Acute pathophysiological effects of muscle-expressed Dp71 transgene on normal and dystrophic mouse muscle

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Wieneke, Sascha, Peter Heimann, Sigalit Leibovitz, Uri Nudel, and Harald Jockusch. Acute pathophysiological effects of muscle-expressed Dp71 transgene on normal and dystrophic mouse muscle. J Appl Physiol 95: 1861–1866, 2003; 10.1152/japplphysiol.00326.2003.—The products of the dystrophin gene range from the 427-kDa full-length dystrophin to the 70.8-kDa Dp71. Dp427 is expressed in skeletal muscle, where it links the actin cytoskeleton with the extracellular matrix via a complex of dystrophin-associated proteins (DAPs). Dystrophin deficiency disrupts the DAP complex and causes muscular dystrophy in humans and the mdx mouse. Dp71, the major nonmuscle product, consists of the COOH-terminal part of dystrophin, including the binding site for the DAP complex but lacks binding sites for microfilaments. Dp71 transgene (Dp71tg) expressed in mdx muscle restores the DAP complex but does not prevent muscle degeneration. In wild-type (WT) mouse muscle, Dp71tg causes a mild muscular dystrophy. In this study, we tested, using isolated extensor digitorum longus muscles, whether Dp71tg exerts acute influences on force generation and sarcolemmal stress resistance. In WT muscles, there was no effect on isometric twitch and tetanic force generation, but with a cytomegalovirus promoter-driven transgene, contraction with stretch led to sarcolemmal ruptures and irreversible loss of tension. In MDX muscle, Dp71tg reduced twitch and tetanic tension but did not aggravate sarcolemmal fragility. The adverse effects of Dp71 in muscle are probably due to its competition with dystrophin and utrophin (in MDX muscle) for binding to the DAP complex.

dystrophin; mdx mouse; sarcolemmal ruptures; eccentric contractions; mechanophysiology

THE DYSTROPHIN GENE of humans and mice codes for five transcripts of different lengths that arise from the activity of internal promoters: full-length dystrophin (Dp427) and the shorter polypeptides Dp260, Dp140, Dp116, and Dp71. Dp427 is a submembraneous cytoskeletal protein that consists of four domains with distinct functions: the NH2-terminal binds to F-actin, the central rod domain confers elasticity on the protein, the cysteine-rich domain binds to a complex of dystrophin-associated proteins, and the COOH-terminal binds to cytoplasmatic syntrophin (5, 14). Because one of the dystrophin-associated proteins, α-dystroglycan, binds to laminin, dystrophin links the actin cytoskeleton to the extracellular matrix. Dystrophin deficiency causes muscular dystrophy, which is severely progressive in affected Duchenne patients but relatively benign in the MDX mouse. In MDX muscle, the autosomal homologue of dystrophin, utrophin, is upregulated and partially compensates for the loss of dystrophin (6, 18). The properties of dystrophin-deficient muscle cells suggests that an important function of dystrophin is to protect the sarcolemma against mechanical stress during contraction (13, 15); yet, an additional signaling role is likely (16).

Dp71 consists of the cysteine-rich and COOH-terminal domains of dystrophin and lacks binding sites for the cytoplasmic microfilaments. It is the major nonmuscle product that is absent from skeletal muscle and particularly abundant in the central nervous system. In neurons and glial cells, it occurs in at least two splice isoforms with different subcellular localizations (12). The cellular function of Dp71 remains to be elucidated.

Dp71 transgene (Dp71tg) expressed in MDX muscle restored the dystrophin-associated protein complex but did not prevent muscle degeneration (4, 7). In a previous study (10), a deleterious effect of a muscle-expressed Dp71tg in wild-type mice was documented by degeneration and regeneration and elevated levels of plasma creatine kinase, i.e., long-term and steady-state symptoms of muscular dystrophy.

In this study, we tested, using both wild-type and dystrophin-deficient MDX mice, the hypothesis that the deleterious effect of Dp71tg involves an increased fragility of the sarcolemma due to the competition of Dp71 with endogenous full-length Dp427, in the case of wild-type transgenic animals, and upregulated utrophin (6), in the case of mdx transgenic animals. We measured the generation of isometric (Iso) tension before, during, and after a series of forced lengthening contractions and subsequently tested the same muscles for membrane ruptures by using the influx of an indicator dye. We find influences of Dp71tg both on sarcolemmal stability and on Iso tension depending on whether dystrophin is present or absent.

MATERIALS AND METHODS

Mutant and control mice. Transgenic MDX mice, generated as previously described (7), were back-crossed on...
C57BL10 wild-type background. To obtain all genetic combinations in the offspring, heterozygous \textit{mdx} female mice were mated with hemizygous \textit{mdx} male mice carrying the skeletal \textit{\alpha}-actin promoter transgene (\textit{aDp71tg}). A second strain carrying a different \textit{Dp71tg} (CMV promoter \textit{Dp71tg} (\textit{cDp71tg})) was generated on wild-type CB6 (Balb/c × C57BL/6) background (cf. Ref. 10). The \textit{cDp71} construct also contains an influenza virus hemagglutinin tag. The hemagglutinin moiety does not seem to contribute to the pathogenicity of the transgene product (10). The breeding and cervical dislocation of mutant and transgenic mice was performed according to German law for the protection of animals with a permit from the local authorities.

Transgenic mice were mated with nontransgenic partners to maintain heterozygosity of the transgene or with transgenic partners to generate homozygous transgenic offspring. In the latter case, transgenic male mice were test mated with two wild-type female mice, and the offspring (15–20 individuals) was analyzed by polymerase chain reaction (PCR). Male mice that produced exclusively transgenic offspring were considered homozygous for the transgene.

Assessment of genotypes. The \textit{mdx} allele was identified by PCR (1). The following primers (5'–3') were used: wild type/\textit{mdx}, sense, AACTCATCAAATATGCGTGTTAGTG; \textit{mdx}, antisense, GTCACTCAGATAGTTGAAGCCATTTAA; wild type, antisense, GTCACTCAGATAGTTGAAGCCATTTAG. Amplification was performed by using a “touchdown” procedure (annealing temperature decreased during the first 10 cycles from 60 to 58°C) followed by 25 standard PCR cycles with a constant annealing temperature (58°C).

For identification of the \textit{Dp71tg}, the following primers (5'–3') were used: \textit{aDp71}, sense, GAAGCTCACTCCTCACCTCGTACCC; \textit{aDp71}, antisense, ATCCCTCCCTGTCCTCCGTTACATCATA; \textit{cDp71}, sense, CGAATTCATGAGGAACCATTGCTCAAAAGGC; \textit{cDp71}, antisense, CAGTCTAGGAGAGGCCGCTTCG. The same touchdown amplification protocol was used for monitoring of \textit{aDp71} and \textit{cDp71}: 10 cycles during which the annealing temperature decreased from 66 to 64°C, followed by 20 standard PCR cycles with a constant annealing temperature of 64°C.

Mechanophysiology and histopathology. Young adult (90–120 days old) transgenic and nontransgenic wild-type and \textit{MDX} male mice were used for experiments. Mice were killed by cervical dislocation, and the hind legs were cut off the trunk. Extensor digitorum longus (EDL) muscles were dissected in a continuously exchanged synthetic interstitial fluid (Tyrode solution), containing (in mM) 125 NaCl, 23.81 NaHCO3, 5.37 KCl, 1.0 MgCl2, 1.8 CaCl2, and 10.09 glucose, saturated with 95% O2 and 5% CO2, adjusted to pH 7.2–7.4.

One tendon of the muscle was attached to a hook in the experimental chamber and the other to the lever arm of a dual-mode servomotor system (300B-LR, Aurora Scientific, Vata Court, Ontario, Canada) via 6-0 surgical silk (ETHICON, D-22851, Norderstedt, Germany), which was tied securely to the proximal and distal tendons. Isometric (Iso)
contractions of freshly prepared EDL muscles were recorded at 25 ± 0.5°C. Muscles were directly stimulated via the chamber fluid with constant supramaximal voltage by using a Grass SD-9 (Grass Instrument, Quincy, MA) stimulator. Muscles were carefully stretched to the length at which single twitches showed the highest amplitude [optimal length (L0)] and then kept at L0 for subsequent measurements.

For evaluation of peak tetanic tension, stimulation was applied for 0.5 s at a rate of 100 pulses/s. To generate high tensions, the voltage applied for 0.5 s at a rate of 100 pulses/s was increased until maximal tetanic tension was achieved. Signals were amplified by a Grass P/24 amplifier and recorded on a data acquisition system (OPTEC). The peak tetanic tension (TTP), the force of maximum isometric tetanus, was applied to the contralateral muscle, serving as an internal control. Signals were amplified and visualized on a HAMEG 205-3 oscilloscope (HAMEG, Frankfurt, Germany). Data were digitalized and visualized on a HAMEG 205-3 oscilloscope (HAMEG, Frankfurt, Germany). Data were digitalized and visualized on a HAMEG 205-3 oscilloscope (HAMEG, Frankfurt, Germany).

Values of the animals heterozygous and homozygous for the transgene were pooled. For immunocytochemistry, cryosections of 8-μm thickness were cut from shock frozen muscles. To suppress background staining by endogenous IgG, sections were blocked for 1 h with papain-digested rabbit-anti-mouse IgG (11) and subsequently stained with DYS1 or DYS2 antibodies (Novocastra) against dystrophin’s rod and COOH-terminus, respectively.

Table 1. Contractile properties of isolated EDL muscles

<table>
<thead>
<tr>
<th></th>
<th>WT (BL10)</th>
<th>WT-aDp71</th>
<th>WT-cDp71</th>
<th>WT-cDp71</th>
<th>MDX</th>
<th>MDX-aDp71</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 14/7)</td>
<td>(n = 8/4)</td>
<td>(n = 16/8)</td>
<td>(n = 12/8)</td>
<td>(n = 13/14)</td>
<td>(n = 17/9)</td>
</tr>
<tr>
<td>Weight, mg</td>
<td>12.0 ± 0.3</td>
<td>11.1 ± 0.2</td>
<td>12.7 ± 0.5</td>
<td>14.6 ± 0.6</td>
<td>15.8 ± 0.6*</td>
<td>15.4 ± 0.3*</td>
</tr>
<tr>
<td>CSA, mm²</td>
<td>1.89 ± 0.03</td>
<td>1.81 ± 0.04</td>
<td>2.00 ± 0.07</td>
<td>2.21 ± 0.08</td>
<td>2.57 ± 0.09*</td>
<td>2.49 ± 0.06*</td>
</tr>
<tr>
<td>P0, N/cm²</td>
<td>5.48 ± 0.19</td>
<td>5.36 ± 0.18</td>
<td>4.82 ± 0.16</td>
<td>4.35 ± 0.21</td>
<td>4.00 ± 0.14*</td>
<td>3.01 ± 0.21*</td>
</tr>
<tr>
<td>TTP, ms</td>
<td>19.3 ± 0.3</td>
<td>19.4 ± 0.4</td>
<td>18.3 ± 0.3</td>
<td>19.1 ± 0.3</td>
<td>19.0 ± 0.4</td>
<td>19.7 ± 0.3</td>
</tr>
<tr>
<td>RT50, ms</td>
<td>22.1 ± 1.0</td>
<td>21.8 ± 1.0</td>
<td>20.3 ± 1.2</td>
<td>22.7 ± 1.3</td>
<td>22.6 ± 0.8</td>
<td>23.0 ± 0.7</td>
</tr>
<tr>
<td>P0, N/cm²</td>
<td>20.45 ± 0.52</td>
<td>21.08 ± 0.79</td>
<td>19.40 ± 0.58</td>
<td>16.84 ± 0.94</td>
<td>14.84 ± 0.47*</td>
<td>11.33 ± 0.74*</td>
</tr>
<tr>
<td>LP0, %</td>
<td>8.06 ± 1.14</td>
<td>9.38 ± 1.18</td>
<td>11.36 ± 1.02</td>
<td>24.14 ± 3.04*</td>
<td>31.94 ± 3.05*</td>
<td>31.03 ± 2.19*</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = number of muscles/number of animals. CSA, cross-sectional area as derived from lengths (12–14 mm) and weights; P0, twitch tension; LP0, irreversible loss of P0 after pliometric contractions; TTP, time to peak for twitch; RT50, half-relaxation time of twitch; WT, wild type; aDp71, α-actin promoter dystrophin 71; cDp71, CMV promoter-driven Dp71. Significantly (P < 0.01, Student’s unpaired t-test) different from *strain-matched WT (BL10 for aDp71, CB6 for cDp71) and/or †nontransgenic WT or MDX controls. ‡Values of the animals heterozygous and homozygous for the transgene were pooled.

RESULTS

Presence of dystrophin antigens in Dp71 transgenic muscles. In accordance with biochemical evidence (10), we show in this study, by using domain-specific antibodies to dystrophin, that submembranous dystrophin is decreased by the presence of ectopic Dp71 in muscle, marginally by the low expressed aDp71tg and drastically by the highly expressed cDp71 (Fig. 1). The deficit in full-length dystrophin seems to be compensated by submembranous Dp71, which links to the dystroglycan complex but does not connect to the actin cytoskeleton (cf. Ref. 10).

Contractile properties of Dp71 transgenic muscle. The mechanophysiological analyses were performed on isolated EDL muscles from 90- to 120-day-old mice. In contrast to MDX muscles, contractions of WT muscles show a progressively higher loss of tension (1-2% per twitch) that reaches its maximum (7-8%) at 6 contractions. In MDX muscles the decrease of tension per twitch is higher and reaches a maximum of 8-9% at 6 contractions (Fig. 2).
the case of \textit{mdx} mice, the muscle fibers at that age have undergone at least one round of degeneration and regeneration (as witnessed by central nuclei).

Two transgenes, aDp71tg and cDp71tg, of which the latter is expressed earlier and at higher levels, were tested on two wild-type backgrounds, the inbred strain C57BL/10 (BL10), and the hybrid strain C57BL/6 × Balb/c (CB6). Characteristics of twitch and tetanic force generation were very similar between these two mouse strains (Table 1). There was no statistically significant effect of Dp71tg expression (either aDp71tg or cDp71tg) on twitch and tetanic Iso force generation by wild-type EDL muscles. However, the variability of the maximum Iso force values was higher with both transgenes (Fig. 2); time to peak and half-relaxation times were unaffected (Table 1).

To measure the susceptibility of isolated EDL muscles to mechanical injury, we have applied series of Plio contractions (the muscle is forcibly stretched on the plateau of an Iso tetanic contraction). Maximum tension (P$_m$) during the imposed stretch (0.5 L$_o$/s, 10% L$_o$) was higher by a factor of 1.6–1.8 than the corresponding Iso tension (maximum Iso force) before stretch. With wild-type muscle, aDp71tg had no significant effect on the loss of Iso tension during Plio contractions, but the expression of cDp71tg more than doubled the irreversible reduction of maximum Iso force (Table 1; Fig. 2), indicating a significant weakening of the sarcolemma.

MDX muscle compared with wild-type muscle showed the well-known hypertrophy and deficiencies in specific twitch and tetanic tension (cf. Ref. 3; Table 1). Expression of aDp71tg did not further increase hypertrophy but significantly lowered specific twitch and tetanic tensions (Table 1; Fig. 2). There was again no effect on the time constants of twitches. During Plio contraction, the ratio of the maximum tension during imposed stretch to the corresponding Iso tension was significantly (P < 0.05) higher in MDX aDp71tg but not in MDX EDL muscles compared with wild-type controls. The reason is probably increased stiffness due to secondary fibrosis (connective tissue replacing muscle fibers) in the Dp71 transgenic mice. Unexpectedly, loss of tension on Plio contraction was not increased by aDp71tg expression in MDX muscle. Expression of cDp71tg in MDX mice gave results similar to those with aDp71tg but with higher variability (not shown).

Sarcolemmal damage in Dp71 transgenic muscle. Acute stress-induced sarcolemmal ruptures, as indicated by the influx of RO, have been evaluated on cross sections of EDL muscles, which had previously undergone Plio or, as a control, Iso contractions (Fig. 3). After Iso contraction, there were only very few RO-positive fibers in EDL muscles of all genotypes. In wild-type and wild-type aDp71tg muscles that had undergone Plio contraction, a few RO-positive fibers (<5%) were detected, whereas a large fraction of RO-positive fibers (5–20%) was detected on cross sections of MDX (Fig. 3) and MDX aDp71tg EDL muscles (Figs. 3 and 4). In accordance with the loss of force, an increased number of RO-positive fibers was observed in wild-type...
cDp71tg (Figs. 3 and 4). No RO-positive fibers were found in contralateral muscles subjected to Iso contraction (not shown); thus the observed damage is not a preparation artifact but is caused by the forced lengthening contractions during Plio contraction. There was a positive correlation of Plio-induced membrane ruptures with loss of tension, with small effects of aDp71tg and strong effects of cDp71tg; the latter, however, were lower than those of dystrophin deficiency (Fig. 4). Intracellular IgG, which is absent from intact fibers, indicates previous transient ruptures of the sarcolemma (cf. Ref. 8). Groups of IgG positive fibers, a characteristic of MDX skeletal muscle, were observed in MDX aDp71tg and in wild-type cDp71tg muscles (not shown).

DISCUSSION

According to a traditional view, cytoskeletal proteins serve mainly structural functions, i.e., they would participate in the assembly of elements of cell architecture and serve functions as building blocks, like actin, or as molecular rulers like nebulin and titin (19), or mechanically stabilize the cell, like intermediate filaments and spectrin. However, with a more detailed analysis of the domain structures, especially of larger cytoskeletal spectrin, additional roles in intracellular signaling were found (16, 17). Chronic deficits in intracellular signaling pathways may contribute to muscular dystrophy, as do repeated sarcolemmal ruptures caused by mechanical stress.

For the distinction between these two causes of muscle fiber degeneration, mechanophysiological measurements on directly stimulated isolated muscles are informative. Although the previous in situ history of an isolated muscle from a mutant or genetically manipulated organism cannot be entirely eliminated, the immediate effect of mechanical stress can be observed compared with nonstressed mutant and stressed wild-type muscle. In contrast to whole animal experiments, acute humoral, neural, and cardiovascular effects are largely excluded. We have applied this rationale to analyze the effects on muscle of ectopically expressed Dp71. In a parallel investigation, this COOH-terminal model for such dominant negative effects of Dp71, with a binding site only for the dystroglycan complex but not aDp71tg had no effect on the acute stability of wild-type muscle (O. Agbulut, G. Butler-Browne, and H. Jockusch, unpublished observations) whereas the low-level-expressed aDp71tg had no effect on the acute stability of wild-type EDL myofibers during Plio contractions. However, although force generation was only marginally affected, the higher levels of cDp71tg significantly increased the susceptibility toward contractile stress, as indicated by an accelerated loss of tension during Plio and by increased numbers of RO-positive fibers.

In addition to the effect on wild-type mice, we found an aggravating rather than ameliorating effect of ectopic Dp71 on dystrophin-deficient MDX muscle. Specific Iso strength, both of twitches and during tetanic contractions, in MDX aDp71tg EDL muscles was reduced by >20% compared with that of nontransgenic MDX controls, and similar, but rather variable, effects were found with high level expressed cDp71tg in MDX muscle (data not shown). Apart from a higher variability in its physiological characteristics, mutant muscle often acquires long-term changes that counteract the acute deleterious effects due to stress. Examples are double mutant myotonic MDX muscles, which “escape” stress damage by shifting to a more resistant fiber type (8), and fibrotic muscles in Dp71 transgenic MDX animals, which, due to their lowered force and increased stiffness, were less susceptible to stress-induced membrane damage than those with a near-normal appearance and force generation. Dp71 transgenic MDX muscles with the best basic performance thus yielded the most clear-cut signals on acute stress-induced damage.

In a previous report (10), our laboratory demonstrated that the levels of utrophin are reduced in muscles of Dp71tg wild-type muscles; in Dp71tg MDX mice, the upregulation observed in standard MDX muscles was partially reversed. In view of the severe pathology of MDX-utrophin-knockout double mutant mice (6), one might speculate that the impaired stability of Dp71 transgenic MDX muscle is caused by an interference with strategically localized utrophin. A molecular model for such dominant negative effects of Dp71, with a binding site only for the dystroglycan complex but not for the microfilament system, has been proposed (10).

Our findings support the view that muscular dystrophies (Duchenne muscular dystrophy and MDX) are predominantly caused by the loss of mechanical protection of muscle fibers in the absence of dystrophin or by its function being compromised by a competing but functionally insufficient protein. Furthermore, our findings provide an explanation of why the expression of Dp71 is specifically repressed in mature skeletal muscle (9).

DISCLOSURES

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