Fast-twitch skeletal muscles of dystrophic mouse pups are resistant to injury from acute mechanical stress

ROBERT W. GRANGE, THOMAS G. GAINER, KRISTA M. MARSCHNER, ROBERT J. TALMADGE, AND JAMES T. STULL

1Department of Human Nutrition, Foods and Exercise, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0430; 2Department of Physiology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9040

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Fast-twitch skeletal muscles of dystrophic mouse pups are resistant to injury from acute mechanical stress. Am J Physiol Cell Physiol 283: C1090–C1101, 2002. First published May 29 2002; 10.1152/ajpcell.00450.2001.—Loss of the dystrophin-glycoprotein complex from muscle sarcolemma in Duchenne’s muscular dystrophy (DMD) renders the membrane susceptible to mechanical injury, leaky to Ca\(^{2+}\), and disrupts signaling, but the precise mechanism(s) leading to the onset of DMD remain unclear. To assess the role of mechanical injury in the onset of DMD, extensor digitorum longus (EDL) muscles from C57 (control), mdx, and mdx:utrophin-deficient [mdx:utrn(−/−)]; dystrophic] pups aged 9–12 days were subjected to an acute stretch-injury or no-stretch protocol in vitro. Before the stretches, isometric stress was attenuated for mdx:utrn(−/−) compared with control muscles at all stimulation frequencies (\(P < 0.05\)). During the stretches, EDL muscles for each genotype demonstrated similar mean stiffness values. After the stretches, isometric stress during a tetanus was decreased significantly for both mdx and mdx:utrn(−/−) muscles compared with control muscles (\(P < 0.05\)). Membrane injury assessed by uptake of procion orange dye was greater for dystrophic compared with control EDL (\(P < 0.05\)), but, within each genotype, the percentage of total cells taking up dye was not different for the no-stretch vs. stretch condition. These data suggest that the sarcolemma of maturing dystrophic EDL muscles are resistant to acute mechanical injury.

Duchenne’s Muscular Dystrophy; mice; membrane damage; procion orange dye; dystrophin; sarcolemma

DUCHENNE’S MUSCULAR DYSTROPHY (DMD) is an X-linked lethal muscle-wasting disease caused by deletions that disrupt the open reading frame of the gene that codes for dystrophin, an integral membrane protein (4, 19). Dystrophin is normally expressed in skeletal, smooth, and cardiac muscle and in brain (19), but its absence in DMD is particularly conspicuous in skeletal and cardiac muscle. DMD is clinically apparent between ages 2 and 3 yr and demonstrates relentless progression that yields muscle weakness and wasting, contractures of the ankles and hips, scoliosis, and premature death, usually from respiratory or cardiac deficiency by age 20 yr (19). Although the genetic reason for this severe disease is known, the mechanism(s) by which the progressive muscle wasting is initiated are not yet clearly defined. One proposed possibility is that the absence of dystrophin leads to a more injury-susceptible sarcolemma (23, 24, 32).

The muscle isoform of dystrophin (427 kDa; see Ref. 4) is localized to the sarcolemma as an integral protein in the dystrophin-glycoprotein complex (DGC; see Ref. 33). The DGC along with additional proteins forms rib-like lattices on the cytoplasmic face of the sarcolemma known as costameres that help stabilize the cytoskeleton to the extracellular matrix (ECM; see Refs. 4 and 29). Costameres act as mechanical couplers to distribute contractile forces generated in the sarcomere laterally through the sarcolemma to the basal lamina (8) and thereby facilitate uniform sarcomere length between fibers of active and nonactive motor units (29). Dystrophin is also found in abundance in the myotendinous junction and is thought to facilitate the transmission of forces from the muscle fibers to the tendon (36). Thus dystrophin is considered a key structural element in the muscle fiber.

The absence of expressed dystrophin in DMD can lead to the complete loss of the DGC (22) and a disrupted costameric lattice (38). Loss of the DGC is considered to render the membrane less stiff and more readily damaged by mechanical stress (23). Thus sarcolemmal structural integrity in DMD could be compromised in early development because of the absence of dystrophin and continue to degrade with time (19), thereby representing a major determinant of the onset of the dystrophic process. Fast fibers are considered most susceptible to damage because of the greater forces they generate (21). Increased membrane damage assessed by uptake of a fluorescent dye after stretch-induced injury has been reported for the fast-twitch extensor digitorum longus (EDL) muscles from adult mdx (24) and mdx:utrophin knockout [mdx:utrn(−/−)]; see Ref. 9] mice compared with control mice. Although...
both of these studies demonstrated that the absence of dystrophin yielded a sarcolemma that was more susceptible to injury in older dystrophic mice, the age of the animals precluded drawing conclusions about the role of dystrophin in the onset of muscle injury. Thus, we considered that, if dystrophin results in structurally weak membranes, and this is the primary cause for the onset of DMD, then a stretch-injury protocol in fast-twitch EDL muscles of young dystrophic mice should induce significant membrane damage. Furthermore, we considered that if utrophin compensated for the absence of dystrophin to stabilize muscle membranes of mdx mice (14, 39), then mice lacking both dystrophin and utrophin (11) might demonstrate greater injury.

In this study, we tested the hypothesis that the sarcolemmal membranes of dystrophic EDL muscles obtained from young mice (mdx and mdx:utrn(−/−)) genotypes are more susceptible to injury after a stretch-injury protocol compared with 1) unstretched dystrophic EDL muscles and 2) stretched or unstretched control EDL muscles. We demonstrate that EDL muscles from both dystrophic models exhibit only modestly greater membrane damage compared with control muscles. We also report that the stretch-injury protocol did not induce greater membrane damage in either the control or dystrophic EDL muscles of young pups aged 9–12 days.

METHODS

Our objective in this report was to determine if the sarcolemma of EDL muscles from young dystrophic mouse pups were susceptible to damage after a stretch-injury protocol and thereby could contribute to the onset of DMD. EDL muscles obtained from control, mdx, and mdx:utrn(−/−) pups aged 9–12 days were subjected to a stretch-injury protocol and muscle membrane damage assessed with the fluorescent dye propidium. In addition, the presence of centralized nuclei to indicate the presence of regenerating fibers and the distribution of myosin heavy chain (MyHC) isoforms in the EDL muscles for each of the genotypes was also determined. The Virginia Tech Animal Care Committee approved all procedures used in this study.

Mouse genotypes. Mice were obtained from our colony at Virginia Tech. Breeder pairs of control C57BL/6 and mdx mice were originally obtained from Jackson Laboratories, whereas those for the mdx:utrn(−/−) mice were a kind gift from Drs. Mark Grady and Joshua Sanes (Washington University, St. Louis, MO). Because the mdx:utrn(−/−) mice are not fertile (11), mdx:utrn(+/−) male and female mice were crossed to generate mice deficient in both dystrophin and utrophin. Mice genotyping was determined by PCR analysis of DNA obtained from tail snips.

DNA isolation. Isolation of DNA followed the methods of Laird et al. (17). Briefly, mouse tail snips (~0.5 cm) were digested overnight at 55° C in 1 ml lysis buffer [5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 mM Tris (pH 8.5), and 0.2 mg/ml protease K]. Samples were then spun at 13,000 revolutions/min for 10 min, the supernatant of each sample transferred to a clean tube, and the DNA was precipitated by adding an equal volume of 100% ice-cold isopropanol. The strands of precipitated genomic DNA for each sample were transferred to a clean tube and resuspended in 10 mM Tris-EDTA (pH 7.4). Samples were stored at −20°C until subsequent PCR analysis.

PCR analysis. All PCR reagents, including primers, were obtained from GIBCO-BRL. Specific forward and reverse primers were used to screen the DNA of each sample for the dystrophin mutation (i.e., mdx; see Ref. 1) and for the utrophin knockout (Dr. Mark Grady, personal communication). The mdx and wild-type genotypes for dystrophin were screened in separate tubes with the following reverse primers: for mdx (2595E), 5′-GTACTCATAGATTGTTAAGCCATT-TAA-3′; and, for wild-type dystrophin (260E), 5′-GTACTCATAGATTGTTAAGCCATT-TA-3′. Both of these reactions used the same forward primer (9427F) 5′-AACCTCAAAAT-ATGCTGTGTTTAGT-3′ (1). The screen for the utrophin genotype of each sample was performed in a single tube using the following primers: forward primer for utrophin knockout (22803F), 5′-TGCACAGTCTAATTCCCATCGAAGCTG-3′; forward primer for wild-type utrophin (553F), 5′-TGCAGGTGT-CTCCTAATTGCAGTAC-3′; and, for wild-type dystrophin (260F), 5′-CTGAGTCACACGCTTGAAAGCCTC-3′ (Dr. Mark Grady, personal communication). PCR reactions were performed on 0.5 μl DNA in a final volume of 25 μl, with final concentrations of 1.5 mM MgCl2, 0.4 mM dNTPs, and 0.025 U/μl platinum Taq in 1× PCR buffer (GIBCO-BRL). The final concentrations of the primers were 200 nM. PCR reactions were run on a PTC-150 Minicycler with Hot Bonnet (MJ Research) under the following running conditions for the utrophin screen: 1) 5 min at 94°C for initial denaturation of double-stranded DNA, 2) 35 cycles with three steps per cycle, each for 25 s (50°C, ligation; 72°C, extension; and 94°C, denaturation), and 3) 5 min at 72°C for final extension. The mdx screen used similar conditions except the ligation temperature was 48°C. The PCR products were run on a 3% agarose gel (GIBCO-BRL) containing ethidium bromide (0.001 mg/ml) in 0.5× Tris-borate-EDTA running buffer (Fisher) at 100 volts for 100 min. Images of the PCR product bands in the gels were visualized under ultraviolet light (Alpha Innotech Imager) and captured using Alpha Imager 2000 software. PCR products for both wild-type and mutated dystrophin yielded a band size of 105 bases; thus, interpretation of the genotype was based on the reverse primer used in the reaction. The presence of a band from both screens indicated a heterozygote (Fig. 1 and Ref. 1). For the utrophin screen, a band of 640 bases indicated wild-type utrophin, and a band of 450 bases indicated the utrophin knockout; the presence of both bands in the same screen indicated a utrophin heterozygote (Dr. Mark Grady, personal communication and Fig. 1).

Muscle preparation. Both EDL muscles were carefully dissected from anesthetized control (C57Bl/6; n = 6), mdx (n = 10), and mdx:utrn(−/−) (n = 5) pups aged 9–12 days (2 mg xylazine-20 mg ketamine/100 g body mass ip). Dissection was performed while viewing the hindlimbs of the pups through a Reichert Stereo Starzoom stereoscope. EDL muscles were similarly obtained from additional control, mdx, and mdx: utrn(−/−) pups aged 9–14 and 20 days for additional experiments as noted below.

All muscles used in the stretch-injury protocol were incubated in an oxygenated (95% O2-5% CO2) physiological salt solution (PSS; pH 7.6) containing (in mM) 120.5 NaCl, 4.8 KCl, 1.2 MgSO4, 20.4 NaHCO3, 1.6 CaCl2, 1.2 NaH2PO4, 1.0 dextrose, and 1.0 pyruvate and were maintained at 30°C. A 6–0 suture was used to secure the muscle from its distal tendon at the myotendinous junction to the arm of a dual-mode servomotor system (Aurora Scientific). A short piece of 6–0 suture was also tied to the proximal tendon at the myotendinous junction to increase grip when the tendon and
suture were fixed in a clamp at the base of the bath. Muscles were stimulated by square pulses of 0.2-ms duration at a voltage and muscle length ($L_o$) to elicit maximal isometric twitch force. The output stimulus was derived from a Voltage Pulse Generator (Directed Energy) triggered at the appropriate frequency and for the correct duration by specialized software (Aurora Scientific). The stimulus was delivered via two platinum-wire electrodes that extended the length of and ~0.5 cm from either side of the muscle. Based on preliminary studies, the maximal isometric twitch response was elicited in EDL muscles of the pups aged 9–12 days with a resting tension of 0.5 g and voltage set at 20 volts.

**Experimental procedures.** One muscle of each pair was maintained at a resting tension of 0.5 g without any electrical stimulation or stretches (“unstretched” muscle) for the same duration required to conduct the contractile measures in the other muscle (“stretched” muscle), including a tension-frequency protocol, five stretches, and the poststretch and tetanic responses obtained at 15 and 30 min after the last stretch. Resting tension for both muscles was continuously monitored and adjusted as necessary to 0.5 g throughout the experiment. In the stretched condition, after the muscle was mounted to the servomotor arm, it was left quiescent for 10 min and then was subjected to a series of three isometric twitches and tetani (150 Hz) spaced 1 min apart. These were elicited to establish consistent contractile responses before any additional experimental procedures were performed. After an additional 5-min quiescent period, the stretched muscle was subjected to a tension-frequency protocol at electrical stimulation frequencies of 1, 30, 50, 80, 100, and 150 Hz, each for a duration of 900 ms and spaced 1 min apart. After the tension-frequency protocol, and an additional 10 min of rest, muscle length at $L_o$ for both muscles was determined to the nearest 0.1 mm using calipers (Sigma), and then muscles were subjected to the stretch protocol.

**Stretch-injury protocol.** The stretch-injury protocol described by Petrof et al. (24) was used. The muscle was stimulated at 80 Hz for 700 ms. During the first 500 ms, contraction was isometric at $L_o$, whereas during the final 200 ms the muscle was stretched at 0.5 $L_o$/s to yield a total displacement of 0.1 $L_o$ (e.g., an eccentric contraction). This stretch was repeated five times, once every 4 min. At 15 and 30 min after the final stretch, isometric twitch and tetanic (150 Hz) responses from the stretch muscle were elicited. After the final tetanic contraction, the muscles were incubated in a 0.2% procion orange/PSS solution (Reactive Orange 14, mol wt 631; lot number 46H0459; Sigma-Aldrich) for 60 min to assess membrane damage. Muscles were then washed two times, each time for 5 min in fresh PSS and lightly blotted; tendons were dissected free, and the mass to the nearest 0.1 mg was determined on an A-200D electronic analytical balance (Denver Instrument). Muscles were then mounted for sectioning as described below. Note that muscles were incubated in the dye after all contractile measures were obtained. This was done because, in preliminary studies, both control and dystrophic EDL muscles obtained from young mice incubated in the dye demonstrated attenuated force responses (data not shown). We found that the dye batch was critical, because force responses in the presence or absence of the procion orange dye originally used by Petrof et al. (24; Sigma-Aldrich Lot no. 89F0671, a kind gift from Lee Sweeney, University of Pennsylvania) were not different. However, the supply of this dye was limited.

**Assessment of membrane damage from handling dystrophic EDL muscles.** The potential effect on membrane damage resulting from handling between excision of the muscle and mounting it to the servomotor followed by isometric stimulation was assessed in paired EDL muscles excised from anesthetized mdx pups aged 11 days ($n = 3$ pairs; 30°C). In addition, EDL muscles obtained from mdx;utrn–/– pups aged 20 days ($n = 3$ pairs; 30°C) were also assessed. This age was selected with the idea that the dystrophic process was in progress, and therefore membrane damage arising from handling the muscles might be more evident. After the muscles were excised from each animal, one muscle of each pair floated free in a beaker of 0.2% procion orange-PSS (“not mounted” condition), whereas the other muscle was mounted in the contractile apparatus at a resting tension of 0.5–1.0 g (“mounted” condition), and then, after determination of $L_o$, was subjected to a tension-frequency but not a stretch protocol. This muscle was then immersed in 0.2% procion orange-PSS. Both muscles were incubated in the dye for 30–60 min and then washed two times with PSS and mounted for sectioning as described below.

**Muscle sectioning, fiber uptake of procion orange dye, fiber morphology, and fiber typing.** Muscles incubated in the procion orange dye from the stretch or no-stretch conditions were mounted on cork in a mixture of gum tragacanth (Sigma-Aldrich) and Histoprep (Fisher) and frozen in isopentane at −80°C until further analysis. Cross sections were obtained from the midbelly of each muscle sample on a cryostat (Microm HM 505 N) at −21°C and transferred to glass slides.

**Procion orange uptake.** EDL muscle sections (10 μm) were viewed under a fluorescent microscope (Nikon Eclipse E400) at ×25 magnification through a Nikon B-2A filter, with the excitation wavelength range set at 450–490 nm and the emission range set at 505–520 nm. Color images were captured on 800 Speed film (Fuji) with a Nikon 35-mm camera (Nikon N6006) fitted to the microscope. To count damaged cells, a plastic sheet was placed over the photographic image, and a dot was marked over each fluorescent cell. Any fluorescent cells noted within a two-cell depth from the border of...
the muscle and extending radially in the cross section were not included in the overall damaged cell count, since these could have resulted from dissection. When all damaged cells were counted, the total number of cells in the cross section was determined in a similar manner. The extent of damage was determined from the ratio of cells taking up dye to the total number of cells in the section and was expressed as a percentage.

Hematoxylin and eosin staining. Muscle cross sections (12 μm) were fixed for 10 min in 10% formalin in PBS and then stained with hematoxylin and eosin (H&E) as described previously (15). Sections were viewed with a Nikon Eclipse E400 microscope, and a single region (entire field of view with a ×20 objective lens) from the middle portion of each muscle was randomly selected and digitized using an image analysis system (Scion) driven by NIH Image analysis software. All of the fibers within the digitized region were counted, and the proportion of fibers with clearly discernible central nuclei (i.e., cytoplasm completely surrounding the nucleus) was determined for each muscle. The average number of fibers analyzed per muscle was 143.

MyHC isoforms. The proportional content of MyHC isoforms was determined using a modified method of Talmadge and Roy (34). Briefly, frozen EDL muscles obtained from control, mdx, and mdx:utrn(--/--) pups aged 9–14 days were thawed on ice in microfuge tubes, homogenized in 200 μl sample buffer (16) using a micropestle, and heated to 60°C for 10 min. Each sample (40 μl) was subjected to SDS-PAGE using 8% separating and 4% stacking gels (34). Samples were run on a 20-cm vertical slab gel unit (CBS Scientific) for 40 h at a constant current of 4.2 mA. Gels were stained with Coomassie blue, destained, and scanned using an image analysis system (Alpha Imager 2000). Band densities for each of six MyHC isoforms were expressed as a percentage of the total MyHC band density.

Contractile data. Contractile data were obtained with specialized software (Aurora Scientific) that controlled the isometric and isotonic modes of the servo arms as well as the timing of pulses that triggered the voltage pulse generator to electrically stimulate the muscles. Contractile responses were collected at a sampling frequency of 1,000 Hz and were stored to disk for subsequent analysis to determine contractile properties using specialized analysis software (Aurora Scientific). Force responses were digitally filtered with a dual-pass Butterworth filter with a cut-off frequency of 60 Hz. The cross-sectional area for each muscle was determined by dividing the mass of the muscle (g) by the product of its length (L, cm) and the density of muscle (1.06 g/cm³; see Ref. 20) and was expressed as square millimeters. Muscle output was expressed as stress (g/mm²) determined by dividing the tension (g) by the muscle cross-sectional area. Twitch contractile parameters [e.g., peak stress, maximum rates of stress development and relaxation, time-to-peak stress (TPS), and half-relaxation time (HRT)], the stress-frequency relation, and the isometric and dynamic stress values resulting from the stretch protocol were also analyzed. An index of stiffness during each eccentric contraction was determined by dividing the difference in stress produced between the 80-Hz isometric response and the peak stress resulting from the imposed stretch (i.e., change in stress) by the change in length of the muscle during the stretch (0.1 L0).

Statistical analysis. The differences in contractile properties between the genotypes and during the series of stretches within each genotype were determined by a two-way ANOVA. Contractile properties before and after the stretch protocol were determined similarly. Differences in stress generation at a given time of contraction and dye uptake between the mouse genotypes were analyzed by a one-way ANOVA. The Newman-Keuls multiple range post hoc test was applied to all significant main effects to determine differences between means. Differences were considered significant at P < 0.05. Data are presented as means ± SE.

RESULTS

Genotyping. Specific primers were used to assay DNA isolated from mouse tail snips of the mice using PCR to discriminate the mdx and mdx:utrn(--/-) genotypes. When the PCR products were run on a 3% agarose gel, the various homozygote and heterozygote genotypes could be readily determined based on the size of the bands, as described in the legend for Fig. 1.

Morphological data. The morphological data are reported in Table 1. The mean ages for the three genotypes were similar as follows: control, 11.0 ± 0.4 days (n = 6), mdx 10.9 ± 0.3 days (n = 10), and mdx:utrn(--/-) 11.4 ± 1.0 days (n = 5). The mdx pups (8.32 ± 0.02 g; P < 0.05) had a mean body mass significantly greater than either the control (6.78 ± 0.34 g) or the mdx:utrn(--/-) (7.14 ± 0.60 g) pups. EDL muscle length was greater for mdx (8.32 ± 0.13 mm; P < 0.05) compared with control (7.31 ± 0.11 mm) but was not different from the values for the mdx:utrn(--/-) (7.90 ± 0.54 mm) pups. The EDL masses were similar between the control (1.1 ± 0.1 mg) and mdx:utrn(--/-) (1.0 ± 0.1 mg) pups but were significantly less than the values for the mdx group (1.7 ± 0.3 mg; P < 0.05). Both the control (0.14 ± 0.02 mm²) and mdx:utrn(--/-) muscle cross-sectional areas (0.13 ± 0.02 mm²) were similar, but both were significantly less than the mdx values (0.20 ± 0.02 mm²; P < 0.05). Within each genotype, there were no differences in the number of fibers per cross section for those EDL muscles in the stretch vs. no-stretch condition; therefore, these were combined by genotype. There were a fewer total number of fibers per muscle cross section in the mdx:utrn(--/-) (666 ± 30 fibers) and mdx (769 ± 42 fibers) compared with control (932 ± 43 fibers; P < 0.05) EDL muscles. There were no significant differ-

Table 1. Morphological data for control, mdx, and mdx:utrophin(--/-) pups

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Age, days</th>
<th>Body Mass, g</th>
<th>EDL Length, mm</th>
<th>EDL Mass, mg</th>
<th>EDL XSA, mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>11.0 ± 0.4</td>
<td>6.78 ± 0.34</td>
<td>7.31 ± 0.11</td>
<td>1.1 ± 0.1</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>mdx</td>
<td>10</td>
<td>10.9 ± 0.3</td>
<td>8.32 ± 0.02†</td>
<td>8.13 ± 0.13³</td>
<td>1.7 ± 0.3†</td>
<td>0.20 ± 0.02†</td>
</tr>
<tr>
<td>mdx:utrophin</td>
<td>--</td>
<td>11.4 ± 1.0</td>
<td>7.14 ± 0.60</td>
<td>7.90 ± 0.54</td>
<td>1.0 ± 0.1</td>
<td>0.13 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. EDL, extensor digitorum longus muscle; XSA, cross-sectional area; mdx:utrophin(--/-), mdx:utrophin-deficient. P < 0.05, significantly different from control values (†) and significantly different from control and mdx:utrophin (--/-) values (³).
ences in the percentage of central nucleated fibers (Fig. 2), nor were there significant differences in myosin isoform distribution among the genotypes (Fig. 3).

Stress frequency relation. The isometric stress produced by EDL muscles from the mdx:utrn(−/−) pups was less than the values obtained from the control EDL muscles at each stimulation frequency (P < 0.05; Fig. 4A). The control and mdx stresses were similar at each stimulation frequency except at 50 and 80 Hz (P < 0.05; Fig. 4A).

Isometric stress at 80 Hz during the stretch protocol. EDL muscles obtained from pups of each genotype were subjected to five stretches. This protocol required that one muscle of each pair not be stretched, whereas the second muscle was stimulated while isometric at 80 Hz for 0.5 s before a stretch of 0.1 L0 imposed at 0.5 L0/s (24). This stretch procedure was repeated five times once every 4 min. There were no differences in the absolute isometric stresses at 80 Hz across the five stretches within each genotype (Fig. 4B), but there were differences between the genotypes (P < 0.05), with stress stimulation of the mdx:utrn(−/−) EDL less than that of the control EDL at each of the five stretches. For example, the mean stress at 80 Hz for the first stretch for control EDL muscles was 16.39 ± 2.93 compared with 5.45 ± 1.93 g/mm² for the mdx: utrn(−/−) muscles (P < 0.05). The mdx EDL isometric responses at 80-Hz stimulation differed from those of control EDL only for stretches 1 and 2 (e.g., for stretch 2, control = 14.76 ± 2.49 and mdx = 8.59 ± 1.86 g/mm²; P < 0.05). The mdx:utrn(−/−) values differed from those for the mdx muscles only for stretches 4 and 5 (Fig. 4B; P < 0.05).

Stiffness. A stiffness value was determined for each stretch response by dividing the difference between the plateau of the isometric response at 80 Hz and the peak stress response during the stretch by 0.1 L0, the imposed length change (Fig. 4C). There were no differences in stiffness within each genotype across stretches 1–5 or between the genotypes. The stiffness values (in g·mm⁻²·L₀⁻¹) for the initial and final stretches for the control EDL were 330.49 ± 17.74 and 371.48 ± 22.82 for control EDL, 250.2 ± 28.2 and 268.9 ± 33.4 for mdx

Figure 3. Myosin isoform distribution in control, mdx, and mdx: utrn(−/−) EDL muscles from pups aged 9–14 days. A: representative gel indicating the bands associated with each isoform for an EDL muscle obtained from each of the genotypes. Lane 1, control; lane 2, mdx; lane 3, mdx: utrn(−/−). B: distribution of myosin isoforms in EDL muscles obtained from each genotype expressed as a percentage of the total fibers in a cross section. The myosin isoform distributions were similar across all genotypes (n = 5–6 muscles for each genotype). MyHC, myosin heavy chain; Emb, embryonic; Neo, neonatal.

Figure 2. Representative hematoxylin- and eosin-stained 12-μm cross sections from control (A), mdx (B), and mdx: utrophin deficient [mdx: utrn(−/−); C] extensor digitorum longus (EDL) muscles obtained from pups 9–14 days of age to determine general morphological features and number of centralized nuclei. None of the samples demonstrated overt evidence of degenerating or regenerating fibers. Fiber regeneration was assessed by determining the number of centralized nuclei in a sample of >100 fibers from a randomly selected region of the cross section. The average percentage of centralized nuclei for each genotype was 0.14 ± 0.28 for control (n = 4 muscles; fibers counted = 160 ± 19); 1.4 ± 1.6 (n = 5 muscles; fibers counted = 123 ± 26) for mdx, and 0.85 ± 0.53 (n = 7 muscles; fibers counted = 147 ± 35) for mdx: utrn(−/−). Arrows in B and C indicate cells with centralized nuclei. Bar in A is 100 μm.
EDL, and 312.0 ± 48.7.4 and 396.5 ± 55.5 for mdx: utrn((–/–) (Fig. 4C).

Contractile properties before and after the stretch protocol. Isometric twitches and tetani were collected before (initial) and at 15 and 30 min after the conclusion of the stretch-injury protocol (post) to assess the effects of stretch on the subsequent stress-generating capability at low (i.e., twitch)- and high (i.e., tetanus at 150 Hz)-frequency electrical simulation.

Twitch. For muscles from control animals, mean isometric twitch peak stress was decreased to 50.4 ± 9.7% (P < 0.05) of the initial twitch peak stress (4.04 ± 0.65 g/mm²) at 15 min post but was not different from the initial value at 30 min post (81.7 ± 8.7%; Fig. 5A). TPS was similar for the initial and 15 and 30 min postcontractions, with values of 20.8 ± 0.5, 22.0 ± 0.3, and 22.1 ± 1.2 ms, respectively (Fig. 5B). Compared with the initial mean HRT (16.3 ± 1.7 ms), the mean values were not different at 15 min (18.8 ± 1.9) or 30 min post (16.5 ± 1.4; Fig. 5C). The maximum rate of stress development (+dS/dt) was depressed at 15 min post (in g·mm⁻²·s⁻¹; 182.5 ± 31.3) compared with both the initial (366.7 ± 64.6) and 30 min post (306.8 ± 32.6) values. The initial and 30 min post values were not different. The maximum rate of stress relaxation (−dS/dt) was depressed at 15 min post (−108.2 ± 14.5 g·mm⁻²·s⁻¹) compared with both the initial (−195.3 ± 26.1) and 30 min post (−173.3 ± 17.8; Fig. 5D) values. The initial and 30 min post values were not different.

For muscles from mdx animals, compared with the initial mean isometric twitch peak stress (3.33 ± 0.32 g/mm²) at 15 min post, mean twitch peak stress was only 46.5 ± 8.9% of initial values (P < 0.05) and had only recovered to 72.1 ± 9.6% of initial values at 30 min post (P < 0.05; Fig. 5A). Twitch TPS values were similar for the initial and 15 and 30 min post contractions with values of 17.8 ± 0.7, 15.8 ± 0.8, and 15.8 ± 0.9 ms, respectively (Fig. 5B). HRT at 15 min (in ms; 15.3 ± 1.5) and 30 min (15.7 ± 0.6) post were less than the initial twitch values (20.0 ± 1.0, P < 0.05; Fig. 5C). Maximum rates of stress development and relaxation were similar at the three time points (in g·mm⁻²·s⁻¹; +dS/dt: 348.8 ± 68.5, 194.0 ± 40.0, and 298.1 ± 73.1, respectively; −dS/dt: −152.9 ± 29.0, −95.2 ± 16.0, and −138.2 ± 28.7, respectively; Fig. 5D).

For muscles from mdx: utrn((–/–) animals, the mean isometric twitch peak stress values at 15 and 30 min post were, respectively, 52.9 ± 10.7% (P < 0.05) and 53.4 ± 12.6% (P < 0.05) of the initial mean twitch peak stress value of 1.35 ± 0.53 g/mm² (Fig. 5A). Twitch TPS values were not different at the initial or 15 and 30 min post time points (16.0 ± 2.5, 17.0 ± 2.1, and 17.5 ± 1.6 ms, respectively; Fig. 5B). HRTs were not different at the three time points (18.0 ± 1.4, 11.7 ± 2.7, and 16.3 ± 1.4 ms, respectively; Fig. 5C). Lack of a significant decrease in HRT at 15 min post was likely because two of the five muscles did not respond to a twitch stimulus; one of these muscles did recover and produce stress at 30 min. Maximum rates of stress development and relaxation were similar at the three time points (in g·mm⁻²·s⁻¹; +dS/dt: 137.5 ± 43.6, 103.3 ± 35.0, and 141.2 ± 51.9, respectively; −dS/dt: −78.0 ± 27.1, −79.6 ± 45.3, and −92.6 ± 46.2, respectively; Fig. 5D).

Tetanus. For control EDL muscles, the initial mean peak tetanic stress of 24.61 ± 2.40 g/mm² was decreased to 41.9 ± 6.4% of initial values (P < 0.05) at 15

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min post and recovered to 77.1 ± 2.9% (P < 0.05) at 30 min post (Fig. 6). For the mdx EDL muscles, at 15 min post, the mean peak tetanic stress was only 27.1 ± 2.6% (P < 0.05) of the initial mean value of 17.66 ± 3.05 g/mm² and had recovered only to 72.7 ± 10.0% at 30 min (P < 0.05; Fig. 6). For the mdx:utrn(−/−) EDL muscles, the initial mean peak tetanic stress of 9.41 ± 2.82 g/mm² was decreased to 34.3 ± 8.9% (P < 0.05) of initial values at 15 min post and showed partial recovery to 53.2 ± 15.8% of initial values at 30 min post (P < 0.05; Fig. 6).

Comparisons between the three genotypes. Initial peak isometric twitch stress generation was similar between control and mdx EDL muscles but was significantly less for the mdx:utrn(−/−) EDL muscles (P < 0.05). The initial and 30 min post mdx:utrn(−/−) values for +dS/dt were significantly attenuated compared with corresponding control and mdx values (P < 0.05; Fig. 5D). Only the initial mdx:utrn(−/−) −dS/dt value was different from the respective control value (P < 0.05). TPS for the mdx and mdx:utrn(−/−) muscles was significantly less compared with control at 15 and 30 min post (Fig. 5B; P < 0.05), but HRT was similar between the three genotypes at each time point (Fig. 5C). Comparisons between the contractile and temporal characteristics of the post and initial twitches within each genotype (and across all genotypes) revealed a consistent pattern. In general, at 15 min after the stretch protocol, twitch contractile properties were attenuated compared with those of the initial twitch and then demonstrated partial or full recovery toward initial values at 30 min post. The pattern of tetanic response was similar to that of the twitches, with attenuated stress generation at 15 min post and partial recovery at 30 min post (Fig. 6). The mean peak stress generated during the initial tetanus by the mdx:utrn(−/−) EDL muscles was significantly less than that of control muscles (P < 0.05) but was not different from the mdx initial values. At 15 min post, peak stress generation (in g/mm²) during a tetanus was depressed.
for both the mdx and mdx:utrn(−/−) EDL compared with control EDL values (P < 0.05). At 30 min post, only the mdx:utrn(−/−) peak stress was different from the respective control value (P < 0.05; see legend for Fig. 6).

**Dye uptake as an index of membrane damage.** There was no apparent effect on membrane damage from handling EDL muscles of mdx:utrn(−/−) pups aged 20 days. The percentage of total fibers in a cross section that were dye positive was not different for the not mounted vs. mounted conditions (n = 3; 14.7 ± 3.8 vs. 9.1 ± 4.4%, respectively; data not shown). Dye uptake in EDL muscles obtained from mdx mice aged 11 days were 5.1 ± 0.1 and 4.4 ± 0.6% for the not mounted and mounted conditions, respectively (data not shown). Both the not mounted and mounted mean values for these mdx muscles were less than the mean for the unstretched mdx muscles (9.2 ± 1.0%; P < 0.05) but were not different from the mean for the stretched mdx muscles (8.0 ± 1.0%).

Independent of genotype, membrane damage in muscles subjected to the stretch-injury protocol, as indicated by the number of dye-positive fibers, was not different from the values for muscles in the unstretched condition (Figs. 7 and 8). Respective unstretched and stretched values for EDL muscles of each genotype were, for control, 2.1 ± 0.2 and 2.1 ± 0.3%, for mdx, 9.2 ± 1.0 and 8.0 ± 1.0%, and for mdx:utrn(−/−), 7.2 ± 2.4 and 6.6 ± 2.5% (Fig. 8). The percentage of dye-positive fibers was significantly greater in the mdx and mdx:utrn(−/−) EDL muscles compared with control muscles independent of the unstretched or stretched conditions (P < 0.05; Fig. 8). The percentage of dye-positive fibers in both the stretch and unstretched conditions was not different between the mdx and mdx:utrn(−/−) genotypes (Fig. 8).

**DISCUSSION**

**Major finding.** The major finding in this study was that sarcolemmal membranes of EDL muscles obtained from dystrophic pups aged 9–12 days subjected to an acute stretch-injury protocol did not demonstrate greater damage compared with unstretched contralateral muscles, as assessed by uptake of the fluorescent dye procion orange. We hypothesized that, if the absence of dystrophin (and utrophin) rendered the membrane more susceptible to injury as has been shown for muscles of older dystrophic mice (9, 24), and this was a primary mechanism initiating the onset of DMD, then muscles from dystrophic pups at this age would show significant membrane injury when stretched eccentrically in vitro, but this did not occur. These data suggest that the early onset of the dystrophic process may be independent of mechanical perturbation to the sarcolemma.

**Stress generation is dramatically reduced in mdx: utrn(−/−) EDL muscles.** Stress generation was depressed in mdx:utrn(−/−) compared with control skeletal muscles obtained from older mice (9). In mdx: utrn(−/−) compared with control pups aged 9–12 days, EDL stress generation was also markedly depressed throughout the stress frequency relation. In contrast, mean stresses at most stimulation frequencies were similar between mdx and control EDL muscles. Depressed stress generation in the mdx:utrn(−/−) muscles could have been the result of increased numbers of regenerating fibers. The dystrophic process in skeletal muscles of mdx:utrn(−/−) and mdx mice occurs at age ~2 and ~3–4 wk, respectively (11, 28). Morphological evidence of dystrophy includes the presence of central nuclei indicative of regenerating fibers (11). However, we found that few fibers in EDL obtained from pups of either dystrophic phenotype exhibited central nuclei based on the H&E stain, suggesting that fiber regeneration was minimal at this early age. In the absence of these influences, the severely attenuated stress generation in the mdx:utrn(−/−) EDL even before stretches were imposed suggests a potential intrinsic deterioration in the contractile apparatus. This decrement could be because of alterations in the distribution of myosin isoforms and/or alterations in myosin function.

Decreases in the proportion of type IIX and increases in the type I MyHC isoform have been reported as a mechanism for depressed contractile function in the dystrophic diaphragm of mdx mice aged 3–4 (25) and 6 (6) months. In mice aged 10 wk, a decrease in MyHC 2B and an increase in MyHC 2X were observed in mdx:utrn(−/−) compared with control EDL muscles (9). However, in the present study, the distribution of MyHC isoforms was similar for EDL muscles obtained from pups of each genotype aged 9–14 days. Thus depressed stress generation in the mdx:utrn(−/−) EDL did not appear to be because of shifts in the MyHC content. As reported for EDL muscles of control, mdx, and mdx:utrn(−/−) mice aged 10 wk (9), twitch temporal properties revealed similar TPS and HRTs between the control, mdx, and mdx:utrn(−/−) EDL mus-
cles of pups aged 9–12 days. In the present study, the mdx:utrn(−/−) muscles were generating less peak stress in approximately the same time as control muscles. This slower rise to peak stress was also reflected in the significantly depressed maximum rate of rise of stress. Taken together, these characteristics suggest an alteration in cross bridge function. Coirault et al. (6) concluded that less force per cross bridge was generated in diaphragm obtained from mdx compared with control mice aged 6 mo. This potential intrinsic depression in myosin cross bridge function could account for the severe attenuation of stress from the mdx:utrn(−/−) EDL noted herein.

Isometric stress at 80 Hz was maintained during the stretch protocol. The mean proportion of maximal stress (150 Hz) elicited at 80 Hz during the stretch protocol was similar between the genotypes [control 66.6%, mdx 65.7%, and mdx:utrn(−/−) 68.5%]. Relative stress over the five stretches was unchanged for control and gradually increased in the mdx EDL but was depressed ~37% in the mdx:utrn(−/−) EDL muscles. However, the decrease in the mdx:utrn(−/−) EDL occurred between the second and first stretches and stabilized thereafter. These data contrast with the ~29% (control) and ~57% (mdx) reductions in isometric force output at 80 Hz for diaphragm between the final and initial stretches in mice aged 90–110 days (24) and the 10, 41, and 76% decreases in tetanic force observed for EDL muscles of control, mdx, and mdx: utrn(−/−) EDL muscles, respectively, from mice aged 10 wk subjected to stretches imposed during tetanic contractions (9). In EDL muscles of adult control and mdx mice aged 90–125 days subjected to five stretches, we observed mean decreases from the initial isometric stress at 80 Hz (~80% of maximum tetanic stress) of ~13% (n = 5) and ~22% (n = 4), respectively [data not shown; note: mdx:utrn(−/−) mice this age were not available]. These results indicate that the magnitude

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**Fig. 7.** Representative procion orange-stained EDL muscle cross sections (10 μm) obtained from control (A and B), mdx (C and D), and mdx:utrn(−/−) (E and F) pups aged 9–12 days subjected to a no-stretch or a 5 eccentric contraction stretch protocol. Paired muscles from each pup are compared: A, C, and E, no stretch; B, D, and F, stretch. See METHODS for details.
ever, stiffness in the absence of both dystrophin and utrophin. How-

teenage tension would likely be borne by the sarcolemma that
could result in injury. However, we did not see an increase in fiber membrane damage. Because of the simi-

lar stiffness values among the genotypes, these data suggest that 1) the dystrophic membrane was capable of ac-

ccommodating the imposed stretch and/or 2) additional elements may stabilize the dystro-

phic muscle membrane and fiber-tendon linkages at this early age that may be subsequently lost during maturation because of the relentless progression of the dystrophic process.

Membrane damage of dystrophic muscles was not increased by a stretch-injury protocol. Membrane in-

jury as assessed by uptake of the fluorescent dye pro-

cion orange after either a no-stretch or stretch condi-
tion was 3.5- to 4.5-fold greater in EDL muscles from both dystrophic mouse models (mdx and mdx:utrn(−/−)) compared with control muscles (∼7–9% vs. ∼2%, respectively). Without stretch (e.g., the mounted and unstretched conditions), the percentage of dye-positive fibers in young mdx muscles was ∼4–9% compared with ∼3% for both adult control (n = 3) and mdx (n = 3) muscles (data not shown; see Ref. 24). This small difference suggests that maturing compared with adult mdx muscles may exhibit slightly greater and more variable fiber damage in vivo that reflects the early onset of the dystrophic pathology, and/or they are more sensitive to handling. Given the small size of the maturing dystrophic muscles we assessed, we cannot discount some fiber damage from handling that possibly masked effects of the stretch; yet, this appeared to be

and tendon are compromised in the absence of dystrophin and utrophin alone, then a greater de-

crease in mechanical coupling between sarcomeres and sarco-

tema. However, if it is true that mechanical coupling be-

 tween sarcomeres, sarcolemma, and between fibers and tendon in the absence of
dystrophin (e.g., mdx EDL; see Refs. 36, 29, and 38) and in the absence of dystrophin and utrophin [e.g.,

mdx:utrn(−/−) EDL]. Surprisingly, stiffness was simi-

lar for all genotypes. Stiffness in the mdx muscles may have been similar to control muscles because of com-

pensation by utrophin (14, 39) in providing stability to the

gastrocnemius and costameres. However, if it is true that

threefold less (e.g., ∼7–9 vs. ∼23%). This suggests that the absence of the DGC does not render the membrane acutely susceptible to mechanical shear stress during early maturation (i.e., >90% of the total fibers were undamaged by stretch).

The sarcolemma of a control muscle fiber is convo-

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radially or eccentrically), the folds can expand to accommodate the expansion or elongation (for review, see Ref. 13). In a dystrophic fiber, not only is there an absence of the DGC, there are fewer sarcolemmal folds (13). Thus, during a concentric contraction, the absence of restraining linkages (e.g., via the DGC) could allow the membrane to push out and eventually tear. During an eccentric contraction, a membrane with fewer folds now takes up the imposed stress, and this likely leads to microtears (13). In young pups, possibly in contrast to older mice, there are sufficient sarcolemmal folds to accommodate the imposed stretch despite the absence of the DGC. With maturation and progression of the dystrophic process, these folds may diminish, the membrane may be further weakened by disruption of its lipid bilayer (13), and/or additional connections between the ECM and sarcolemma may be lost that render the membrane more susceptible to damage from shear stresses.

**Nature of the dystrophic process.** The significant depression in stress-generating capability of the mdx: utrn(−/−) EDL independent of membrane damage suggests the presence of an active dystrophic process. This observation could be interpreted in at least three ways. 1) The absence of dystrophin does not initially render the membrane more susceptible to injury because the membrane is elastic enough to tolerate and recover from eccentric contractions; thus, the dystrophic process is dependent on one or more disrupted signaling pathways. Our data indicate that the dystrophic process may have been evident in a small percentage of the total fibers but was not exacerbated by the acute injury protocol. 2) The acute perturbation of the muscle used herein was not enough to exacerbate the structural weakness of the muscle (i.e., the muscle was not stressed enough or long enough). Thus an increase in the percentage of maximal isometric stress before the stretch, the extent of stretch, the number of stretches, or chronic activity may be necessary to induce the dystrophic process. These possibilities, however, must be tempered with the caveat that the imposed challenge(s) on the muscle reasonably reflects those experienced by pups aged 9–12 days in vivo. 3) Alternatively, the onset of dystrophy could become evident several hours or even days later after an acute injury (e.g., muscle injury associated with eccentric work), but this may also favor a signaling hypothesis to account for the onset of DMD (e.g., triggering of inflammatory and immune responses).

Rando (27) recently proposed a “2 Hit” hypothesis to explain the onset of DMD. In this model, a dystrophic muscle may undergo some perturbation to the sarcolemma resulting from mechanical influences (e.g., an eccentric contraction) that is exacerbated by ischemia (e.g., decrease in nitric oxide (NO); Hit 1), as well as additional signal disruption in the DGC (e.g., integrins; Hit 2). The result is the injured fiber or a group of fibers undergo necrosis rather than recovery, as would be expected in a normal fiber similarly perturbed. Thus the initial perturbation is amplified by the absence of one or more signal pathways. Interpreted from a mechanical viewpoint, the results from the EDL muscles of young dystrophic pups subjected to an acute stretch-injury protocol argue against a simple membrane structural deficiency in the absence of dystrophin. Instead, the absence of the DGC may also disrupt the signaling pathways on both the extracellular and/or cytosolic sides of the sarcolemma to account for the onset and/or the progressive nature of DMD.

Integrins are critical signaling proteins composed of α- and β-chains localized to the extracellular side of the sarcolemma (2) that facilitate communication between the ECM and the cytosol (2) and also form a mechanical linkage between the cytoskeleton and the ECM via the costamere (29). Increased expression of the αβ1-integrin rescues the severe dystrophic phenotype of mdx: utrn(−/−) mice, yielding longer-living mice with attenuated kyphosis and limited muscle dystrophy (3). Nevertheless, muscles of the rescued mice still demonstrated cycles of degeneration/regeneration (3), suggesting that the increased content of the αβ1-integrin was not enough to eliminate the dystrophic process entirely, either by stabilizing the membrane and/or by providing some signaling function. Incomplete rescue of the dystrophic phenotype despite overexpression of the αβ1-integrin is also noted in mdx mice and DMD patients (2). This outcome suggests the absence of additional compensatory stabilization and/or signaling mechanisms.

Neuronal nitric oxide synthase (nNOS) is a protein associated with the DGC via interactions with syntrophin (31). In healthy skeletal muscle, NO produced by nNOS could regulate functional hyperemia through an NO- and cGMP-mediated pathway that leads to relaxation of vascular smooth muscle (7, 12, 18) and could act as an anti-inflammatory molecule within skeletal muscle fibers (37). In the absence of dystrophin or α-dystrobrevin, the membrane-associated nNOS is decreased, and there is a decrease in the cGMP produced during muscle contraction (5, 10, 18), implying a decrease in the production of NO within skeletal muscle. Under conditions of decreased nNOS at the sarcolemma, and reduced NO production, vascular regulation is compromised in contracting dystrophic mouse muscle (35). In addition, Sander et al. (30) reported increased muscle ischemia in contracting muscles of young Duchenne’s patients, supporting the idea that, in the absence of nNOS, this NO-mediated signaling pathway may be compromised. Furthermore, overexpression of an nNOS transgene in mdx mice was recently reported to diminish the dystrophic pathology in soleus muscles, likely by providing NO as an anti-inflammatory molecule (37). These findings support our general conclusion that factors other than mechanical perturbation to the dystrophic membrane (e.g., disrupted NO signaling) may contribute to the onset of the dystrophic pathology.

In summary, our data do not discount the importance of the structural role of dystrophin in preventing injury to the sarcolemmal membrane but do suggest that this may not be its only role during early maturation, particularly if dystrophin-deficient membranes at this developmental stage are more compliant and
resilient to mechanical perturbation. Given the limited understanding at present regarding the mechanism(s) responsible for the onset of the dystrophic process, our mechanical data support the idea that signaling mechanisms may be responsible. It is clear that identifying these potential signaling pathways will greatly advance our understanding of the mechanisms responsible for DMD.

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