Stimulation of calcineurin Aα activity attenuates muscle pathophysiology in *mdx* dystrophic mice

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Stimulation of calcineurin Aα activity attenuates muscle pathophysiology in *mdx* dystrophic mice. Am J Physiol Regul Integr Comp Physiol 294: R983–R992, 2008. First published January 16, 2008; doi:10.1152/ajpregu.00375.2007.—Calcineurin activation ameliorates the dystrophic pathology of hindlimb muscles in *mdx* mice and decreases their susceptibility to contraction damage. In *mdx* mice, the diaphragm is more severely affected than hindlimb muscles and more representative of Duchenne muscular dystrophy. The constitutively active calcineurin Aα transgene (*CnAα*) was overexpressed in skeletal muscles of *mdx* (*mdx CnAα*) mice to test whether muscle morphology and function would be improved. Contractile function of diaphragm strips and extensor digitorum longus and soleus muscles from adult *mdx CnAα* and *mdx* mice was examined in vitro. Hindlimb muscles from *mdx CnAα* mice had a prolonged twitch time course and were more resistant to fatigue. Because of a slower phenotype and a decrease in fiber cross-sectional area, normalized force was lower in fast- and slow-twitch muscles of *mdx CnAα* than *mdx* mice. In the diaphragm, despite a slower phenotype and a ~35% reduction in fiber size, normalized force was preserved. This was likely mediated by the reduction in the area of the diaphragm undergoing degeneration (i.e., mononuclear cell and connective and adipose tissue infiltration). The proportion of centrally nucleated fibers was reduced in *mdx CnAα* compared with *mdx* mice, indicative of improved myofiber viability. In hindlimb muscles of *mdx* mice, calcineurin activation increased expression of markers of regeneration, particularly developmental myosin heavy chain isoform and myocyte enhancer factor 2A. Thus activation of the calcineurin signal transduction pathway has potential to ameliorate the *mdx* pathophysiology, especially in the diaphragm, through its effects on muscle degeneration and regeneration and endurance capacity.

The loss of dystrophin in Duchenne muscular dystrophy (DMD) disrupts the linkage between the muscle fiber cytoskeleton and the extracellular matrix, which increases the susceptibility of muscle fibers to contraction-induced injury (29). Ongoing muscle degeneration and ineffective regeneration lead to a progressive loss of muscle fibers and infiltration of fat and connective tissue (61). Fast-twitch type IIb muscle fibers are preferentially susceptible to damage in DMD (57). The mechanisms underlying the impairment in regeneration are not well understood, although exhaustion of satellite cells (9), impaired myoblast differentiation (35), and increased expression of TNF-α (20), TGF-β1 (19), and insulin-like growth factor-binding protein type 5 (31) have been implicated. As a consequence of the progressive muscle degeneration, exercise performance is limited in boys with DMD, even in the early stages of the disease. Endurance capacity and muscle strength are lower in boys with DMD than in age-matched controls (45), and their respiratory muscles are more susceptible to fatigue (28).

Because of a mutation in the dystrophin gene in *mdx* mice, they do not express the functional protein. The hindlimb muscles undergo a bout of severe degeneration at 2–4 wk of age, but a high regenerative capacity ensures good functional and structural recovery. In contrast to patients with DMD, adult *mdx* mice have a normal life span, and, although centrally nucleated fibers are prevalent in the hindlimb muscles, there is only minor inflammatory cell and connective and adipose tissue infiltration, such that the normalized force-producing capacity of soleus and extensor digitorum longus (EDL) hindlimb muscles is reduced by only 10–15% compared with wild-type mice (26). In contrast, the diaphragm of *mdx* mice exhibits a more severe and progressive pathophysiology, with ongoing degeneration and ineffective regeneration resulting in a loss of viable muscle fibers and an infiltration of inflammatory cells and connective tissue (22, 25, 50). The normalized force-producing capacity of the diaphragm is decreased by ~40% in adult *mdx* compared with wild-type mice (27). Hence, the diaphragm muscle of *mdx* mice provides a suitable model for evaluating the efficacy of experimental interventions for DMD. The aim of the present study was to investigate whether calcineurin activation would attenuate myofiber damage, improve regeneration, enhance force-producing capacity, and reduce fatigability of *mdx* diaphragm and hindlimb muscles. Calcineurin phosphatase enzyme activity and protein levels of dephosphorylated (activated) nuclear factor of activated T cells (NFATc1) are greater in diaphragm than in tibialis anterior (TA) muscles from *mdx* and C57BL/10 mice (50), and the calcineurin signal transduction pathway appears to be most active in diaphragm muscles of *mdx* mice (50). Given that endogenous calcineurin signaling and CnAα transgene expression are affected by skeletal muscle fiber type composition, a secondary aim of the present study was to compare the responsiveness of muscles with different phenotypes to genetic calcineurin activation.

Utrophin expression is regulated by calcineurin and is greater in slow- than in fast-twitch muscles (3, 7). In hindlimb muscles of *mdx CnAα* mice, calcineurin activation increased utrophin levels, which improved sarcolemmal stability, attenuated myofiber damage, and decreased muscle susceptibility to resistance to contraction-induced injury (6, 51). Plasma creatine kinase levels, inflammatory cell infiltration, and infrafiber...
staining of the plasma proteins were reduced in muscles from \textit{mdx} CnA\textsuperscript{\text{*}} compared with \textit{mdx} mice. Associated with decreased muscle damage was a reduction in the proportion of centrally nucleated fibers in muscles from \textit{mdx} CnA\textsuperscript{\text{*}} mice (6, 51). Fewer damaged and regenerating myofibers would be expected to improve the force-producing capacity of diaphragm muscle strips from \textit{mdx} mice (14, 17).

The calcineurin signal transduction pathway is essential for successful muscle regeneration (41, 42). Calcineurin can regulate myoblast differentiation (1), transcription and/or activation of myoregulatory and growth factors [e.g., myocyte enhancer factor 2A (MEF-2A), MyoD, myogenin, and insulin-like growth factor 1] (2, 13, 60), fusion of myoblasts to nascent myofibers (8), and expression of the developmental MyHC isoform (39). In hindlimb muscles of young \textit{mdx} mice, pharmacological inhibition of calcineurin increased degeneration, impaired regeneration, and compromised muscle force-producing capacity, effects that appear to be dose dependent (49). Conversely, calcineurin activation may enhance muscle regeneration. After myotoxic damage, recovery of fiber size and force-producing capacity was hastened in TA muscles of wild-type mice expressing the CnA\textsuperscript{\text{*}} transgene compared with control mice, an effect associated with differential expression of myoregulatory factors in regenerating muscles (52). Calcineurin also regulates the contractile and metabolic properties of skeletal muscle by activating the expression of slow oxidative fiber-specific genes (21, 37). In mouse skeletal muscles, calcineurin activation enhanced lipid oxidation and attenuated glycolysis (21), increased the proportion of type I muscle fibers (6, 54), and reduced fatigability of fast-twitch TA muscles (51, 52). This is the first study to specifically compare the potentially therapeutic effects of calcineurin activation on the structural and functional phenotype of different muscles from \textit{mdx} dystrophic mice, with particular emphasis on the severely affected diaphragm. We tested the hypothesis that, in \textit{mdx} mice, expression of the constitutively active CnA\textsuperscript{\text{*}} transgene would improve diaphragm and hindlimb muscle function and morphology by promoting the slow oxidative phenotype and improving muscle endurance, attenuating degeneration, and enhancing muscle fiber regeneration.

METHODS

Transgenic mice. All experiments were approved by the Animal Experimentation Ethics Committee of the University of Melbourne. Female \textit{mdx} mice homozygous for the X-linked mutation in the dystrophin gene were mated with male transgenic mice (MCK-CnA\textsuperscript{\text{*}} expressing a constitutively active form of calcineurin A (CnA\textsuperscript{\text{*}}) (36). The CnA\textsuperscript{\text{*}} transgene is driven by the mitochondrial creatine kinase (MCK) promoter, which is induced during myogenic differentiation, and its activity is maximal in committed myotubes (47). The MCK-CnA\textsuperscript{\text{*}} mice were developed at the Department of Molecular Biology of the University of Texas Southwestern Medical Center at Dallas (34). The MCK-CnA\textsuperscript{\text{*}} male mice were bred on a B6C3F1 background and the female \textit{mdx} mice on a C57BL/10 ScSn background. The expression level of the transgene in the male parent MCK-CnA\textsuperscript{\text{*}} mice is such that basal calcineurin activity levels are ~10-fold greater than in wild-type mice (59). Others have measured protein levels of the CnA\textsuperscript{\text{*}} transgene and report that constitutively active calcineurin protein accounts for ~34% and 21% of the total calcineurin pool in EDL and soleus muscles, respectively (40). All the F1 male pups were dystrophic, having inherited the X chromosome with the dystrophin mutation from their mothers, and ~50% expressed the autosomal CnA\textsuperscript{\text{*}} transgene (mdx CnA\textsuperscript{\text{*}}), which was inherited from their fathers (51). Male pups that expressed the transgene were identified through PCR screening of DNA extracted from tail tissue (34). Their \textit{mdx} littersmates served as experimental controls. Mice were maintained on a 12:12-h light-dark cycle, with standard mouse chow and water provided ad libitum. EDL, soleus, and diaphragm muscle function was assessed in 3-mo-old \textit{mdx} CnA\textsuperscript{\text{*}} and \textit{mdx} mice (n = 12–15 per muscle per mouse strain).

Assessment of skeletal muscle function. Mice were anesthetized with ketamine (100 mg/kg; Parnell Laboratories, Alexandria, NSW, Australia) and xylazine (10 mg/kg; TROY Laboratories, Smithfield, NSW, Australia), such that they were unresponsive to tactile stimuli. Isometric contractile properties were evaluated in vitro, as described in detail previously (17, 48). Briefly, EDL and soleus muscles were tied at the proximal and distal tendons with braided surgical silk, excised surgically, and transferred to a custom-built Plexiglas bath filled with Ringer solution (in mM: 137 NaCl, 24 NaHCO\textsubscript{3}, 11 tris-glucose, 5 KCl, 2 Ca\textsubscript{2}\textsuperscript{+}, 1 Na\textsubscript{2}HPO\textsubscript{4}, 1 H\textsubscript{2}O, 1 MgSO\textsubscript{4}, and 0.025 \textit{d}-tubocurarine chloride), bubbled with 5% CO\textsubscript{2} in O\textsubscript{2} (Carbogen, BOC Gases, Preston, Australia), and the thermostatically maintained at 25°C. The distal tendon of the muscle was tied to an immovable pin, and the proximal tendon was attached to the lever arm of a dual-mode servomotor-force transducer (model 300B-LR, Aurora Scientific, Aurora, ON, Canada). The still deeply anesthetized mice were killed after cardiac excision. For functional analysis of the diaphragm muscle strips, the entire diaphragm and surrounding rib cage were carefully excised and placed in a dish filled with oxygenated Krebs-Ringer solution. Diaphragm strips (~0.5 cm wide) composed of longitudinally arranged full-length muscle fibers were tied with braided surgical silk (6-0) at the central tendon at one end and sutured through a portion of rib at the other end (17).

The EDL, soleus, and diaphragm muscles were stimulated by supramaximal square-wave pulses that were amplified (Eagle EP500B Audio Assemblies, Campbellfield, Victoria, Australia) and delivered via two platinum electrodes that flanked the length of the muscle to prepare to produce a maximum isometric contraction (15). All stimulation parameters and contractile responses were controlled and measured using custom-built applications (D. R. Stom, Ann Arbor, MI) of LabView software (National Instruments, Austin, TX) driving a personal computer with an onboard controller (model PCI-MIO-16XE-10, National Instruments) interfaced with the transducer-servomotor control/feedback hardware (Aurora Scientific) (15, 23).

Optimum length (\textit{L}\textsubscript{o}) for EDL, soleus, and diaphragm muscles was determined from micromaneuverizations of muscle length and a series of isometric twitch contractions. Maximum isometric tetanic force (\textit{P}\textsuperscript{\text{\text{t}}}) production was determined from the plateau of the frequency-force relationship: 10, 20, 30, 50, 70, 90, 100, and 120 Hz for EDL and diaphragm muscles and 10, 20, 30, 50, 70, 90, 100, and 120 Hz for soleus muscles. To assess fatigability, \textit{P}\textsubscript{\text{\text{t}}} was determined once every 5 s for 4 min. Recovery of \textit{P}\textsubscript{\text{\text{t}}} was determined 5, 10, and 15 min after the fatigue protocol.

After testing, the muscles were trimmed of their tendons and nonmuscle tissue, weighed on an analytic balance, and stored at ~80°C. EDL, soleus, diaphragm, and TA muscles were then mounted in embedding medium, frozen in thawing isopentane, and stored at ~80°C. EDL, soleus, and TA muscles were then mounted in embedding medium, frozen in thawing isopentane, and stored at ~80°C for histological and immunohistochemical analysis. Overall muscle cross-sectional area (CSA) was determined by dividing the muscle mass by the product of optimum fiber length (\textit{L}\textsubscript{o}) and 1.06 mg:mm\textsuperscript{3}, the density of mammalian muscle. \textit{L}\textsubscript{o} was determined by multiplying \textit{L} by previously determined muscle length-to-fiber length ratios: 0.44 for the EDL, 0.71 for the soleus, and 1.0 for the diaphragm. Since \textit{P}\textsuperscript{\text{\text{t}}} is dependent on muscle size, \textit{P}\textsuperscript{\text{\text{t}}} values were normalized for CSA (\textit{P}\textsubscript{\text{\text{t}}} was divided by the calculated total muscle CSA) and expressed as specific force (\textit{sP}\textsuperscript{\text{\text{t}}} in kN/m\textsuperscript{2}) (25). \textit{P}\textsubscript{\text{\text{t}}} and whole muscle CSA are reported for EDL and soleus muscles. For diaphragm muscle strips, the width and thickness and, consequently, mass vary unpredictably among animals at the discretion of the surgeon excising them.
Therefore, absolute forces developed by diaphragm muscle strips have no physiological meaning, and comparisons can be made only after the forces have been normalized for total muscle fiber CSA (kN/m²) (27). The CSAs of diaphragm muscle strips were also determined by dividing muscle mass (mg) by the product of \(L_t\) (mm) and 1.06 mg/mm², the density of mammalian skeletal muscle (27).

**Histology and immunohistochemistry.** Five 5-μm-thick sections were cut from the middle region of each muscle on a cryostat at \(-20^\circ\)C (\(n = 8–10\) per muscle per mouse strain). The sections were air-dried overnight and stored at \(-80^\circ\)C. Hematoxylin-eosin stain was used for determination of fiber CSA, proportion of centrally nucleated fibers, and areas of degeneration; Van Gieson’s stain for quantification of muscle collagen content; and immunohistochemistry for analysis of myogenin, MEF-2A, and developmental MyHC expression as markers of regeneration. Muscle sections were reacted with antibodies against myogenin (catalog no. sc-576, Santa Cruz Biotechnology, Santa Cruz, CA; 1:60 dilution), MEF-2A (catalog no. sc-313, Santa Cruz Biotechnology; 1:60 dilution), and the developmental MyHC isoform (catalog no. NCL-MHCD, Novocastra; 1:20 dilution), as described previously (50, 52). The number of MEF-2A- and myogenin-positive nuclei in each section was expressed per square millimeter of muscle. The number of muscle fibers that reacted with anti-development MyHC antibody was expressed as a proportion of the total number of muscle fibers, and their individual fiber CSA was also determined (≈250–750 fibers examined per section, depending on the muscle studied). Images of muscle cross sections were captured with a digital camera (Spot, version 2.2, Diagnostic Instruments, Sterling Heights, MI) mounted on an upright microscope (model BX-51, Olympus, Tokyo, Japan) and analyzed, as required, using computerized image analysis software (AIS, version 6.0, Imaging Research, St. Catherine’s, ON, Canada).

The proportion of centrally nucleated fibers and mean fiber CSA were calculated by analysis of up to 150 fibers per section. To assess variability in fiber size, the coefficient of variation (CV) for mean fiber CSA was calculated for each muscle (51). For cross sections of diaphragm muscle, areas of degeneration, including mononuclear cell, connective, and adipose tissue infiltration, were identified, outlined using computerized image analysis software (AIS, version 6.0; Imaging Research, St. Catherine’s, ON, Canada). The proportion of centrally nucleated fibers and mean fiber CSA were calculated by analysis of up to 150 fibers per section. To assess variability in fiber size, the coefficient of variation (CV) for mean fiber CSA was calculated for each muscle (51). For cross sections of diaphragm muscle, areas of degeneration, including mononuclear cell, connective, and adipose tissue infiltration, were identified, outlined using computerized image analysis software (AIS, version 6.0; Imaging Research, St. Catherine’s, ON, Canada).

**Statistics.** Values are means ± SE. All statistical analyses were performed using a statistics software package (Minitab, State College, * and relaxation time; \(P_t\), maximum tetanic force; \(TPT\), time to peak twitch; \(1/2\)\(RT\), one-half relaxation time; \(sPo\), specific force or force normalized to overall muscle CSA; \(N/A\), not applicable. †Significantly different from \(mdx\) mice (P < 0.05), whereas EDL muscle mass and whole muscle CSA were similar.

**Twitch force (\(P_t\)) and \(P_o\) were similar in soleus muscles of \(mdx\) CnA* and \(mdx\) mice. Although \(P_t\) was not different, \(P_o\) was reduced in EDL muscles from \(mdx\) CnA* compared with \(mdx\) mice (P < 0.05). When \(P_o\) was normalized for overall muscle CSA, \(sPo\) of EDL and soleus muscles of \(mdx\) CnA* mice was reduced by 20% and 8%, respectively, compared with \(mdx\) mice (Table 1; P < 0.05). In the diaphragm, \(sPo\) is the only valid measure of force-producing capacity, and, in contrast to hindlimb muscles, expression of the CnA* transgene was not associated with a decrease in \(sPo\). Characteristic of a slower phenotype, time to peak twitch tension (TPT) was prolonged in EDL, soleus muscles, and diaphragm muscles, and one-half relaxation time (1/2 RT) was prolonged in EDL and diaphragm, but not in soleus, muscles from \(mdx\) CnA* mice compared with \(mdx\) littermates (P < 0.05; Table 1).

**Muscle fatigue.** After 2, 3, and 4 min of intermittent maximal stimulation, the relative force deficit of EDL muscles of \(mdx\) CnA* mice was ≈10–20% less than that of \(mdx\) mice (P < 0.05, strain \(\times\) time interaction). The relative loss of force-producing capacity after fatigue in diaphragm muscles was less at 3 and 4 min into the fatigue protocol in \(mdx\) CnA* than \(mdx\) mice (P < 0.05, strain \(\times\) time interaction). EDL and diaphragm muscles of \(mdx\) CnA* mice not only fatigued less, but their relative recovery of force after fatigue was also superior at all time points tested (P < 0.05, strain \(\times\) time interaction). In soleus muscles of \(mdx\) mice, calcineurin activation also reduced fatigability and improved force recovery (P < 0.05, main effect; Fig. 1).

| Table 1. Body mass, muscle mass, and contractile properties of EDL and soleus muscles and diaphragm muscle strips from \(mdx\) and \(mdx\) CnA* mice |
|-----------------|-----------------|-----------------|-----------------|
|                 | \(mdx\)         | \(mdx\) CnA*    | \(mdx\)         |
| **Body mass, g**| 32.8 ± 0.8      | 31.4 ± 0.7      | 32.8 ± 0.8      |
| **Muscle mass, g**| 15.7 ± 0.6      | 16.4 ± 0.7      | 12.0 ± 0.5      |
| **Muscle CSA, mm²**| 2.72 ± 0.09     | 2.85 ± 0.50     | 1.53 ± 0.06     |
| \(P_t\), mN     | 156.2 ± 10.8    | 164.1 ± 10.7    | 52.4 ± 2.7      |
| **TPT, ms**     | 10.2 ± 0.5      | 26.0 ± 0.67     | 333.3 ± 0.8     |
| \(1/2\) \(RT\), ms| 28.0 ± 1.7      | 38.0 ± 1.61     | 42.0 ± 1.2      |
| \(P_o\), mN     | 479.4 ± 17.1    | 401.1 ± 16.6†   | 286.6 ± 9.2     |
| **sPo, kN/m²**  | 177.5 ± 6.1     | 142.5 ± 7.2†    | 189.6 ± 5.4     |
| \(Δ\%\)        | \(\downarrow 20†\) | \(\downarrow 8†\) | \(\downarrow 4\) |

Values are means ± SE. EDL, extensor digitorum longus; CSA, cross-sectional area; \(P_t\), maximum twitch force; TPT, time to peak twitch; \(1/2\) \(RT\), one-half relaxation time; \(P_o\), maximum tetanic force; \(sPo\), specific force or force normalized to overall muscle CSA; \(N/A\), not applicable. †Significantly different from \(mdx\) (P < 0.05).
Muscle morphology and degeneration. Expression of the CnAα* transgene in mdx mice reduced mean fiber CSA in all muscles examined (P < 0.05, main effect; Table 2, Fig. 2A). This effect was most pronounced in TA muscles, where mean fiber CSA of muscles of mdx CnAα* mice was reduced by ~50% compared with mdx mice (P < 0.05, strain × muscle interaction; Table 2). Expression of the CnAα* transgene had no significant effect on fiber size variability in any of the muscles examined (Table 2). Others have reported a reduction in fiber size variability with calcineurin activation after analysis for standard deviation, rather than CV (6, 51). We believe that relying on standard deviation to assess fiber size variability can be misleading (51), because expression of the CnAα* transgene reduced mean fiber CSA in all muscles examined. This reduction of mean fiber CSA was accompanied by a decrease in the standard deviation of ~35% in diaphragm, ~7.5% in soleus, ~20% in EDL, and ~50% in TA muscles. Although smaller fibers can have a smaller standard deviation than larger fibers, this does not equate to a reduction in fiber size variability. The CV for all muscles examined in mdx mice was similar and ranged from ~52% to 66%.

As expected, collagen infiltration was greater in diaphragm than hindlimb muscles, but there was no difference between muscles of mdx and mdx CnAα* mice (Table 2). Although muscle degeneration can be associated with increased collagen levels, staining for collagen with Van Gieson’s stain may not be as specific or sensitive enough for identifying collagen in degenerating skeletal muscle. Van Gieson’s stain will identify collagen in the endomysium surrounding healthy skeletal muscle fibers, but it will not stain all areas of degenerating muscle, such as infiltrating mononuclear and inflammatory cells. Hence, the severe pathophysiology of the diaphragm cannot be attributed solely to an increase in collagen content. Muscle degeneration (connective tissue and mononuclear cell infiltration) was assessed in greater detail and sensitivity in hematoxylin-eosin-stained sections of diaphragm muscles from mdx and mdx CnAα* mice. Expression of the CnAα* transgene improved diaphragm muscle morphology by reducing the area of muscle undergoing degeneration by 20% from ~22% in mdx mice to ~17% in mdx CnAα* mice (P < 0.05; Fig. 2C).

The proportion of fibers with central nuclei was nearly twofold greater in hindlimb than diaphragm muscles in mdx and mdx CnAα* mice. Indicative of a reduced susceptibility to contraction-induced injury, the proportion of centrally nucleated fibers was reduced in muscles from mdx CnAα* compared with mdx mice (P < 0.05, main effect; Fig. 2B). In TA, diaphragm, and soleus, but not EDL, muscles, the presence of the CnAα* transgene reduced the proportion of centrally nucleated fibers by 10–20% (Fig. 2B).

Myogenin, MEF-2A, and developmental MyHC protein expression. The number of myogenin-positive nuclei was two- to fivefold greater in diaphragm than hindlimb muscles from mdx and mdx CnAα* mice (P < 0.05, main effect; Fig. 3). MEF-2A and developmental MyHC protein levels were statistically similar in the hindlimb muscles and diaphragm.

No significant differences in nuclear localization of myogenin were observed in diaphragm or hindlimb muscles of mdx and mdx CnAα* mice. However, the effect of calcineurin activation on myogenin protein levels was variable, with in-
creased expression in TA muscles, reduced expression in EDL and diaphragm muscles, and similar expression in soleus muscles (not significant). The number of MEF-2A-positive nuclei was greater in muscles from \textit{mdx CnA}* than \textit{mdx} mice (\(P < 0.05\), main effect; Fig. 4). In the fast-twitch EDL and TA muscles of \textit{mdx CnA}* mice, the number of MEF-2A-positive nuclei was nearly twofold greater than in \textit{mdx} mice. In the soleus and diaphragm muscles, MEF-2A nuclear localization was similar in \textit{mdx} and \textit{mdx CnA}* mice (Fig. 4). The proportion of fibers expressing the developmental MyHC isoform was higher in muscles of \textit{mdx CnA}* than \textit{mdx} mice (\(P < 0.05\), main effect; Fig. 5).

<table>
<thead>
<tr>
<th></th>
<th>Mean Fiber CSA, (\mu m^2)</th>
<th>dMyHC-Positive Fiber CSA, (\mu m^2)</th>
<th>%Collagen Infiltration</th>
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<tr>
<td></td>
<td>\textit{mdx}</td>
<td>\textit{mdx CnA}*</td>
<td>\textit{mdx}</td>
</tr>
<tr>
<td>EDL</td>
<td>1,213 ± 125 (CV = 67%)</td>
<td>1,053 ± 53† (CV = 60%)</td>
<td>728 ((n = 20))</td>
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<td>Soleus</td>
<td>1,282 ± 78 (CV = 52%)</td>
<td>1,027 ± 70† (CV = 60%)</td>
<td>423 ((n = 123))</td>
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<tr>
<td>TA</td>
<td>2,265 ± 249 (CV = 63%)</td>
<td>1,076 ± 63† (CV = 67%)</td>
<td>1,037 ((n = 120))</td>
</tr>
<tr>
<td>DIA</td>
<td>637 ± 44 (CV = 66%)</td>
<td>468 ± 32† (CV = 58%)</td>
<td>425 ((n = 144))</td>
</tr>
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</table>

Values are means ± SE. TA, tibialis anterior; DIA, diaphragm; CSA, cross-sectional area; dMyHC, developmental myosin heavy chain; \(n\), number of fibers; CV, coefficient of variation. †Significantly different from \textit{mdx} (\(P < 0.05\), main effect).

Table 2. Myofiber size and collagen content of muscles from \textit{mdx} and \textit{mdx CnA}* mice

Fig. 2. Morphology of hindlimb and diaphragm muscles of \textit{mdx CnA}* and \textit{mdx} mice. A: representative hematoxylin-eosin-stained sections of EDL, soleus, tibialis anterior (TA), and diaphragm muscles of \textit{mdx CnA}* and \textit{mdx} mice. Mean fiber cross-sectional area of TA muscles in \textit{mdx} mice was 2- to 3.5-fold greater than that of EDL, soleus, and diaphragm muscles. Very large (hypertrophied) fibers in TA muscles in \textit{mdx} mice contributed to the difference in fiber size. Such hypertrophied fibers were not observed in these other muscles. Scale bar, 100 \(\mu m\). B: proportion of centrally nucleated fibers was reduced in muscles of \textit{mdx CnA}* compared with \textit{mdx} mice. *\(P < 0.05\). C: expression of the CnA* transgene reduced degeneration (including connective tissue and mononuclear cell infiltration) in diaphragm muscles of \textit{mdx} mice. *\(P < 0.05\).
transgene increased developmental MyHC expression by two- to fourfold. The CnAα* transgene had little effect on developmental MyHC protein levels in diaphragm muscles. In mdx CnAα* and mdx mice, fibers expressing the developmental MyHC were smaller than average (Table 2).

DISCUSSION

There is considerable research interest in the activation of calcineurin and promotion of a slow oxidative phenotype to ameliorate the pathophysiology of muscular dystrophy (6, 16, 51). In mdx mice, the pathology of the diaphragm is more severe than that of hindlimb muscles, making the diaphragm well suited to investigate whether calcineurin activation can reduce degeneration and enhance regeneration in a mouse model of DMD. In the present study, we report that genetic calcineurin activation in mdx mice enhanced the endurance of hindlimb and diaphragm muscles and had positive effects on contractile properties in slow-twitch muscles (54). Although fiber type proportions were not assessed in the diaphragm in the present study, calcineurin activation would be expected to produce similar effects in mdx and nondystrophic wild-type mice, since fiber type proportions are similar in these mice (15). Despite a slower phenotype and a reduction in mean fiber size, factors that are known to decrease specific (normalized) force, sPo, was similar in mdx CnAα* and mdx mice, a finding attributed to the 20% reduction in the area of degenerating muscle. The potential benefits of calcineurin activation in diaphragm and hindlimb muscles are not easily discerned and are likely confounded by heterogeneous and ongoing fiber damage and repair. Nevertheless, in hindlimb muscles of mdx mice, calcineurin activation may have the potential to enhance muscle regeneration by increasing MEF-2A and developmental MyHC protein expression.

Because calcineurin regulates the expression of slow-twitch fiber-specific genes in hindlimb and diaphragm muscles (34, 54), contractile properties were slower in muscles of mdx CnAα* mice than mdx mice. The increase in TPT in muscles of mdx CnAα* mice may be attributed, in part, to greater expression of slow(er) MyHC isoforms: type I in slow-twitch muscles and type I and IIa in fast-twitch muscles (54). The prolonged 1/2 RT in muscles of mdx CnAα* mice could be indicative of a slower rate of Ca2+ uptake by the sarcoplasmic reticulum and increased expression of the slow sarco(endo)plasmic Ca2+. ATPase (SERCA2a) isoform (4). In other experiments where function was assessed in situ (i.e., with nerve and blood supply intact), TPT and 1/2 RT were similarly prolonged in TA muscles of adult mdx CnAα* and nondystrophic MCK-CnAα* mice (51, 52). Consistent with a slower phenotype was the smaller relative force decrement and improved recovery of force after repeated contractile activity in EDL, soleus, and diaphragm muscles of mdx CnAα* compared with mdx mice. Impaired muscle endurance is characteristic of many neuromuscular disorders (12), and boys with DMD are especially susceptible to respiratory muscle fatigue (28). Since slow-twitch muscles are resistant to fatigue compared with fast muscles, a shift to a slower muscle phenotype may have therapeutic benefits for DMD patients.

In hindlimb and diaphragm muscles, expression of the CnAα* transgene was associated with 20–50% reduction in mean fiber size, depending on muscle phenotype. Because of an inverse relationship between fiber size and oxidative capacity, the reduction in fiber size could be related to the increase in hindlimb and diaphragm muscles, expression of the CnAα* transgene was associated with 20–50% reduction in mean fiber size, depending on muscle phenotype. Because of an inverse relationship between fiber size and oxidative capacity, the reduction in fiber size could be related to the increase in

![Fig. 3. Myogenin expression in hindlimb and diaphragm (DIA) muscles of mdx CnAα* and mdx mice. Representative soleus muscle sections from mdx CnAα* and mdx mice reacted with an antibody against myogenin. Arrows indicate myogenin-positive nuclei, which stained red. *Significantly different (~3-fold greater) than hindlimb muscle (P < 0.05, main effect). Myogenin protein levels were not statistically different in muscles of mdx CnAα* and mdx mice. Scale bar, 100 μm.](http://ajpregu.physiology.org/)

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in oxidative capacity (33, 54). Po and sPo were lower for EDL and soleus muscles of mdx CnAα* than mdx mice, a finding attributed to the decrease in mean fiber CSA and a slower muscle phenotype. In contrast to hindlimb muscles, the normalized force-producing capacity (sPo) of diaphragm muscles of mdx CnAα*/H9251 mice was preserved, which we attribute, in part, to the ~20% reduction in the muscle area affected by degeneration (e.g., inflammatory cell and connective tissue infiltration). Given that the pathophysiology of the mdx diaphragm more closely resembles that of muscles in DMD (11), the findings highlight the therapeutic potential of calcineurin activation for dystrophic muscles. The cellular mechanisms underlying the reduction in connective tissue and mononuclear cell infiltration likely include improved myofiber viability due to utrophin up-regulation (51) and enhanced muscle regeneration (52).

Calcineurin activation can improve sarcolemmal stability and protect dystrophic myofibers from contraction-induced damage by upregulating utrophin expression (6, 51). A reduction in the proportion of albumin-staining fibers after lengthening contractions in TA muscles of mdx CnAα* compared with mdx mice supports the contention that muscle fibers from mdx CnAα* mice are protected from damage and is consistent with other reports of reduced intramuscle fiber Evans blue, albumin, and IgM staining in hindlimb muscles, as well as lower plasma creatine kinase values in mdx CnAα*/H9251 than mdx mice (6). The assumption that calcineurin activation reduces sarcolemmal damage specifically in mdx diaphragm muscles warrants further testing using Evans blue, albumin, or IgM staining. Central nuclei are a hallmark of recently regenerated myofibers, and expression of the CnAα* transgene decreased the proportion of centrally nucleated fibers, indicating a reduced incidence of contraction-induced muscle fiber damage. This was most pronounced in soleus, diaphragm, and TA muscles, where the proportion of centrally nucleated fibers was reduced by 10–15%. Previously, we also observed a ~10% reduction in the proportion of centrally nucleated fibers in TA

Fig. 4. Myocyte enhancer factor 2A (MEF-2A) expression in hindlimb and diaphragm muscles of mdx CnAα* and mdx mice. Representative TA and diaphragm muscle sections from mdx CnAα* and mdx mice reacted with an antibody against MEF-2A. Arrows indicate MEF-2A-positive nuclei, which stained red. Scale bar, 100 μm. *P < 0.05.
Fig. 5. Developmental myosin heavy chain (MyHC) expression in hindlimb and diaphragm muscles of mdx CnAα* and mdx mice. Representative EDL muscle sections from mdx CnAα* and mdx mice reacted with an antibody against the developmental MyHC isoform. Fibers expressing developmental MyHC isoform stained red. Note greater number of fibers expressing developmental MyHC protein in EDL muscle sections of mdx CnAα* than mdx mice. Scale bar, 100 μm.

Muscles of 9- to 10-mo-old mdx CnAα* compared with mdx mice (51). Calcineurin activation had a minimal effect on the proportion of centrally nucleated fibers in EDL muscle, highlighting the differential effects of genetic calcineurin activation based on muscle phenotype (54).

Greater myogenin expression and a ~2.5-fold decrease in the proportion of centrally nucleated fibers in diaphragm compared with hindlimb muscles of mdx CnAα* and mdx mice suggest an impairment in that muscle regeneration downstream of myogenin, leading to a loss of viable muscle fibers and concomitant connective tissue and inflammatory cell infiltration (50). We hypothesized that calcineurin activation has the potential to ameliorate this impairment (1) and enhance regeneration, and not merely to decrease susceptibility to contraction-induced injury. It is difficult to evaluate the effect of calcineurin activation on muscle regeneration in mdx mice, because of the muscle phenotype-dependent dystrophic pathology and CnAα* transgene expression and the heterogeneous nature of uninjured, injured, and regenerating muscle fibers. The stimulatory effect of calcineurin activation on MEF-2A nuclear localization and developmental MyHC expression reported in the present study indicates that calcineurin activation can enhance regeneration in dystrophic muscles. The number of MEF-2A-positive nuclei was higher in muscles from mdx CnAα* than mdx mice, and this was most evident in fast-twitch EDL and TA muscles. Calcineurin-MEF-2A signaling occurs in all muscle fibers, but more so in fibers that are recruited infrequently, i.e., fast-twitch type IIx and IIb, than in slow-twitch fibers (10). Dephosphorylation of MEF-2A by calcineurin promotes MEF-2A translocation to the nucleus and increases its protein stability by protecting it from caspase-mediated degradation, leading to increased transcription of MEF-2A target genes (30). MEF-2A can regulate myogenesis and the expression of slow muscle phenotype-specific genes (5). Relevant to the present study is the observation that, in myocyte cultures, calcineurin and MEF-2A together regulate expression of the developmental MyHC isoform (39). In the present study, developmental MyHC levels were higher in muscles from mdx CnAα* than mdx mice. The proportion of fibers expressing the developmental MyHC isoform was two- to fourfold higher in EDL, soleus, and TA muscles of mdx CnAα* mice than in hindlimb muscles of mdx mice. However, because of the minimal effect of the CnAα* transgene on MEF-2A and developmental MyHC protein content in the diaphragm, it is difficult to state conclusively that calcineurin activation can enhance regeneration in diaphragm muscles of mdx mice.

Diaphragm muscle structure and function deteriorate progressively in mdx mice, and regeneration becomes increasingly impaired (25). In 3-mo-old mdx mice, there was greater collagen infiltration in diaphragm than hindlimb muscles, and the proportion of centrally nucleated fibers was reduced, despite higher myogenin expression. Here we report that, in diaphragm muscles of young (3-mo-old) mdx mice, 3.7% of fibers expressed developmental MyHC protein and 25% of fibers were centrally nucleated, but in diaphragm muscles of 6-mo-old mdx mice, developmental MyHC expression was decreased ~2.5-fold and the proportion of fibers with central nuclei was reduced by one-third (50). The possibility exists that the potentially beneficial effect of increased calcineurin activity on diaphragm muscle regeneration might become apparent in older mdx mice only when the diaphragm is severely compromised.

The effect of the CnAα* transgene on muscle function, morphology, and regeneration was strongly dependent on the initial muscle phenotype, a finding attributed to the fact that fast-twitch muscles might be more responsive to calcineurin signaling than the highly recruited slow-twitch muscles (10) and that activation of the MCK promoter is dependent on initial
muscle phenotype (47). Effects of calcineurin stimulation were greater in fast-twitch muscles, particularly the TA, than in slow-twitch muscles. In TA muscles, expression of the CnA transgene reduced mean fiber size by 50% compared with ~20–35% in the other muscles examined. Calcineurin activation also increased MEF-2A expression to a greater extent in fast- than in slow-twitch muscles, a finding not unexpected, since MCK promoter activity is greater in fast- than in slow-twitch muscles (47). In another study, the diaphragm was reported to be most responsive to the CnA transgene, but TA muscles were not examined (54). To eliminate the confounding effect of the MCK promoter on calcineurin activation, future studies investigating the potential therapeutic effects of calcineurin activation on dystrophin-deficient muscles should make use of compounds known to activate the calcineurin signal transduction pathway, such as Arg8-vasopressin (44) and prednisolone (46).

**Perspectives and Significance**

The present study contributes to a growing body of literature describing the potential of calcineurin stimulation to alleviate the pathology of dystrophin-deficient mdx mouse muscles (6, 51). It is important to determine whether the benefits of calcineurin stimulation are maintained during the life span of the mdx mouse, especially in the diaphragm, where dystrophic pathophysiology is further aggravated by aging (27). The effects of calcineurin activation on the slow-twitch muscle phenotype and utrophin expression in vivo have been well characterized (3, 6, 51, 54). Whether calcineurin activation can enhance muscle regeneration in hindlimb and diaphragm muscles of mdx mice requires further investigation and a better understanding of the associated signal transduction pathways. In particular, NFATc2 and NFATc2c3 are downstream transcription factors important in muscle regeneration and maintenance of muscle mass, which are activated through dephosphorylation by calcineurin and inhibited through phosphorylation by glycogen synthase kinase (GSK)-3β (18, 55). GSK-3β can antagonize calcineurin signaling and suppress myogenic differentiation through the negative regulation of NFATc3 (55). Calcineurin (50) and GSK-3β (56) enzyme activities are elevated in muscles of mdx compared with wild-type mice, and, with respect to myogenesis (and likely muscle fiber regeneration), GSK-3β is the dominant pathway. Therefore, the effects of genetic calcineurin stimulation on NFATc2 and NFATc3 phosphorylation and transcriptional activity and GSK-3β signaling should be investigated in regenerating muscle fibers of mdx mice.

The practical relevance of these findings to DMD is unknown, but a recent report of a significant decrease in serum and lymphocyte calcineurin activity in DMD (53) supports the hypothesis that calcineurin is a potential therapeutic target for DMD. It is important to recognize that the therapeutic benefits of calcineurin activation may be quite specific to DMD, since in a mouse model of limb girdle muscular dystrophy, calcineurin activation exacerbated and calcineurin inhibition ameliorated the dystrophic pathology (38). Previous studies have highlighted the importance of identifying and evaluating any potentially deleterious side effects of calcineurin activation, particularly if this strategy is to find clinical application. Of particular concern is the effect of excessive calcineurin signaling on cardiac hypertrophy, given that the cardiac pathology is already aggravated in mdx mice and boys with DMD (32, 43, 58).

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