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Contraction-induced injury to single permeabilized muscle fibers from \textit{mdx}, transgenic \textit{mdx}, and control mice

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Lynch, Gordon S., Jill A. Rafael, Jeffrey S. Chamberlain, and John A. Faulkner. Contraction-induced injury to single permeabilized muscle fibers from \textit{mdx}, transgenic \textit{mdx}, and control mice. \textit{Am J Physiol Cell Physiol} 279: C1290–C1294, 2000.—Muscle fibers of \textit{mdx} mice that lack dystrophin are more susceptible to contraction-induced injury, particularly when stretched. In contrast, transgenic \textit{mdx} (tg-\textit{mdx}) mice, which overexpress dystrophin, show no morphological or functional signs of dystrophy. Permeabilization disrupts the sarcolemma of fibers from muscles of \textit{mdx}, tg-\textit{mdx}, and control mice. We tested the null hypothesis stating that, after single stretches of maximally activated single permeabilized fibers, force deficits do not differ among fibers from extensor digitorum longus muscles of \textit{mdx}, tg-\textit{mdx}, or control mice. Fibers were maximally activated by Ca\textsuperscript{2+} (pCa 4.5) and then stretched through strains of 10\%, 20\%, or 30\% of fiber length (\textit{Lf}) at a velocity of 0.5 \textit{Lf/s}. Immediately after each strain, the force deficits were not different for fibers from each of the three groups of mice. When collated with studies of membrane-intact fibers in whole muscles of \textit{mdx}, tg-\textit{mdx}, and control mice, these results indicate that dystrophic symptoms do not arise from factors within myofibrils but, rather, from disruption of the sarcolemmal integrity that normally provides protection from contraction-induced injury.

Duchenne muscular dystrophy (DMD), a severe X chromosome-linked myopathy, is caused by a variety of mutations and deletions in the dystrophin gene (13). In the absence of dystrophin expression, the skeletal muscles of patients with DMD undergo continuous cycles of degeneration and regeneration of muscle fibers that lead to a progressive wasting of the skeletal muscles (12). Patients are confined to wheelchairs by their early teens and die of respiratory or heart failure by their early twenties (12). Dystrophin is also absent in \textit{mdx} mice, an animal model for DMD (25). In the limb muscles of \textit{mdx} mice, the cycles of degeneration and regeneration produce hypertrophied, but weak, muscles (8). In contrast, the diaphragm muscles of \textit{mdx} mice show progressive degeneration of fibers analogous to the dystrophic process observed in human beings (6, 15, 21, 26).

Dystrophin links actin in the cytoskeleton through the transmembrane dystrophin-associated glycoprotein (DAG) complex to laminin in the extracellular matrix (ECM) (11). The precise functional role of dystrophin has not been described definitively, but the presence of dystrophin results in the protection of the sarcolemma from injury during muscle contractions (1, 3, 5, 21, 26) and even in quiescent muscle fibers (27). Increased permeability of the sarcolemma in \textit{mdx} mice is indicated by the increased serum concentrations of muscle enzymes and elevated intracellular Ca\textsuperscript{2+} concentration (9, 24). An uptake of Evans blue dye by fibers in muscles of \textit{mdx}, but not control, mice provides further support for an increased permeability of the sarcolemma of fibers lacking dystrophin (27).

A number of different protocols (1, 3, 5, 21) have demonstrated that skeletal muscles of \textit{mdx} mice have a greater susceptibility to injury, particularly when maximally activated muscles are stretched (pliometric contractions). The few reports that muscles of \textit{mdx} and control mice do not differ in their susceptibility to contraction-induced injury involved protocols with hundreds of pliometric contractions (8). These arduous protocols appear to have produced such severe damage to muscles in both \textit{mdx} and control mice that the assay did not discriminate the differences between the two.

Protocols of single stretches of maximally activated single permeabilization fiber segments have identified differences in the susceptibility to contraction-induced injury.
injury of fibers from muscles of old compared with adult mice and rats (2) and of fast compared with slow fibers from muscles of rats (17). Furthermore, the process of permeabilization of muscle fibers disrupts the sarcolemma severely (7). Our rationale was that the process of permeabilization of the membranes of fibers from muscles of mdx and control mice is so severe (7) that any protection provided to intact fibers from muscles of control mice would be eliminated, and the susceptibility of single permeabilized fibers from muscles of mdx, transgenic mdx (tg-mdx), and control mice to contraction-induced injury should not differ. The purpose was to study the magnitude of force deficit to single permeabilized fibers from extensor digitorum longus (EDL) muscles of mdx, tg-mdx, and control mice. We included the tg-mdx mice because the muscle fibers in these mice overexpress dystrophin and show no morphological or functional signs of dystrophy (23). We tested the null hypothesis stating that, following single pliometric contractions of increasing strain, no differences would be observed among the force deficits of single permeabilized fiber segments from the EDL muscles of mdx, tg-mdx, and control mice.

METHODS

Animals. The tg-mdx mice were used in the present study because the muscles of these transgenic mice express sufficient levels of a truncated dystrophin construct such that they do not exhibit histological or functional signs of muscular dystrophy like those of muscles from mdx mice (15). The generation of the tg-mdx mice has been described previously in detail (23). Briefly, the major dystrophin transcript in brain lacks exons 71–74 and encodes an isoform not observed in skeletal muscle. The capacity of this truncated isoform to function in muscle was tested by generating transgenic mice expressing a murine dystrophin minigene missing exons 71–74, termed “A71–74” (22, 23). Wild-type hybrid embryos from C57BL/6J × SJL/J parents were microinjected with the purified insert from the A71–74 transgene. Mice from the F0 generation were screened by polymerase chain reaction with primers to the first intron of the mouse muscle creatine kinase promoter construct and to the first exon of the mouse dystrophin cDNA. Dystrophin transgene-positive males were mated with mdx females to generate transgenic/mdx F1 males. These males were mated with mdx females, and the transgenic progeny were used for further analysis (22, 23). The mdx, tg-mdx, and control mice were housed in a barrier facility in the Unit for Laboratory Animal Medicine at the University of Michigan. The mice were provided with free access to standard laboratory chow and water. All experiments on the mice were conducted in accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. 85-23 (NIH), Revised 1985, Office of Science and Health Reports, Bethesda, MD 20892).

Muscle fiber preparation. The methods used for the preparation of chemically skinned muscle fibers, attachment of the fibers to the force-recording equipment, and activation of the fiber segments as well as the procedures used to induce injury to contracting muscle fibers were described in detail previously (14). Experiments were performed on single permeabilized muscle fibers obtained from EDL muscles of young (6 mo old) male mdx, tg-mdx, and control (C57BL/10ScSn) mice that were euthanized by cervical dislocation. The EDL muscles were excised tendon-to-tendon from one hindlimb and blotted on filter paper. The muscles were tied with silk suture to capillary tubes (at approximately resting length) and placed in a chemical skinning solution (14) and stored at −20°C for up to 3 mo until required. The attachment of isolated single fiber segments to the force-recording equipment, including issues of fiber end compliance, were described in detail previously (14). Single fiber segments were attached between a sensitive force transducer (model 403; Aurora Scientific, Aurora, Ontario, Canada) and a servomotor (model 300; Aurora Scientific). Sarcomere length was estimated from measurements of 100 sarcomeres from Polaroid photomicrographs (×400), and fiber segment length (L0) was determined by using an ocular reticule. Fibers were set to a sarcomere length of −2.7 µm, which is within the optimal range for maximum force production of mammalian skinned muscle fibers. Fiber width was measured by using a stereomicroscope (Wild MZ2, Wild Heerbrugg) coupled with a high-power objective and camera system (models MPS 51S and MPS45; Wild Heerbrugg), and fiber cross-sectional area (CSA) was estimated from a previously established relationship between fiber width and CSA (14). All displacements of the servomotor lever arm and force sampling of single fibers were controlled by a microcomputer running ASYST software (Macmillan Software, New York, NY). Experiments were performed at 15°C.

Contractile activation and pliometric contraction protocol. Fibers were activated maximally by CaCl2 (pCa 4.5). To maintain structural stability, we cycled fibers between an isometric contraction and short periods of isovelocity shortening (2 L0/s), followed by a rapid return to initial L0 (14). For the collection of experimental data, fibers were stretched between cycles. The maximum isometric tetanic force (Pt) was measured at L0 immediately before each stretch. In each experiment, a fiber was maximally activated, and when force had plateaued at Pt, the fiber was subjected to a single stretch of 10%, 20%, or 30% strain (%L0) imposed at a velocity of 0.5 L0/s. The magnitude of injury was assessed immediately after the stretch by the force deficit, defined as the difference between Pt after stretch and the initial Pt before the stretch expressed as a percentage of the initial Pt. During a stretch, the highest force developed during the period of stretch was defined as the peak force (mN). The average force (mN) developed during a stretch was calculated from the area under the force curve during the period of the stretch divided by the elapsed time (17). The values for Pt, average force, and peak force were normalized (kN/m2) to the estimated CSA of each fiber (14). After each pliometric contraction, the work done to stretch a fiber was calculated from the product of the average force developed during the stretch and the displacement. Fiber mass was calculated from the product of L0 and the CSA of the segment, assuming a density of 1 mg/mm3. Values for the work done (J/kg) and the power absorbed (W/kg) during the stretch were normalized to the mass of the fiber (kg).

Statistical analysis. Data are presented in the tables as means ± SE unless otherwise indicated. Differences in the contractile parameters of single fiber segments from the EDL muscles among the three groups of mice were assessed with the use of a General Linear Model and analysis of variance (ANOVA) with animal strain and stretch magnitude as variables. If a significant F ratio was detected, a Fisher's least significant difference test was performed. The contractile variables assessed by the ANOVA were peak force during stretch, the ratio of peak force to the prestretch isometric force, average force during stretch, work imposed on the fiber during stretch, and power absorbed by the fiber during...
stretch. Each contractile variable was compared separately for each of the three magnitudes of strain. Differences were considered significant if $P < 0.05$.

**RESULTS**

The CSA and specific $P_o$ of the single permeabilized fibers from the EDL muscles of mdx, tg-mdx, and control mice did not differ, and the values were similar to those of previous reports (2, 14, 16, 17).

Contractile characteristics of single fibers during stretch. For maximally activated single permeabilized fiber segments, each stretched through one of the strains tested (10%, 20%, and 30% strain), no differences were observed among the three groups in the magnitude of the peak force attained during stretch, the ratio of the peak force during the stretch to the $P_o$ before the stretch, the specific average force during stretch, the work required to stretch fibers, or the power absorbed by the fibers during stretch (Table 1).

Force deficit in single fibers following a single pliometric contraction. For the single permeabilized fiber segments obtained from EDL muscles from each of the three groups of mice, the force deficits increased linearly as a function of the magnitude of the strain imposed (Fig. 1). At any given magnitude of strain, the force deficit of maximally activated single fiber segments from EDL muscles of control, mdx, or tg-mdx mice did not differ.

**DISCUSSION**

Whether whole muscles are studied in vitro (21, 26), in situ (1, 5), or in vivo (3), the overwhelming evidence indicates that whole skeletal muscles of mdx mice show a greater susceptibility to contraction-induced injury than muscles of control mice. The most compelling data indicate a 20% greater force deficit for EDL muscles of mdx mice compared with those of control mice with both studied in situ (1). The force deficits measured 1 h after 10 stretches of 20% strain relative to $L_f$ were performed with one contraction every 10 s with activation to 90% of $P_o$. In stark contrast to the greater force deficit for intact muscles of mdx, compared with control mice exposed to pliometric contractions in situ, is the lack of a difference among the force deficits for maximally activated single permeabilized fibers from EDL muscles of mdx, tg-mdx, and control mice stretched through a single strain in vitro. The use of single permeabilized fibers to study contraction-induced injury assumes that the permeabilization process eliminates the permeability barrier and disrupts the integrity of the basal lamina and the sarcolemma (2, 7). The observation that there is no difference in the force deficits of single permeabilized fibers from EDL muscles of control, mdx, and tg-mdx mice after a single pliometric contraction of 10%, 20%, or 30% strain. $L_f$, fiber length. No significant differences were observed in the force deficits among the groups of mice for any strain tested. For each of the 3 groups of mice, the magnitude of the force deficit was directly proportional to the magnitude of the imposed stretch. Table 1 lists the number of fibers tested from each group.

**Table 1. Comparison of some contractile parameters of single permeabilized fibers from EDL muscles from control, mdx, and tg-mdx mice during a single pliometric contraction at three different strains**

<table>
<thead>
<tr>
<th>Strain, %$L_f$</th>
<th>Group</th>
<th>n</th>
<th>CSA, $\mu$m$^2$</th>
<th>$P_o$, kN/m$^2$</th>
<th>$P_{pk}$, kN/m$^2$</th>
<th>Ratio of Peak Force During Stretch to Force Before Stretch</th>
<th>Average $P_o$, kN/m$^2$</th>
<th>Work, J/kg</th>
<th>Power, W/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Control</td>
<td>5</td>
<td>2,326 ± 217</td>
<td>102 ± 9</td>
<td>270 ± 17</td>
<td>2.86 ± 0.07</td>
<td>127 ± 10</td>
<td>12.7 ± 1.0</td>
<td>63.5 ± 5.1</td>
</tr>
<tr>
<td>10</td>
<td>mdx</td>
<td>5</td>
<td>2,890 ± 482</td>
<td>107 ± 7</td>
<td>319 ± 41</td>
<td>2.93 ± 0.12</td>
<td>158 ± 21</td>
<td>15.7 ± 2.3</td>
<td>78.8 ± 10.8</td>
</tr>
<tr>
<td>10</td>
<td>tg-mdx</td>
<td>8</td>
<td>2,874 ± 234</td>
<td>119 ± 10</td>
<td>337 ± 21</td>
<td>2.89 ± 0.11</td>
<td>167 ± 11</td>
<td>16.7 ± 1.1</td>
<td>83.5 ± 5.3</td>
</tr>
<tr>
<td>20</td>
<td>Control</td>
<td>5</td>
<td>2,963 ± 250</td>
<td>112 ± 7</td>
<td>319 ± 24</td>
<td>2.89 ± 0.09</td>
<td>152 ± 11</td>
<td>30.3 ± 2.2</td>
<td>75.9 ± 5.5</td>
</tr>
<tr>
<td>20</td>
<td>mdx</td>
<td>3</td>
<td>3,768 ± 291</td>
<td>134 ± 9</td>
<td>410 ± 25</td>
<td>3.19 ± 0.07</td>
<td>187 ± 13</td>
<td>37.4 ± 2.6</td>
<td>93.6 ± 6.4</td>
</tr>
<tr>
<td>20</td>
<td>tg-mdx</td>
<td>5</td>
<td>3,855 ± 257</td>
<td>129 ± 11</td>
<td>361 ± 23</td>
<td>2.89 ± 0.07</td>
<td>171 ± 12</td>
<td>34.1 ± 2.4</td>
<td>85.3 ± 6.1</td>
</tr>
<tr>
<td>30</td>
<td>Control</td>
<td>3</td>
<td>3,812 ± 285</td>
<td>110 ± 14</td>
<td>357 ± 35</td>
<td>3.34 ± 0.17</td>
<td>168 ± 17</td>
<td>42.4 ± 6.9</td>
<td>83.7 ± 8.6</td>
</tr>
<tr>
<td>30</td>
<td>mdx</td>
<td>5</td>
<td>2,820 ± 209</td>
<td>141 ± 20</td>
<td>477 ± 66</td>
<td>3.49 ± 0.29</td>
<td>227 ± 35</td>
<td>68.1 ± 10.3</td>
<td>113.4 ± 17.2</td>
</tr>
<tr>
<td>30</td>
<td>tg-mdx</td>
<td>5</td>
<td>2,841 ± 247</td>
<td>140 ± 12</td>
<td>401 ± 34</td>
<td>2.91 ± 0.18</td>
<td>194 ± 15</td>
<td>58.0 ± 4.5</td>
<td>93.4 ± 7.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$ = no. of fibers. EDL, extensor digitorum longus; tg-mdx, transgenic mdx; strain, magnitude of stretch imposed on fiber [%fiber segment length (%$L_f$)]; CSA, fiber cross-sectional area; $P_o$, specific force; $P_{pk}$, maximum (peak) force attained during stretch; average $P_o$, average force during stretch; work, normalized work imposed on fiber during stretch; power, normalized power absorbed by fiber during stretch. Fibers were stretched at a velocity of 0.5 $L_f$/s. No differences were observed for any variables.
fibers from muscles of *mdx*, *tg-mdx*, and control mice provides indirect evidence that the process of permeabilization eliminates specifically any protection conferred by dystrophin and the DAG complex on intact control fibers exposed to pliometric contractions. Our working hypothesis is that the protection conferred on skeletal muscle fibers by dystrophin and the DAG complex is a stabilization in the alignment of sarcomeres through the lateral transmission of force from the myofilaments to the laminin 2 and, eventually, collagen IV in the ECM. For control mice, a single strain of 20% *L* produced a force deficit for whole muscles that was indistinguishable from zero and a force deficit for single permeabilized fibers of 20% (2). The 20% greater force deficit for single permeabilized fibers than for whole muscles indicates the significance of the overall protection from injury afforded the myofibrils by the linkages among the myofibers, the sarcolemma, and the ECM (9, 11, 19, 28). Similarly, the force deficit for EDL muscles of *mdx* mice was 20% greater than that for muscles of control mice after ten 20% strains, although the values for force deficits were 70% and 50%, respectively (1). The equivalency of the 20% difference in the force deficit between permeabilized and intact fibers and between whole muscles from *mdx* and control mice, even though obtained under different circumstances, supports the often cited premise that the dystrophin and DAG complex are major factors in the stabilization of the membrane (11, 20), the lateral transmission of force (28), and the alignment of sarcomeres, particularly during stretches of activated muscles (1, 2, 16).

Single strains of single permeabilized fibers have been used successfully to identify differences in the magnitude of the force deficit of fast compared with slow fibers (17) and fibers from muscles of old compared with young rats (2). In each case, fast compared with slow fibers and fibers from muscles of old compared with young rats showed an ~100% greater force deficit for a given strain. The identification of a difference in the force deficit for single permeabilized fibers for different fiber types and rats of different ages supports our premise that when differences are observed for whole muscles, at least a portion of the difference in force deficit is at the level of the myofibrils (2, 17). In the present study, the support of the null hypothesis that the force deficits, or any other aspect of force development during isometric or pliometric contractions, did not differ among the three groups, indicates a normal functioning of the myofibrils in fibers of muscles from *mdx* mice. Whether this observation indicates that the dystrophin within myofilaments, particularly within the sarcomere at regions overlaying the I band and the M lines (4), is of no functional significance or that the permeabilization process removes the dystrophin from these sites in *tg-mdx* and control mice cannot be resolved in the present experiments.

The structural models that display dystrophin as a link between the cytoskeleton and the laminin 2 in the ECM (9, 11) have led to the hypothesis that dystrophin maintains the integrity of the sarcolemma, both in quiescent fibers (10, 27) and during muscle contractions, particularly when maximally activated fibers are stretched (1, 3, 5, 26). This hypothesis has been supported by reports of differences between fibers in muscles of *mdx* compared with control mice in the release of enzymes from fibers (9), the stiffness of sarcolemma (20), response to osmotic shock (18), and the magnitude of the damage after pliometric contraction protocols (1, 3, 5, 26). The results of the present study on single permeabilized fibers together with the studies of membrane-intact fibers in whole muscles of *mdx*, *tg-mdx*, and control mice (1, 3, 5, 26) indicate that dystrophic symptoms do not arise from factors within the myofilibrillar structure of fibers but, rather, through a disruption of sarcolemmal integrity that normally confers significant protection from pliometric contraction-induced injury.

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