Severe muscle dysfunction precedes collagen tissue proliferation in mdx mouse diaphragm

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IN THE MDX MOUSE, AS WELL AS in human Duchenne muscular dystrophy (DMD), the devastating muscle degeneration is caused by a mutation in the gene encoding dystrophin (20, 43). Mechanisms whereby the lack of dystrophin leads to functional failure of muscle fibers have not been fully elucidated. Dystrophin forms part of a macromolecular complex that links cytoskeletal actin to components of the extracellular matrix (43). This arrangement has lent considerable support to the idea that muscle fiber necrosis during DMD and in mdx mice is a result of compromised structural integrity of the sarcolemma during repetitive contractions (26). More recently, it has been proposed that disruption of key signaling processes mediated by the dystrophin complex could be involved in muscle degeneration (4, 29, 39). For instance, changes in nitric oxide (NO) production in dystrophic muscle may contribute to the pathology (30, 36, 39).

The diaphragm muscle of mdx mice undergoes a progressive degeneration similar to that occurring in human muscles (17, 34). As the muscle tissue is no longer capable of maintaining homeostasis during repeated cycles of degeneration and regeneration, excessive proliferation of connective tissue leads to progressive loss of contractile tissue and ensuing muscle weakness (17, 27, 34). However, in 3-mo-old mdx diaphragm, the 50% decline in force-generating capacity largely exceeds that predicted solely from the cross-sectional loss of muscle tissue (27). Moreover, our laboratory (10, 11) recently reported abnormal myosin function in the diaphragm from 6- and 9-mo-old mdx mice. This indicates that the contractile apparatus of regenerated muscle fibers may be intrinsically dysfunctional in mdx diaphragm. However, at present, only a limited amount of data are available on the mechanical performance of mdx mouse diaphragm in the early stages of the disease (17), i.e., before extensive fibrosis of the muscle.

The first aim of our study was to determine whether intrinsic diaphragm muscle function was impaired in 6-wk-old mdx mice. Between 3 and 4 wk, the mdx diaphragm exhibits extensive muscular necrosis followed by regeneration (32). Therefore, whereas individual necrotic fibers can still be detected in 6-wk-old mdx mouse diaphragm, deposits of connective tissue are still expected to be low. In addition, at this age, maturational changes occurring in the diaphragm in the early postnatal period are expected to be complete (9, 37). The second aim of our study was to determine whether potential muscle weakness was associated with changes in the number, kinetics, and single force of cross bridges (CB). Huxley’s equations (21) were
used to calculate the single force of CB, total number of CB, rate constant for attachment and detachment, and total duration of the CB cycle (10, 22) in control and mdx mouse diaphragm. Two hypotheses were tested: 1) decreased intrinsic diaphragm muscle function is already present in 6-wk-old dystrophic mice; and 2) changes in the mechanical properties are associated with changes in CB properties.

MATERIALS AND METHODS

Animals

Experiments were conducted on 12 six-wk-old male mdx mice and 11 age-matched control mice (C57BL/10ScSn) obtained from Charles River Laboratories (St-Aubin-les-Es-Beufs, France). One subgroup of eight mdx and eight controls was used for mechanical analysis. Another subgroup of four myopathic mice and three control mice was used for morphometric analyses. Ideally, both the mechanical and the morphological experiments should have been conducted on the same animal. However, the mdx mouse is a highly reproducible model of muscular dystrophy. Therefore, no significant differences are expected between the different groups of animal. Care of the animals conformed to the Helsinki declaration, and the study was approved by our institution (Institut National de la Santé et de la Recherche Médicale). After brief ether anesthesia, the animals were laparotomized and then thoracotomized.

Mechanics

Diaphragm muscle strips. A strip of the ventral part of the costal diaphragm was carefully dissected out from the muscle in situ. The insertions on the central tendon and ribs were kept intact. The diaphragm strip was rapidly mounted in a tissue chamber containing a Krebs-Henseleit solution (in mM): 118 NaCl, 24 NaHCO3, 4.7 KCl, 1.2 MgSO4, 1.1 KH2PO4, 2.5 CaCl2·6H2O, 4.5 glucose. The solution was bubbled with 95% O2-5% CO2 and maintained at pH 7.4 and 26°C, so as to ensure good mechanical stability. The costal end of the muscle strip was held in a stationary clip at the bottom of the chamber, whereas the central tendon end was attached to an electromagnetic force-transducer device (9). After a 15-min equilibration period, the muscle was supramaximally stimulated via two platinum electrodes arranged longitudinally on either side of the muscle (electrical field stimulation: 30 V/cm). A force-frequency curve was determined by stimulating muscle strips at 33, 50, 75, 100, and 150 Hz (1-ms pulse duration, 300-ms train duration, 10 per minute) (Fig. 1). Maximum isometric tension was generally achieved at a stimulation frequency of 100 Hz. Experiments were carried out at the initial resting length corresponding to the apex of the initial length-active tension curve (L0). At the end of the experiment, the cross-sectional area (CSA; in mm2) was calculated from the ratio of muscle weight to muscle length at L0, assuming a muscle density of 1.06 (7). Characteristics of the studied muscle strips (n = 8 in each group) were as follows: L0, 6.8 ± 0.2 and 5.7 ± 0.3 mm in control and mdx diaphragm, respectively (P = not significant); CSA, 0.5 ± 0.1 mm² in both control and mdx diaphragm.

Mechanical Parameters

Maximum unloaded shortening velocity (Vmax in L0/s) was measured by means of the zero-load clamp technique (6). Peak isometric tension, i.e., peak force normalized per CSA (Po in mN/mm²), was measured from the fully isometric contraction. The tension-velocity relationship (P·V) (19) was derived from the peak velocity (V) of 8–10 isotonic after-loaded contractions, plotted against the isotonic force level normalized per CSA (P) and by successive load increments from zero load up to the total isometric tension (Po). The P-V relationship was fitted according to Hill’s equation (P + a(V + b) = (Po + a)b (19), where –a and –b are the asymptotes of the hyperbola as determined by multilinear regression. For each muscle strip, the P-V relationship was accurately fitted by a hyperbola (each r > 0.98). The curvature of Hill’s equation (G) is equal to PJo = Vmax/b (19, 42).

CB characteristics. Force and shortening are generated by cyclic interactions between myosin and actin, driven as one molecule of ATP is hydrolyzed (21). According to Huxley’s theory (21), the most widely accepted theory of muscle contraction, Po is the product of the number of cycling CB/mm² and the force of a single CB. Huxley’s model makes it possible to calculate the total number and the elementary force of cycling CB and the kinetics of the main steps of the CB cycle from mechanical data (10, 21, 22).
The maximum value of the rate constant for CB attachment, \( f_1 \) (in s\(^{-1}\)), is given by

\[
f_1 = \frac{-g_1 + \sqrt{g_1^2 + 4g_1g_2}}{2}
\]

where \( g_1 \) (in s\(^{-1}\)) is the maximum value of the rate constant for CB detachment during the power stroke, and \( g_2 \) (in s\(^{-1}\)) is the maximum value of the rate constant for CB detachment after the power stroke.

\[
g_1 = \frac{2wb}{ehG}
\]

where \( w \) is the maximum mechanical work of a single CB, \( h \) is the molecular step size (\( h \) is assumed to be equal to 11 nm), and \( e \) is the free energy required to split one ATP molecule per contraction site (\( e = 5.1 \times 10^{-20} \) J) (21, 42)

\[
g_2 = \frac{2V_{\text{max}}}{h}
\]

The elementary force per CB, \( \pi \) (in pN), is given by

\[
\pi = \frac{w}{l} \frac{f_1}{f_1 + g_1}
\]

where \( l \) is the length between two actin binding sites (\( l = 36 \) nm) (42). The total number of CB per millimeter squared at \( P_o \) (\( \Psi \)) is

\[
\psi = ab \left( \frac{h}{2l} \frac{f_1g_1}{f_1 + g_1} \right)
\]

The total time cycle (tc; in ms) is equal to

\[
tc = \frac{2fl_1 + g_1}{h} \frac{f_1}{f_1 + g_1}
\]

The mean CB velocity during the stroke, \( \bar{v}_o \) (in \( \mu \text{m/s} \)), is given by (22)

\[
\bar{v}_o = \frac{ab}{L_o\psi \pi}
\]

**Morphometry**

Tissues from ventral costal hemidiaphragm were held in an extended position by pinning them to a piece of cork. Then they were snap-frozen in isopentane precooled in liquid nitrogen and stored at \(-80^\circ\)C. Transverse cryosections of the diaphragm (10 \( \mu \text{m} \)) were stained with Masson’s trichrome to determine the percentage of centrally and peripherally located nuclei, the percentage of necrotic fibers, and the percentage of fibrosis (determined by the area of stained collagen fibers). Central nuclei in fibers usually indicate that these fibers have regenerated at least once. A total of 15 sections per sample were analyzed. Therefore, for each diaphragm, fields were randomly chosen in each of the 15 sections, and a total of 1,500 fibers were counted per diaphragm (i.e., 100 fibers per section). The percentages are thus relative to the total number of fibers counted. Sections were observed with an Olympus BX 60 microscope. The area of stained collagen fibers was determined by using an image-analysis software (MetaView image analysis).

**Statistical Analysis**

Data are expressed as means ± SE. After ANOVA, comparisons of mechanical parameters between \( \text{mdx} \) and control groups were performed by using Student’s unpaired \( t \)-test. Comparisons of histological parameters were performed by using two-way ANOVA with repeated measurements. A \( P \) value <0.05 was considered statistically significant.

**RESULTS**

**Diaphragm Morphometry**

Limited areas of necrotic muscle fibers were observed in 6-wk-old \( \text{mdx} \) mice (Fig. 2). Percentages of fiber necrosis represented 2.8 ± 0.6 and 0.1 ± 0.1% of the total muscle fibers in the \( \text{mdx} \) and control diaphragm, respectively (\( P < 0.05 \)). Very little fibrosis was observed in the diaphragm muscles of 6-wk-old \( \text{mdx} \) mice, although the collagen surface area was significantly larger than in controls (3.6 ± 0.7 compared with 1.1 ± 0.1%, \( P < 0.05 \)). The percentage of fibers having central nuclei was 24.9 ± 2.8% in the \( \text{mdx} \) and 0.3 ± 0.1% in the control mice.

**Contractile Performance of the Diaphragm Muscle**

Mechanical parameters of the diaphragm muscle are presented in Fig. 3. \( P_o \) was 37% lower in 6-wk-old \( \text{mdx} \) than in controls (\( P < 0.01 \)). Moreover, compared with controls, there was an almost 32% decline in \( V_{\text{max}} \) in the \( \text{mdx} \) group (\( P < 0.001 \)). Compared with controls,
the $G$ of the force-velocity hyperbola was significantly lower in $mdx$ mice (5.9 ± 0.7 compared with 3.6 ± 0.4, $P < 0.01$; Fig. 4).

Diaphragm CB Properties

CB number and force in $mdx$ and control diaphragm are presented in Fig. 5. Compared with controls, the 6-wk-old $mdx$ diaphragm exhibited an ~30% reduction in the total number of active CB per millimeter squared ($P < 0.01$). The elementary force per CB was significantly lower in $mdx$ than in controls (percent difference between $mdx$ and controls ≈18%, $P < 0.01$). CB kinetics in control and $mdx$ mice are presented in Fig. 6. The rate constant for CB detachment $g_2$ was significantly lower in $mdx$ than in controls ($P < 0.05$). There was no difference in the rate constant for CB attachment $f_1$ between groups. The total duration of the CB cycle was significantly shorter in $mdx$ than in controls. Compared with controls, the mean CB velocity during the power stroke was significantly higher in $mdx$ ($P < 0.01$). In both $mdx$ and control groups, there was a strong linear relationship between $P_o$ and the total number of active CB (Fig. 7): the higher the CB number, the higher the $P_o$. Conversely, there was no

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**Fig. 3.** Mechanical parameters in control (C) and 6-wk-old $mdx$ mouse diaphragm. A: peak isometric force normalized per cross-sectional area ($P_o$). B: maximum unloaded shortening velocity ($V_{max}$). Values are means ± SE ($n = 8$ in each group). †$P < 0.01$, ‡$P < 0.001$ compared with control. $L_o$ is the initial muscle length at which active tension is maximum.

**Fig. 4.** Force-velocity relationships in control and 6-wk-old $mdx$ mouse diaphragm. A: absolute tension ($P$) and shortening velocity ($V$) values were used to determine the $P$-$V$ relationships. B: normalized $P$ and $V$ values were used to determine the $G$ curvature of the hyperbola. $P_{max}$, maximum absolute tension.

**Fig. 5.** Total number of cross bridges (CB) (10⁹/mm²; A) and elementary force per CB ($\tau$; B) in control and 6-wk-old $mdx$ mouse diaphragm. Values are means ± SE ($n = 8$ in each group). *$P < 0.05$, †$P < 0.001$ compared with control.
significantly relation between $P_0$ and the CB single force (Fig. 7).

DISCUSSION

The main result of the present study is that diaphragm muscle performance was markedly impaired in 6-wk-old $mdx$ mice, i.e., at a stage at which muscle necrosis and/or fibrosis remained limited. As CB interactions were accounted for, our study suggested early CB abnormalities in the dystrophic diaphragm. Such abnormalities may well play a role in repetitive cycles of diaphragm muscle fiber degeneration, a point that deserves further studies.

The genetic similarities between $mdx$ and DMD have made the $mdx$ mouse an extremely attractive model for the study of human muscular dystrophy. Previous studies have described the time course of progressive muscle weakness and deterioration with age throughout the life of $mdx$ mice (17, 24). In 2-wk-old $mdx$ mice, few abnormalities have been detected in leg (24) and diaphragm muscles (17). Although it would be of interest to analyze diaphragm CB interactions before 3 wk of age, maturational changes occur in the diaphragm in the early postnatal period (37). These result from quantitative and/or qualitative changes in actomyosin interactions (9) that may complicate the interpretation of the data. After extensive muscle necrosis at the age of 3 wk, early imbalance between degeneration and regeneration leads to progressive fibrosis and severe diaphragm muscle weakness (17, 34). On the one hand, the percentage of muscle tissue to total diaphragm CSA averages only 72 and 44% of control values in 3- and 22-mo-old $mdx$ diaphragm, respectively (27). On the other hand, diaphragm tetanic strength in the $mdx$ mouse shows a 35–50% (14, 27), 50% (10), and 80% reduction (27, 34) at 3, 6, and 16 mo of age, respectively. However, it has been suggested that the force-generating capacity of the diaphragm is more impaired in 3-mo-old $mdx$ than that expected from loss of con-

Fig. 6. CB kinetics in control and 6-wk-old $mdx$ mouse diaphragm. $A$: peak value for rate constants of detachment ($g_2$). $B$: total duration of the CB cycle. $C$: peak value for the rate constant for CB attachment ($f_1$). $D$: mean velocity of CB during the power stroke ($\langle v_0 \rangle$). Values are means $\pm$ SE ($\bar{n}=8$ in each group). * $P<0.05$, ‡ $P<0.001$ compared with control. Only significant difference is figured.

Fig. 7. A: relationship between $P_0$ and the total number of CB ($\Psi\times 10^6$ mm$^{-2}$). Within groups, there was a close linear relationship between $P_0$ and $\Psi$ in both control ($P_0=6.9\Psi+23.9; r=0.979, P<0.001$) and $mdx$ ($P_0=8.4\Psi-13.7; r=0.970, P<0.001$) diaphragm. $B$: conversely, in both control and $mdx$ diaphragm, there was no simple relationship between maximum isometric tension and $\pi$. 

Significant relations between $P_0$ and the CB single force (Fig. 7).
tractile tissue (27). In 6-wk-old mdx mice, we found that diaphragm muscle function was severely impaired, despite limited necrotic and/or fibrotic lesions (2.8 and 3.6%, respectively). Thus, although progressive diaphragm fibrosis undoubtedly impairs muscle strength by 3 mo of age, our results pointed to severe muscle dysfunction as an intrinsic characteristic in regenerated mdx diaphragm, which cannot be accounted for by necrosis and fibrosis.

According to Huxley’s equations, muscle force depends on the elementary force produced per CB and the total number of CB generating contractile force (21, 22). Therefore, a severe decline in muscle strength, despite limited morphometric muscle damage, strongly suggests that CB recruitment and/or unitary force generated per CB are altered in 6-wk-old mdx diaphragm. We found that both active CB and the elementary force generated per actomyosin interaction were significantly lower in dystrophic than in control diaphragm (Fig. 5). Associated changes in CB kinetics, as manifested by the shorter duration of the CB cycle and longer time for CB dissociation, i.e., lower $g_2$ value, further attested to qualitative modifications in CB cycling in dystrophic diaphragm (Fig. 6). However, the finding that total isometric tension ($P_0$) was strongly related to the total number of CB, but not to unitary CB force (Fig. 7), indicated that the 30% decrease in active CB played a preponderant role in the impaired respiratory muscle force in the dystrophic mouse. These results are consistent with those previously reported in 6-mo-old mice, although the reduction in force produced per myosin head between control and mdx mouse diaphragm appeared higher in mdx mice from 6 wk of age (~18% at 6 wk compared with ~5% at 6 mo) (10).

Abnormal CB recruitment and qualitative changes in CB cycling suggest functional alterations leading to impaired CB activation and/or intrinsic molecular modifications of the myosin head structure (33). Recent studies have suggested that the dystrophin complex has a scaffold function that recruits signaling proteins to the membrane (29, 38). The absence of dystrophin and its associated proteins causes redistribution of neuronal NO synthase from the membrane to the cytosol in muscle cells (5). These abnormalities are associated with both a reduction in NO-mediated protection against ischemia (36) and an increase in cellular susceptibility to oxidant challenges (16, 18, 30). A number of studies have provided evidence that muscle pathology in DMD and mdx mice is, in part, mediated by free-radical species (28). Oxidative damage may induce lipid peroxidation of membranes (3, 12), a mechanism that could favor the development of diaphragm sarcolemmal injury in dystrophic mice. Moreover, oxidative stress may induce oxidative modifications of thiol residues involved in excitation-contraction coupling (1) and direct inhibition of CB interactions by modulating critical thiols on the myosin head (23). Modifications in isometric force, CB recruitment, and CB cycling observed in the dystrophic diaphragm were fairly consistent with previously reported effects of oxidative stress in skeletal muscle (25). Subtle changes in amino acid integrity within the myosin molecule itself may, in turn, contribute to the reduced CB unitary force, given that in vitro motility assays have previously revealed abnormal myosin function in purified actin and myosin molecules from the mdx diaphragm (11). Therefore, it seems likely that oxidative stress may contribute to severe diaphragm dysfunction in 6-wk-old mdx mice. Oxidative-stress-mediated myosin damage combined with sarcolemmal vulnerability to mechanical stress would be predicted to favor the development of diaphragm muscle injury.

Alternatively, the absence of the dystrophin complex leads to disorganization of the costameric cytoskeleton, the cytoskeletal lattice that links the sarcomeric apparatus to the sarcolemma (41). This, in turn, may favor nonhomogeneity in sarcomere length within muscle fibers and/or abnormal orientation of the myosin molecules relative to the actin filament axis, thereby altering interactions between the myosin head and actin filament (13, 35). One might also hypothesize that diaphragm dysfunction is linked to the regenerative process itself. Indeed, transitory expression of embryonic and neonatal myosin heavy chain (27, 40) and immature Ca$^{2+}$-transient in fibers undergoing regeneration (8, 15) may influence diaphragm contractile properties (31). However, several findings argue against such a hypothesis. First, limb muscles from 6-wk-old mdx mouse exhibit functional recovery (17). Therefore, impaired force-generating capacity appears to be specific, either to the diaphragm or to chronically active muscle (2). Second, it has been previously reported that progressive transitions in the relative expression of the different myosin isoforms are not associated with changes in CB unitary force value during postnatal development, at least in the diaphragm muscle of the hamster (9) and the rat (23). These observations strongly suggest that factors other than myosin heavy chain isoforms contribute to the impaired CB kinetics in mdx diaphragm.

In conclusion, our study demonstrates that, in 6-wk-old mdx mice, diaphragm weakness cannot be related to the presence of extensive necrotic or fibrosis areas. Irrespective of the cause, the reduced diaphragm muscle weakness may render the muscle more prone to degeneration, as only a fraction of active CB is forced to carry the ventilatory workload of the diaphragm.

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1750 EARLY DIAPHRAGM MUSCLE DYSFUNCTION IN Mdx MICE


