effect on force production, supra-physiological nNOS may directly contribute to muscular dystrophy. In this regard, Sunada *et al* have found that nNOS overactivation is involved in caveolin-3 mutation-related limb-girdle muscular dystrophy [55]. A recent study by Wehling-Henricks *et al* also suggests that dietary supplementation of arginine, a NO donor, exacerbates fibrosis in mdx mice [56].

In dystrophin-deficient muscle, nNOS μ is lost from the sarcolemma but it is not eliminated from muscle cells (Figures 1A–1C). Actually, it has been shown that the relative cytosolic NOS activity is elevated in dystrophin-null muscle [5–12]. While the loss of sarcolemmal nNOS μ causes ischaemic muscle injury, it remains controversial whether delocalized nNOS μ contributes to muscle disease in DMD [4,9,10,18].

The loss of muscle force is a major clinical manifestation of DMD. However, the molecular mechanisms underlying muscle force reduction are not completely understood. Stamler and co-workers have provided convincing evidence that NO inhibits muscle force under high oxygen tension [50,51]. Recent studies suggest that NO impairs skeletal muscle contractility via nitrosylative inhibition of RyR1 [33,53,54]. In DMD muscle, delocalized nNOS μ , high cytosolic free calcium, and elevated oxidative stress seem to have provided a great milieu for RNS-mediated RyR1 hyper-nitrosylation and force inhibition (Figure 5).

To determine whether delocalized nNOS μ has indeed contributed to force loss in dystrophin-deficient mice, we used two independent approaches. First, we completely removed nNOS μ by crossing mdx4cv mice to the nNOS μ -null background. Second, we inactivated nNOS activity in mdx mice with 7-NI, a pharmacological nNOS inhibitor. In both cases, we observed a significant increase of specific muscle force (Figure 1).

Our hypothesis would predict nNOS μ delocalization as the initiating event that starts the chain reaction leading to nitrosative inhibition of muscle force (Figure 5). In the absence of significant nNOS μ delocalization, muscle force should be better preserved. The results from δ -sarcoglycan-null mice supported this argument. First, δ -sarcoglycan-deficient mice retained significant levels of sarcolemmal nNOS μ expression (Figures 2A and 2B). Second, δ -sarcoglycan-null mice yielded higher specific forces than mdx4cv mice despite the fact that both dystrophic models had similar muscle pathology such as elevated calcium,

myofibre necrosis, and inflammation [19,57–61]. Third, genetic elimination of nNOS μ from δ -sarcoglycan-deficient mice had nominal impact on specific muscle force (Figure 2C).

To study the molecular mechanism(s) underlying nNOS μ delocalization-associated force loss, we compared muscle pathology and utrophin expression between mdx4cv and n-dko mice. We did not find any evidence suggesting that improved muscle force in n-dko mice was due to histopathology amelioration or utrophin up-regulation (Figures 1A, 1C, and 4A, and Supporting information, Supplementary Methods, Supplementary Figure 2). Interestingly, several previous studies also revealed a disconnection between muscle force and the histological lesion [19,22,54,62,63]. Hack *et al* first reported that muscle degeneration in γ -sarcoglycan-deficient mice did not lead to specific force reduction [63]. Bogdanovich *et al* found that myostatin inhibition therapy increased muscle strength in γ -sarcoglycan-deficient mice but did not improve muscle pathology [62]. We also found discordance between the severity of muscle pathology and muscle force in mdx3cv, mdx4cv, and δ -sarcoglycan-null mice [19,22]. Bellinger *et al* further show that the disconnection between force and histology may happen in normal mice. After 3 weeks of chronic exercise, the authors observed a significant reduction of the EDL muscle specific force but they did not detect any morphological change [54].

The primary cellular function of nNOS is to generate NO. Interaction of NO with reactive oxygen species (ROS) produces highly reactive RNS such as peroxynitrite [13,15]. Hence, we investigated whether nNOS μ delocalization-associated force reduction is mediated by nitrosative stress. nNOS is activated by free calcium. While the cellular free calcium level is abnormally elevated in dystrophin-deficient muscle, recent studies suggest that the abbreviated synthetic dystrophin genes can rectify calcium defects [4,20,64–69]. We generated a pair of micro-dystrophin transgenic mdx mice. One strain had normal nNOS μ localization at the sarcolemma, while the other strain contained delocalized cytosolic nNOS μ (Figure 3). We have previously shown that these microgenes can effectively ameliorate muscular dystrophy in mouse models of DMD [4,20]. We expected that a correction of muscle disease would normalize calcium overload and oxidative stress. Consequently, it would prevent abnormal nNOS activation, RNS production, and nitrosative force inhibition even if there was delocalized nNOS μ . Indeed, we obtained the same muscle forces from both transgenic strains (Figure 3).

To further demonstrate the involvement of RNS hypernitrosylation, we measured nitrosative stress markers in mdx4cv and n-dko mice. Compared with mdx4cv mice, aberrant nitrotyrosination and RyR1 S-nitrosylation were corrected in n-dko mice (Figures 4B and 4C). Evaluation of protein nitrotyrosination in micro-dystrophin transgenic mdx mice lent additional support to our hypothesis. Irrespective of subcellular nNOS μ localization, minimal 3-nitrotyrosine bands were detected in these mice (Figure 4D).

In summary, we propose a model of RNS-mediated force inhibition in DMD (Figure 5). In dystrophin-deficient muscle, elevated free calcium activates delocalized nNOS μ and generates excessive NO. In the presence of ROS, NO is converted to RNS. Subsequent nitrosative modification of contractile proteins (in particular, S-nitrosylation of RyR1) further reduces the already compromised force (Figure 5). Genetic elimination of nNOS μ or administration of the nNOS inhibitor 7-NI blocks the pathway and improves muscle force. In δ -sarcoglycan-deficient mice, the pathway is not initiated due to the absence of nNOS μ delocalization. The Δ R4–23/ Δ C micro-dystrophin therapy corrects calcium mishandling and reduces ROS. As a result, nitrosative stress is prevented and muscle force is preserved despite the failure of this microgene to anchor nNOS μ to the sarcolemma.

One potential confounding factor that is not studied here is inducible NOS (iNOS). As a consequence of muscle inflammation, the iNOS level is elevated in dystrophin-deficient muscle. This likely represents another important source of NO and RNS production [33]. Assessing muscle force in iNOS/dystrophin double-deficient mice in the future may provide a clue on the relative contribution of iNOS. Nevertheless, the results from our δ/n -dko mice suggest that participation from iNOS may be limited. Muscle inflammation is observed in both δ -sarcoglycan-null mice and dystrophin-null mice. Genetic elimination of nNOS μ should not affect the iNOS level. If iNOS, rather than delocalized nNOS μ , plays a determining role in NO/RNS-mediated force inhibition, then as in the case of δ/n -dko mice we would expect n-dko muscle to yield similar force to dystrophin-deficient muscle. This is contradictory to our experimental results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Note: References 1–9 are cited in the supporting information to this article.

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Figure 1.

Removing nNOS μ improves muscle force in dystrophin-deficient mice. (A) Representative whole muscle lysate western blot for dystrophin, nNOS μ , and utrophin. α -Tubulin was used as the loading control. n-Dko = nNOS μ /dystrophin double knockout mice. Dystrophin (C), an anti-dystrophin C-terminal domain specific antibody, was used in the study to reveal the full-length dystrophin protein. (B) Representative microsomal preparation western blot for dystrophin, nNOS μ , and α -sarcoglycan. Rapid blue staining was used as the loading control. (C) Representative immunostaining photomicrographs for dystrophin (top row), utrophin (second row), and nNOS (bottom two rows). The third row shows representative photomicrographs of *in situ* NOS activity staining. nNOS IF = nNOS immunofluorescence staining. Arrow indicates neuromuscular junction. (D) *Ex vivo* analysis of the EDL muscle force in young adult mice. $N = 19$ for BL6 mice; $N = 12$ for mdx4cv mice; $N = 8$ for nNOS μ KO mice; $N = 14$ for n-dko mice. (E) *In situ* analysis of the TA muscle force in young adult mice. $N = 6$ for BL6 mice; $N = 4$ for mdx4cv mice; $N = 3$ for nNOS μ KO mice; $N = 6$ for n-dko mice. (F) *Ex vivo* analysis of the EDL muscle force in aged mdx4cv and n-dko mice. $N = 5$ for mdx4cv mice and $N = 4$ for n-dko mice. (G) *Ex vivo* analysis of the EDL muscle force in adult mdx mice with or without 7-NI treatment. 7-NI = 7-nitroindazole. $N = 10$ for untreated mdx mice and $N = 8$ for 7-NI-treated mdx mice. *Significantly different from the other strain(s) in the same group. † Significantly lower than that of nNOS μ KO.

Figure 2.

nNOS μ expression and muscle forces in δ -sarcoglycan knockout mice and δ /n-dko mice. (A) Representative photomicrographs of nNOS immunofluorescence staining and NOS activity staining in δ SG KO and δ /n-dko muscles. (B) Representative whole muscle lysate and microsomal preparation western blots for nNOS μ . α -Tubulin and rapid blue staining were used as the loading controls for whole lysate and microsomal western blots, respectively. (C) Specific twitch force and tetanic forces of the EDL muscle. $N = 8$ for δ -SG KO mice and $N = 3$ for δ /n-dko mice. δ SG KO = δ -sarcoglycan knockout; δ /n-dko = δ -sarcoglycan/nNOS μ double knockout.

Figure 3. Analysis of nNOS μ expression and EDL muscle force in micro-dystrophin transgenic mdx mice. (A) Schematic outline of the micro-dystrophin genes used in the study (not drawn to scale). Only the R16–17/ ΔC microgene contains the dystrophin nNOS μ -binding domain. (B) Representative whole muscle lysate western blot for full-length dystrophin, micro-dystrophin, nNOS μ , and utrophin. α -Tubulin was used as the loading control. Micro-dystrophin (N), an anti-human dystrophin N-terminus specific antibody, was used to reveal the micro-dystrophin protein. Full-dystrophin (C), an anti-dystrophin C-terminal domain specific antibody, was used to reveal the endogenous full-length murine dystrophin protein. (C) Representative microsomal preparation western blot for full-length dystrophin, micro-dystrophin, nNOS μ , and α -sarcoglycan. Rapid blue staining was used as the loading control. (D) Representative photomicrographs of micro-dystrophin immunofluorescence staining and NOS activity staining. (E) Specific twitch and tetanic forces of the EDL muscle. $N = 7$ for $\Delta R4-23/\Delta C$ transgenic mice and $N = 6$ for R16–17/ ΔC transgenic mice.

Figure 4.

Characterization of histopathology, serum creatine kinase, and nitrosative stress. (A) Left panel: central nucleation quantification. $N = 6$ for BL6 mice, $N = 7$ for mdx4cv mice, $N = 6$ for nNOS μ KO mice, and $N = 7$ for n-dko mice. Right panel: serum creatine kinase levels. $N = 9$ for BL6 mice, $N = 27$ for mdx4cv mice, $N = 3$ for nNOS μ KO mice, and $N = 6$ for n-dko mice. (B) Representative western blot analysis of 3-nitrotyrosine in BL6, mdx4cv, nNOS μ knockout, and n-dko mice. α -Tubulin was used as the loading control. (C) Representative western blots for total RyR1 (left panel) and S-nitrosylated RyR1 (middle and right panels) in BL6, mdx4cv, nNOS μ knockout, and n-dko mice. Rapid blue staining was used as the loading control. (D) Representative western blot analysis of 3-nitrotyrosine in BL10, mdx, and the Δ R4–23/ Δ C and R16–17/ Δ C strains of micro-dystrophin transgenic mice. α -Tubulin was used as the loading control.

Figure 5.

nNOS μ delocalization leads to nitrosative inhibition of muscle force in dystrophic mice. Dystrophin deficiency leads to the loss of sarcolemmal nNOS μ . In the presence of excessive cytosolic free calcium in dystrophic muscle, delocalized nNOS μ is abnormally activated and generates nitric oxide. Oxidative stress is another feature of muscular dystrophy. Elevated reactive oxygen species (ROS) interacts with nitric oxide and produces pathogenic levels of reactive nitrogen species (RNS). While the primary factor leading to specific muscle force reduction may have come from sarcolemmal damage and other yet to be defined mechanisms, our results suggest that nNOS μ may partially contribute to specific force reduction via inducing nitrosative stress. Complete elimination of nNOS μ (nNOS μ KO) or pharmacological inhibition of NOS activity (7-NI) improves specific force in dystrophin-null mice. The R16–17/ Δ C micro-dystrophin gene restores sarcolemmal nNOS expression. Both R16–17/ Δ C and Δ R4–23/ Δ C microgenes correct sarcolemmal leakage, calcium misregulation, and oxidative/nitrosative stress. Consequently, they improve specific muscle force. In δ -sarcoglycan knockout (δ -SG KO) mice, nNOS μ is not delocalized and specific muscle force is better preserved.

Table 1

Anatomic properties of the EDL and TA muscles

Strain	Muscle	Age (months)	N	Weight (mg)	CSA (mm ²)
BL6	EDL	5	19	10.84 ± 0.27	1.78 ± 0.40
Mdx4cv	EDL	5	12	16.47 ± 0.70*	2.67 ± 0.09*
nNOS μ KO	EDL	5	8	8.55 ± 0.70*	1.38 ± 0.11*
n-Dko	EDL	5	14	12.59 ± 0.26	1.99 ± 0.04
Mdx4cv	EDL	18	5	18.02 ± 0.77*	2.65 ± 0.07*
n-Dko	EDL	18	4	15.13 ± 0.20	2.21 ± 0.02
Mdx	EDL	8	10	18.78 ± 0.59*	2.88 ± 0.08*
7-NI-treated mdx	EDL	8	8	13.23 ± 0.51	2.07 ± 0.06
Δ R4-23/ Δ C	EDL	8	7	10.74 ± 0.30	1.68 ± 0.05
R16-17/ Δ C	EDL	8	6	10.75 ± 0.20	1.71 ± 0.04
δ SG KO	EDL	4	8	17.38 ± 0.65*	2.60 ± 0.09*
δ /n-Dko	EDL	4	3	11.27 ± 0.29	1.84 ± 0.05
BL6	TA	4	6	48.62 ± 0.93	5.96 ± 0.13
Mdx4cv	TA	4	4	91.20 ± 3.96*	10.61 ± 0.43*
nNOS μ KO	TA	4	3	46.47 ± 1.64	5.57 ± 0.10
n-Dko	TA	4	6	57.50 ± 5.70	6.90 ± 0.61

* Significantly different from other strain(s) of the same group. n-Dko = nNOS μ /dystrophin double knockout; 7-NI = 7-nitroindazole; δ SG = δ -sarcoglycan; δ /n-Dko = δ -sarcoglycan/dystrophin double knockout; AV = adeno-associated virus.