Short Communication

Making Fast-Twitch Dystrophic Muscles Bigger Protects Them from Contraction Injury and Attenuates the Dystrophic Pathology

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The lack of functional dystrophin protein in Duchenne muscular dystrophy (DMD) renders muscle fibers highly fragile and susceptible to damage during contractions. Contraction-mediated injury is a major contributor to the progressive degeneration and etiology of muscle wasting in DMD. The prevailing understanding is that large fibers are highly susceptible to contraction damage and are affected preferentially, whereas smaller fibers are relatively spared in DMD. We tested the hypothesis that a pharmacological treatment that caused myofiber hypertrophy would increase the susceptibility of muscles from dystrophin-deficient *mdx* mice to contraction-induced injury, and thus aggravate the dystrophic pathology. The β -agonist formoterol (100 μ g/kg per day, i.p.) was administered to *mdx* mice for 28 days. Formoterol increased muscle mass, fiber cross-sectional area, and maximum force producing capacity by 30%, 23%, and 21%, respectively, in fast-twitch tibialis anterior muscles of *mdx* mice. Myofiber hypertrophy and increased maximum force producing capacity were also observed in the predominantly slow-twitch soleus muscles of *mdx* mice. Our original hypothesis was rejected since tibialis anterior muscles from formoterol-treated *mdx* mice had lower cumulative force deficits, indicating that they were less susceptible to contraction-induced injury. Formoterol treatment did not affect injury susceptibility in soleus muscles. These findings indicate that making dystrophic muscles bigger protects them from contraction damage and does not aggravate the dystrophic pathophysiology. These novel results further support the contention that anabolic agents have therapeutic potential for muscle wasting conditions including DMD. (Am J Pathol 2010, 176:29–33; DOI: 10.2353/ajpath.2010.090760)

Duchenne muscular dystrophy (DMD) is a severe and progressive muscle wasting disorder caused by mutations in the dystrophin gene that result in the complete absence of the membrane-stabilizing protein dystrophin.^{1–3} The loss of this integral muscle protein renders dystrophic muscles fragile and highly susceptible to damage from everyday contractions.^{4,5} What would normally be considered innocuous contractions in healthy muscle causes tears in muscle fibers and a subsequent influx of Ca^{2+} that activates degenerative pathways in dystrophic muscle.⁶ Repeated injurious events eventually exhaust the regenerative capacity of dystrophic muscles and infiltration of adipose and connective tissue ensues leading to progressive functional impairments in affected patients.⁶

Although the most likely cure for DMD will come from gene therapies, either by restoration or replacement of the mutated dystrophin gene, several significant hurdles must be overcome before such treatments become available and accepted.⁷ Until then, alternative therapies are needed that can attenuate the severity and progression of muscle wasting and enhance the quality of life for DMD patients. One of the most widely trialed therapeutic strategies for DMD has been the administration of anabolic agents such as anabolic steroids, myostatin-blocking antibodies/peptides, and β -adrenoceptor agonists (β -agonists).^{8–11} These approaches have shown improvements in muscle function in some studies,^{9,10,12} but others have shown little or no effect.¹¹

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Although bigger muscles generally produce more force, it has been suggested that enhancing muscle size in *mdx* mice would increase their susceptibility to contraction-induced injury.¹³ Previous studies have shown that large, fast type II fibers are damaged selectively after lengthening contractions^{14,15} and these fibers are preferentially affected in DMD.^{16–19} Conversely, smaller caliber fibers such as those in extraocular and intrinsic laryngeal muscles are spared in DMD patients and animal models of muscular dystrophy.^{20–23} Since contraction-induced injury contributes to the dystrophic pathology, it is imperative that potential therapeutic strategies do not adversely affect the susceptibility of dystrophic muscles to self-injury.

Using the well-characterized effect of β -agonists to induce skeletal muscle hypertrophy, we tested the hypothesis that making dystrophic muscles bigger would increase their susceptibility to contraction-mediated injury and thus aggravate the dystrophic pathology.

Materials and Methods

Experimental Animals

All procedures were approved by the Animal Experimentation Ethics Committee of The University of Melbourne and conformed to the Guidelines for the Care and Use of Experimental Animals described by the National Health and Medical Research Council (Australia). Male C57BL/10ScSn (BL/10) and *mdx* dystrophic mice (8–10 weeks old; $n = 8$ per group; 32 total) were obtained from the Animal Resource Centre (Canning Vale, WA, Australia) and housed in the Biological Research Facility at The University of Melbourne under a 12-hour light/dark cycle. The animals were provided access to drinking water and standard chow *ad libitum*. Mice received daily injections of formoterol (100 μ g/kg in saline, i.p.) or an equivalent volume of isotonic saline for 28 days.

Functional Properties

The methods for measuring contractile function of mouse tibialis anterior (TA) muscles *in situ* have been described in detail elsewhere.²⁴ Briefly, TA muscles from BL/10 and *mdx* mice were stimulated by supramaximal 0.2 ms square wave pulses of 350 ms duration, delivered via two wire electrodes adjacent to the peroneal nerve. The contractile properties of the soleus muscle were determined *in vitro*, as described previously.¹² Briefly, soleus muscles were stimulated by supramaximal 0.2 ms square wave pulses of 1200 ms duration, delivered via platinum plate electrodes that flanked the muscle. For both muscles, optimum muscle length (L_o) was determined from maximum isometric twitch force (P_o), and maximum isometric tetanic force (P_o) was recorded from the plateau of a complete frequency-force relationship. Specific force (sp_o) was determined by normalizing P_o to muscle cross-sectional area (CSA) and expressed in kN/m^2 . The protocol for assessment of contraction-induced injury has been described previously.^{25,26} Briefly, muscles were

activated maximally and then lengthened at a velocity of two fiber lengths per second ($2 L_f/s$) at progressively greater magnitudes of stretch (beyond L_f). Maximum isometric force was determined after each lengthening contraction. All muscles were subjected to a 2 minute rest period between successive contractions. Cumulative contraction-induced muscle damage was quantified by determining the force deficit—the difference in P_o after each successive lengthening contraction expressed as a percentage of maximum P_o before commencing the injury protocol.^{24,25}

Immunohistochemical Analyses

TA and soleus muscles were excised, blotted, and weighed on an analytical balance and then embedded in optimal cutting temperature compound, and frozen in isopentane cooled in liquid nitrogen for later immunohistochemical analyses. Multiple serial sections (5 μ m) were thaw-mounted on uncoated, pre-cleaned glass slides. Muscle fiber type-specific oxidative capacity and CSA were estimated from the determination of succinate dehydrogenase activity (SDH) combined with immunolabeling of laminin, myosin heavy-chain I, and myosin heavy-chain IIa in the muscle cross-sections, as described previously.²⁷ Other sections were stained with Mayer's hematoxylin and eosin to visualize general muscle architecture.

Digital images of stained sections (four images per muscle section) were obtained using an upright microscope ($\times 20$ objective) with camera (Axio Imager D1, Carl Zeiss, Wrek Göttingen, Germany), controlled by AxioVision AC software (AxioVision AC Rel. 4.7.1, Carl Zeiss Imaging Solutions, Wrek, Göttingen, Germany). Images were quantified using AxioVision 4.7.1 software for average fiber CSA, fiber type, and SDH activity as described previously.²⁷ In brief, the signal derived from the antibody against laminin was used to select single muscle fibers. Antibodies against myosin heavy-chains I and IIa, were used to differentiate between fiber types. Since the TA muscles consisted of only negligible numbers of type I muscle fibers, these were not used in the analysis. Approximately 360 muscle fibers were quantified in each section.

The minimal Feret's diameter is the minimum distance between opposing parallel tangents of a muscle fiber. Dystrophic muscles display a high degree of variability in the myofiber size, which can be quantified as the minimal Feret's variance coefficient. This parameter is highly sensitive and is used routinely for detecting differences between dystrophic and healthy muscles.²⁸

Statistical Analysis

All values are expressed as mean $\pm$ SEM unless specified otherwise. Groups were compared using one-way analysis of variance (analysis of variance) where appropriate. Bonferroni's *post hoc* multiple comparison procedure was used to determine significant differences be-

Figure 1. Properties of tibialis anterior (TA) muscles from formoterol-treated and untreated BL/10 and *mdx* mice. Muscle mass (A), maximum isometric force (B), specific (normalized) force (C), and susceptibility to contraction-induced injury (D) in formoterol-treated BL/10 and *mdx* mice. Note the greater susceptibility to contraction-induced injury in muscles from *mdx* mice compared with wild-type control mice and the reduced injury susceptibility exhibited by muscles from treated *mdx* mice. Significantly different from respective control strain (* $P < 0.05$); *mdx* significantly different from BL/10 (** $P < 0.05$).

tween groups. The level of significance was set at $P < 0.05$ for all comparisons.

Results

Pathophysiology of TA Muscles

Consistent with the results of our previous investigations on the effect of β -agonists on skeletal muscles, formoterol treatment increased muscle mass in BL/10 and *mdx* mice by 22% and 30%, respectively ($P < 0.05$, Figure 1A). Maximal P_o was increased by 19% and 21% in formoterol treated BL/10 and *mdx* mice, respectively ($P < 0.05$, Figure 1B). Specific (normalized) force (sP_o) was higher in muscles from BL/10 mice, as compared with *mdx* mice ($P < 0.05$, Figure 1C), but formoterol treatment did not affect sP_o in either strain.

Muscles from BL/10 mice were less susceptible to contraction-induced damage than those from *mdx* mice ($P < 0.05$, Figure 1D). Formoterol treatment significantly reduced the susceptibility to contraction-induced damage in muscles from *mdx* mice as evidenced by lower cumulative force deficits from the 10% stretch onwards in the injury protocol ($P < 0.05$, Figure 1D). Muscles from BL/10 mice displayed small, but significant, force deficits of 10% to 15% after the contraction-induced injury protocol.

Representative H&E-stained sections and CSA data for the TA muscle are presented in Figures 2, A and B. The mean CSA of myofibers from TA muscles of formoterol-treated *mdx* mice was 23% greater than in muscle from untreated *mdx* mice ($P < 0.05$, Figure 2B). SDH staining intensity, an indirect measure of oxidative capacity, was 36% lower in muscles from *mdx* than BL/10 mice ($P < 0.05$, Figure 2C). Formoterol did not affect SDH

staining intensity or fiber type composition (data not shown).

Minimal Feret's variance was significantly lower in muscles from BL/10, as compared with *mdx* mice ($P < 0.05$). Formoterol treatment had no effect on this measure

Figure 2. Representative photomicrographs of H&E-stained sections of TA muscle from formoterol-treated and untreated BL/10 and *mdx* mice. Magnification $\times 20$ (A). Bar graph representations of mean myofiber cross-sectional area (CSA; B), and mean succinate dehydrogenase (SDH) intensity (C). Significantly different to untreated control (* $P < 0.05$). *mdx* significantly different to BL/10 (** $P < 0.05$).

Figure 3. Properties of soleus muscles from formoterol-treated and untreated BL/10 and *mdx* mice. Muscle mass (**A**); maximum isometric force (**B**); specific (normalized) force (**C**); and susceptibility to contraction-induced injury in formoterol-treated BL/10 and *mdx* mice (**D**). Significantly different from respective control strain (* $P < 0.05$); *mdx* significantly different from BL/10 (** $P < 0.05$).

of the dystrophic pathology (315 ± 7 vs. 300 ± 8 in *mdx* control and formoterol treated, respectively).

Pathophysiology of the Soleus Muscle

Soleus muscle mass was increased by 29% in formoterol-treated *mdx* mice ($P < 0.05$, Figure 3A). In BL/10 mice the difference in soleus muscle mass after treatment (16%) was

of borderline significance ($P = 0.053$, *t*-test). Formoterol treatment increased maximal P_o in soleus muscles of BL/10 and *mdx* mice by 15% and 11%, respectively ($P < 0.05$, Figure 3B,C). Susceptibility to contraction-induced injury was neither different between soleus muscles of BL/10 and *mdx* mice, nor different within strains after formoterol treatment, as evidenced by the similar cumulative force deficits after each magnitude of strain (Figure 3D). Formoterol treatment did not alter fiber type composition in the soleus muscles of BL/10 or *mdx* mice (data not shown).

Minimal Feret's variance²⁸ was significantly lower in soleus muscles of BL/10 compared with *mdx* mice ($P < 0.05$). Formoterol treatment had no effect on this measure of the dystrophic pathology (260 ± 6 vs. 253 ± 6 for untreated and formoterol treated *mdx* mice, respectively).

Discussion

The most important finding of this study was that larger dystrophic skeletal muscles after treatment with the β -agonist, formoterol, were protected from contraction-mediated damage. Based on the prevailing knowledge that smaller caliber muscle fibers were protected in muscular dystrophy, we originally proposed that hypertrophied muscles would be more susceptible to injury and that this would aggravate the dystrophic pathology. In contrast, despite formoterol treatment increasing muscle mass and force production, the cumulative force deficit was actually lower in TA muscles of treated than untreated *mdx* mice. The findings support the contention that enhancing muscle size confers protection to fast-twitch dystrophic muscles and thus anabolic agents have therapeutic potential for skeletal muscle diseases.

In DMD, the absence of dystrophin renders muscles highly susceptible to contraction-induced injury.²⁹ This injury contributes to the etiology of the dystrophic pathology, ultimately leading to a loss of functional tissue. As a consequence, the extra workload imposed on the fewer remaining functional muscle fibers causes them to hypertrophy and be more vulnerable to contraction-mediated damage.^{25,30} Pharmacological approaches that cause muscle fiber hypertrophy have been suggested as being detrimental for muscular dystrophy if they aggravated the pathology. For example, anabolic steroids were shown in one study to have beneficial effects on muscle strength but exacerbated the dystrophic pathology.³¹ These seemingly contradictory results can be reconciled with an understanding that although bigger myofibers might produce more force, they might also be more easily damaged by their own contractions.

Previous studies from our laboratory have demonstrated that formoterol can increase muscle mass and muscle fiber CSA in rats and mice.^{12,32} Similarly, in the present study, formoterol treatment increased muscle mass in BL/10 and *mdx* mice.

Formoterol treatment did not affect oxidative capacity in either *mdx* or BL/10 mice. However, muscle fiber CSA was significantly greater in formoterol-treated mice, which was mediated primarily by hypertrophy of type IIb fibers, with no change in mean CSA of type IIa fibers. The absence of

changes in fiber type and oxidative capacity indicate that the hypertrophic response of type IIb fibers to formoterol treatment was the major factor responsible for conferring protection from contraction-induced injury. These findings have major implications for DMD patients since large type IIb fibers bear considerable loads, and so preventing their loss and/or improving their contractile protein content could help preserve mobility and quality of life in affected patients.¹⁶

Since hypertrophy of the type IIb fibers was the major factor protecting the TA muscle from contraction-induced injury in formoterol treated *mdx* mice, we also examined the susceptibility to contraction-induced damage in a muscle with a highly oxidative phenotype. Interestingly, we found no difference in the susceptibility to contraction-induced damage in soleus muscles from *mdx* compared with control mice. The high degree of resistance to injury in soleus muscles from lengthening contractions has been demonstrated elsewhere,^{25,33} but the mechanisms for protection are not well understood. It is reasonable to suggest that the obvious difference in fiber types within TA and soleus muscles would explain the differences in their relative pathology and susceptibility to injury. In the present study, the TA muscles in *mdx* mice were comprised of >90% of type IIb fibers, while the soleus muscle had fewer than 10% type II fibers. These differences in fiber type proportions may also contribute to the lack of substantial fiber hypertrophy in the soleus muscles.

Taken together, our findings revealed that β -agonist-induced hypertrophy conferred protection to fast-twitch dystrophic muscles from contraction-induced injury and therefore remains a valid and important therapeutic approach for the treatment of muscular dystrophies.

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