Enhanced Dystrophic Progression in mdx Mice by Exercise and Beneficial Effects of Taurine and Insulin-Like Growth Factor-1

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Received July 10, 2002; accepted September 20, 2002

ABSTRACT

A preclinical screening for prompt-to-use drugs that are safer than steroids and beneficial in Duchenne muscular dystrophy was performed. Compounds able to reduce calcium-induced degeneration (taurine or creatine 10% in chow) or to stimulate regeneration [insulin-like growth factor-1 (IGF-1); 50 or 500 μg/kg s.c.] were administered for 4 to 8 weeks to mdx mice undergoing chronic exercise on a treadmill, a protocol to worsen dystrophy progression. α-Methyl-prednisolone (PDN; 1 mg/kg) was used as positive control. The effects were evaluated in vivo on forelimb strength and in vitro electrophysiologically on the macroscopic chloride conductance (gCl), an index of degeneration-regeneration events in mdx muscles, and on the mechanical threshold, a calcium-sensitive index of excitation-contraction coupling. The exercise produced a significant weakness and an impairment of gCl, by further decreasing the already low value of degenerating diaphragm (DIA) and fully hampering the increase of gCl typical of regenerating extensor digitorum longus (EDL) mdx muscle. The already negative voltage threshold for contraction of mdx EDL was also slightly worsened. Taurine > creatine > IGF-1 counteracted the exercise-induced weakness. The amelioration of gCl was drug- and muscle-specific: taurine was effective in EDL, but not in DIA muscle; IGF-1 and PDN were fully restorative in both muscles, whereas creatine was ineffective. An acute effect of IGF-1 on gCl was observed in vitro in untreated, but not in IGF-1-treated exercised mdx muscles. Taurine > PDN > IGF-1, but not creatine, significantly ameliorated the negative threshold voltage values of the EDL fibers. The results predict a potential benefit of taurine and IGF-1 for treating human dystrophy.

The absence of dystrophin is responsible for the life-threatening progressive skeletal muscle degeneration in Duchenne muscular dystrophy (DMD) (Hoffman and Dressman, 2001). No treatment is available; in fact, gene therapy is not feasible yet, and beneficial glucocorticoids have severe side effects (Hoffman and Dressman, 2001; Blake et al., 2002). The identification of prompt-to-use drugs to ameliorate DMD is delayed by the partial understanding of the pathological cascade leading to death of dystrophin-deficient fibers (Hoffman and Dressman, 2001; Blake et al., 2002). Also, the mdx mouse, the most used model of DMD, has a limited usefulness for preclinical drug studies, because a successful muscle regeneration leads to a mild progression of the pathology (Hoffman and Dressman, 2001; Blake et al., 2002).

Dystrophin is a subsarcolemmal component of the dystrophin-glycoprotein complex linking the contractile machinery to the extracellular matrix and likely confers mechanical reinforcement to sarcolemma. Its absence may facilitate contraction-induced focal membrane damage with increases in cytosolic Ca2+ level and consequent activation of Ca2+-dependent proteases and necrosis (Alderton and Steinhardt, 2000; Blake et al., 2002). As a mark of altered calcium homeostasis, the excitation-contraction coupling mechanism of mdx muscle fibers is affected, the voltage for contraction being more negative versus that of control (De Luca et al., 2001). The mechanical role of dystrophin is supported by the susceptibility of dystrophin-deficient fibers to the workload the muscle withstand. The diaphragm of mdx mice, continuously active throughout life, progressively degenerates simi-
ularly to DMD muscles (Petrof et al., 1993; Dupont-Versteegden et al., 1994; Blake et al., 2002). Low-intensity swimming as well as immobilization of hind limb muscle seem to ameliorate muscle performance (Hayes and Williams, 1998; Mokhatarian et al., 1999), whereas forced treadmill running, especially with episodes of eccentric contractions, induces severe muscle damage with even fatal consequences (Sandri et al., 1997; Vilquin et al., 1998). The treadmill-exercised mdx mice have been used by Granchelli et al. (2000) for a large drug screening, considering drug effectiveness the ability to prevent the loss of forelimb muscle strength occurring after 4 to 5 weeks of exercise. However, the use of in vivo muscle strength as the sole endpoint to state both muscle function and drug effectiveness is rather elusive, because this parameter is also influenced by nervous reflexes and cognitive function. A cellular parameter useful to functionally assess the degeneration and regeneration events occurring in dystrophic fibers is the resting conductance to chloride ions (gCl), the parameter that controls the electrical stability of sarclemma (Klocke et al., 1994; De Luca et al., 1997). gCl is significantly impaired in progressively degenerating mdx diaphragm (DIA), whereas in the hind limb extensor digitorum longus (EDL) muscle, gCl rises over the control values between the 8th and the 12th week of age, the period of mouse life in which the spontaneous regeneration is still ongoing (De Luca et al., 1997, 1999; McIntosh et al., 1998). The change in gCl may work as a fine-tuning of the mechanical-stress by modulating sarclemma excitability (De Luca et al., 1997). In turn, gCl can be a sensitive mark of the dystrophic progression due to increased workload such as exercise. We presently used the exercised mdx mice for preclinical drug screening. Three- to four-week-old mdx animals were made running on a treadmill twice a week for 4 to 8 weeks and the effect of either exercise or exercise plus drug treatment, were evaluated both in vivo on forelimb strength and in vitro, at the end of the protocol, on gCl of EDL and DIA muscles, and on mechanical threshold (MT), the calcium-sensitive index of excitation-contraction (e-c) coupling (De Luca et al., 1996, 2001). The drugs used were creatine, taurine, and insulin-like growth factor-1 (IGF-1). Taurine and creatine levels, normally high in striated fibers, fluctuate in mdx muscle in relation to pathology progression (McIntosh et al., 1998). Creatine stimulates muscle metabolism in vivo, whereas either compounds seem to control calcium-handling mechanisms in vitro, with potential ability to counteract calcium-induced degeneration (Pulido et al., 1998; De Luca et al., 2001). IGF-1 stimulates muscle regeneration, a failing process in DMD patients (Hoffman and Dressman, 2001). In support of its therapeutic potential, we found higher levels of IGF-1 in hind limb muscle and plasma during regeneration period of mdx mouse, and recently transgenic mdx mouse over-expressing IGF-1 showed an amelioration in force and muscle mass (De Luca et al., 1999; Barton et al., 2002). Steroids are beneficial in dystrophic patients (Anderson et al., 2000; Dubowitz, 2002); thus, a treatment with α-methyl-prednisolone (PDN) was also used as positive control.

Materials and Methods

Animal Groups and Drug Treatment. Fifty-four mdx and 28 wild-type (C57/B10) male mice 3 to 4 weeks old (IFFA Credo, Lyon, France; The Jackson Laboratories, Bar Harbor, Maine) were used. Initially, all the animals were weighed and forelimb force measured by means of a grip strength meter (see below). Each group was then subdivided into two further groups: exercised and sedentary. For drug treatments, a total of 45 exercised mdx mice were used, subdivided into three groups as follows: group 1, five untreated, six creatine-treated, and six taurine-treated; group 2, five untreated and six PDN-treated; and group 3, five untreated, six treated with IGF-1 at 50 μg/kg, and six treated with IGF-1 at 500 μg/kg. No significant differences were found between the untreated exercised mdx mice of the three groups. For this reason, and for giving a global view of the effect of exercise between the two strains (control and mdx), the data regarding the untreated exercised mdx mice have been pooled from the three groups. However, to have a more strict statistical evaluation of the drug effectiveness, each group of drug-treated mice has been compared with its own related untreated mdx group. All treatments started a few days before the beginning of the exercise protocol, and continued until the day of sacrifice. Creatine and taurine were given every day with amino acid-enriched chow (10% in weight). The daily amount of food eaten by each animal ranged between 2 and 4 g. The chow enriched with PDN (0.00125% in weight) was given 6 days a week up to the daily amount for the dose of 1 mg/kg. Then, the mice received ad libitum the standard drug-free chow to avoid PDN overdose. Both standard and drug-enriched chow made under request were purchased from Eberle Nafag AG (Gossau, Switzerland). IGF-1 (recombinant human; Chiron, Emeryville, CA) at 50 and 500 μg/kg was administered s.c. and untreated mdx mice belonging to the same group received daily the same amount of solvent. The nine mdx mice, taken as sedentary, were left free to move in the cage, without additional exercise. The sedentary mice were monitored for in vivo and in vitro studies at the same time points of exercised counterparts, as needed.

Similar drug regimes on control animals are important to estimate pure drug effect on tested parameters without interference with pathology. However, the information available helped us to limit such a control treatment as much as possible, in agreement with ethical laws. In fact, systemic use of steroids is known to produce severe myopathy, which implies a possible risk of misinterpretation of their therapeutic potential if used as control treatment in healthy subjects (Mitsui et al., 2002). Previous experiments performed in our laboratory have shown that taurine, either in vivo and in vitro, does not modify the electrical and contractile parameters of skeletal muscle, unless in the presence of a taurine deficiency (De Luca et al., 1996, 2001). Creatine and IGF-1 are both known for their anabolic activity, but may possibly affect muscle function in pathological condition (Semsarian et al., 1999; Tarnopolsky and Beal, 2001). Among these two compounds we have chosen to perform a treatment with IGF-1, because previous data suggest that this somatomedin physiological modulates the function of muscle chloride channel (De Luca et al., 1998), one of the main parameters presently used to monitor drug effectiveness on mdx muscles. For this reason five exercised control mice were treated daily with 50 μg/kg for 4 to 8 weeks and the effects were evaluated both in vivo and in vitro on chloride conductance.

Exercise Protocol and in Vivo Studies. The wild-type and mdx (both untreated and treated) mice belonging to the exercised groups underwent a 30-min run on a horizontal treadmill (Columbus Instruments, Columbus, OH) at 12 m/min, twice a week, for 4 to 8 weeks (Granchelli et al., 2000). The training protocol started at the mouse age of 3 to 4 weeks. About half of the mdx mice showed an avoidance behavior with respect to exercise, with a higher tendency to fatigue, and had to be gently stimulated, or left resting, to complete the 30-min running session. This behavior, never observed in wild-type animals, was not modified by either exercise or drugs. Every week all the exercised mice were monitored for body weight and compared with related sedentary counterparts. The force for exercised mice (both controls and mdx) was evaluated before each training session by means of a grip strength meter (Columbus Instruments). For this measurement, the mice were allowed to grasp a triangular ring connected to a force transducer and then gently pulled away until the...
grip was broken. The transducer saved the force value at this point, which was a measure of the maximal resistance the animal can use with its forelimbs. Five measurements were taken from each animal. An independent-samples t-test was used to evaluate the statistical significance between the groups. The intergroup variance was considered acceptable for such analyses. All data are expressed as the mean ± S.E.M. The S.E. estimate for gCl was obtained as described previously (De Luca et al., 1997, 1998).

Results

Effect of Exercise on mdx Mice

Effect of Exercise on Body Weight Gain and Forelimb Strength. At the beginning of the training protocol 3- to 4-week-old mdx mice were significantly lighter (16.4 ± 0.57 g; n = 24) than age-matched wild-type animals (18 ± 0.47 g; n = 23) (by Student’s t test; p < 0.05). The two groups were subdivided each into two further groups, exercised and sedentary, of similar body weight. An age-dependent increase in body weight was observed with no significant differences between groups and strain. In fact, after 4 weeks of exercise protocol, the mean of body weight gain in exercised mdx mice was 4.93 ± 0.68 g (n = 15) versus 4.79 ± 0.79 g (n = 9) of sedentary ones. Similarly, the weight gain was 4.28 ± 0.47 g (n = 13) and 4.10 ± 0.48 g (n = 10) in exercised and sedentary wild-type mice, respectively. At the beginning of the training period the mdx mice were significantly weaker with respect to age-matched wild-type, the forelimb strength being 0.069 ± 0.004 kg (n = 24) and 0.084 ± 0.002 kg (n = 23), respectively. Figure 1 shows the effect of 4 weeks of exercise on the increment of forelimb strength in the two strains. A significant difference between groups was found by ANOVA test on either absolute increment (Fig. 1A; F = 5.85; p < 0.005) or on strength increment normalized for body weight (Fig. 1B; F = 3.66; p < 0.05). In fact, a similar, not significant, forelimb muscle strength increment was observed in both sedentary and exercised wild-type animals as well as in sedentary mdx mice, whereas the exercised mdx mice were not significantly different from the corresponding sedentary ones (Fig. 1C; F = 1.64; p = 0.16).
muscle fibers of 8- to 12-week-old mdx animals were characterized by a significantly higher value of Rm with respect to age-matched wild types (Table 1). This high Rm value was due to the 30% decrease of gCl, the gK being almost unchanged. Four to 8 weeks of exercise produced a slight increase of Rm of mdx DIA fibers, due to a further, albeit not significant, 10% lowering of gCl, whereas gK showed no significant increase. Interestingly, the exercise produced similar effects on DIA fibers of wild-type animals, significantly decreasing gCl (Table 1), suggesting a particular susceptibility of DIA to the increase of workload.

As previously observed (De Luca et al., 1997, 1998) and as opposite to DIA muscle fiber, EDL muscle fibers of 8- to 12-week-old mdx mice differed significantly from age-matched wild-type mice for the significantly lower value of Rm, mostly due to a significantly higher gCl value (Table 1). The exercise significantly increased the Rm value of mdx EDL muscle, as a result of a dramatic 35% drop in gCl, whereas gK was not modified by exercise (Table 1). The gCl value of exercised mdx EDL muscle fibers was also significantly lower with respect to the value of wild-type animals, and approached the values typically recorded in the degenerating mdx DIA. Thus, the increase of gCl, typically observed in sedentary mdx mice aged 8 to 12 weeks, likely related to regeneration events (De Luca et al., 1997, 1998), was completely hampered by the chronic exercise. In fact, exercise did not produce any significant change of either gCl or gK of EDL muscle fibers of wild-type animals.

**Effect of Exercise on Mechanical Threshold of EDL Muscle Fibers.** EDL muscle fibers of mdx mice, in spite of the active regeneration, maintains an alteration in e-c coupling mechanism, likely in relation to the proposed increase in cytosolic calcium levels, the MT being typically shifted toward more negative potentials irrespective of mouse age (De Luca et al., 2001). This alteration of MT was also observed in the present study; in fact, at any pulse duration, the voltage threshold for contraction was significantly more negative in the mdx versus wild-type animals (Table 2), with rheobase values of $-72.2 \pm 0.8$ and $-66.4 \pm 0.4$ mV, respectively ($p < 0.001$; Student’s t test). The mdx EDL muscle fibers also showed a significantly smaller rate constant (1/τ) with respect to wild type ($0.114 \pm 0.006$ versus $0.143 \pm 0.006$ s$^{-1}$; $p < 0.001$; Student’s t test), accounting for a longer time constant to reach the rheobase. The chronic exercise produced significant changes when multiple comparison between groups was performed at level of both voltage threshold values (Table 2) and fitted rheobase voltages ($F = 13.6$; $p = 0.0005$ vs. sedentary wild type; $p = 0.035$ vs. exercised wild type).

**Effect of Exercise on Component Ionic Conductances on DIA and EDL Muscle Fibers.** In agreement with previous observations (De Luca et al., 1997), the DIA and EDL muscle fibers showed a marked impairment of such a force increment ($0.0005 < p < 0.029$ by Bonferroni’s t test).

**TABLE 1**

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>EDL</th>
<th>Diaphragm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N/n</td>
<td>Rm [Ω cm$^2$]</td>
</tr>
<tr>
<td>Sedentary wild type</td>
<td>10/88</td>
<td>442 ± 13</td>
</tr>
<tr>
<td>Exercised wild type</td>
<td>13/81</td>
<td>440 ± 12</td>
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<tr>
<td>Sedentary MDX</td>
<td>9/72</td>
<td>364 ± 11$^{a,b}$</td>
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<tr>
<td>Exercised MDX</td>
<td>15/172</td>
<td>533 ± 15$^{b,c}$</td>
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</tbody>
</table>

Significant differences by Bonferroni t test vs. $^{a}$ sedentary wild type, $^{b}$ exercised wild type, and $^{c}$ sedentary mdx ($p < 0.05$).


table 2

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Duration (ms)</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>250</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary wild-type</td>
<td>-44.7 ± 0.8 (31)</td>
<td>-57.3 ± 0.5 (33)</td>
<td>-64.5 ± 0.5 (31)</td>
<td>-66.2 ± 0.4 (35)</td>
<td>-66.6 ± 0.4 (35)</td>
<td>-67.2 ± 0.4 (33)</td>
<td>-67.2 ± 0.4 (33)</td>
<td>-66.7 ± 0.4 (33)</td>
<td></td>
</tr>
<tr>
<td>Exercised wild-type</td>
<td>-48.4 ± 0.7* (49)</td>
<td>-59.7 ± 0.5* (48)</td>
<td>-67.5 ± 0.6* (48)</td>
<td>-70.3 ± 0.5* (56)</td>
<td>-70.4 ± 0.4* (55)</td>
<td>-70.9 ± 0.5* (48)</td>
<td>-71.5 ± 0.5* (63)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sedentary mdx</td>
<td>-50.4 ± 0.5b (31)</td>
<td>-60.7 ± 0.6* (33)</td>
<td>-68.9 ± 0.6* (30)</td>
<td>-72.4 ± 0.3* (37)</td>
<td>-72.8 ± 0.3* (36)</td>
<td>-72.7 ± 0.3* (36)</td>
<td>-72.8 ± 0.3* (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exercised mdx</td>
<td>-50.9 ± 0.3b (63)</td>
<td>-61.6 ± 0.5b (65)</td>
<td>-69.9 ± 0.4b (60)</td>
<td>-72.6 ± 0.3b (69)</td>
<td>-73.5 ± 0.2b (71)</td>
<td>-73.7 ± 0.2b (65)</td>
<td>-73.7 ± 0.3b (78)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Significant differences by Bonferroni's t test with respect to *sedentary wild type and #exercised wild type (p < 0.05). No significant difference was revealed by the statistical analysis between sedentary and exercised mdx mice.

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$ p < 0.0001 $. Exercise produced a further shift of MT of mdx EDL muscle toward more negative potentials, the fitted parameters being $ -73 \pm 0.7 \text{mV} $ for the rheobase and $ 0.110 \pm 0.008 \text{s}^{-1} $ for $ \lambda $. Interestingly, the exercise caused a significant $ 4.5 \text{mV} $ shift of the MT of wild-type muscle fibers toward more negative potentials ($ -70.3 \pm 0.7 \text{mV} $), along with a small reduction of $ \lambda $ ($ 0.123 \pm 0.008 \text{s}^{-1} $), suggesting that e-c coupling mechanism of normal muscle fibers is sensitive to the increase in muscle workload (Table 2). However, from a statistical point of view, the voltage threshold parameters of exercised wild-type muscles did not reach the severity of mdx ones. Also, preliminary histological evaluation allowed to exclude any damaging effect of exercise in hind limb muscles of wild-type animals (see below).

**Effect of Exercise on Histopathology.** To ascertain the effect of exercise on pathology progression of dystrophin-deficient muscle, tibialis anterior (TA) muscle, removed after the 4 to 8 weeks of exercise protocol from both wild-type and professionally trained mice was used as placebo for exercise. Histological evaluation showed a significant decrease in the number of necrotic and infiltrates cells in exercised mdx mice compared to sedentary mdx mice. A representative section from a tibialis anterior muscle of a wild-type and mdx mouse is shown in Fig. 2. The histological features of the TA muscle from sedentary and exercised mdx mice are similar, with a more uniform structure of the TA muscle in the exercised group. Bottom, quantification of the number of necrotic and infiltrates cells in each experimental condition. This evaluation has been performed over a comparable number of fibers per section (see text) and showed significant differences by ANOVA between the four groups. Significant difference by Bonferroni’s t test with respect to wild-type groups (*) and sedentary mdx mice (**).
mdx animals was analyzed for histopathological signs in comparison with sedentary counterparts. Figure 2 shows representative sections for each group, along with the main differences found in the muscle histology as a consequence of exercise. The analysis was performed by evaluating morphological differences over comparable number of fibers per section (NF/S) for each experimental condition. In wild-type muscle, the exercise only produced an increase in number of mitochondria, without substantial morphological changes (NF/S: 649.3 ± 68, n = 3 sedentary and 690 ± 124, n = 3). In line with classical description of dystrophin-less muscle (Blake et al., 2002), TA muscles of mdx mice were histologically distinguishable from wild type for the more disorganized structure due to the presence of necrosis, centronucleated fibers, regenerating fibers, and infiltration of mononuclear inflammatory cells. Over 622.8 ± 37 (n = 5) and 680.3 ± 52 (n = 5) NF/S for sedentary and exercised mdx muscles, it was possible to verify that the histopathological signs of mdx TA muscles were worsened by exercise. This was particularly evident for the number of necrotic fibers (F = 46.1; p < 1 × 10^{-5}) and for the number of infiltrates (F = 68.3; p < 1 × 10^{-5}), both clear indexes of increased muscle damage and inflammatory reaction in the dystrophic muscle as a consequence of the increased contractile stress. Other parameters were also present but not significantly changed by exercise.

**Effect of Drug Treatments on Exercised mdx Mice**

**Effect of Drugs on Body Weight and Forelimb Strength.** Taurine treatment almost doubled the body weight gain of exercised mdx mice, whereas all other drugs were ineffective on this parameter. After 4 weeks of treatment, all the compounds were significantly effective in fully counteracting the deleterious effect of exercise on the absolute force increment (Fig. 1A; 9.26 > F > 3.73; p < 0.02). Surprisingly, taurine produced an increase in muscle strength larger than that observed in both sedentary mdx mice and wild-type animals, followed by creatine that also was highly effective. To rule out any possible effect of body weight in the drug-induced increase in strength, for each mouse we normalized the forelimb strength to body weight at the beginning (time 0) and at the end of 4 weeks of exercise (time 4) and we considered the difference as normalized force increment (Fig. 1B). Also by this approach taurine and creatine were similarly highly effective, followed by IGF-1, which was effective in a dose-dependent manner (7.43 > F > 3.33; p < 0.03). Surprisingly, PDN was the less beneficial compound, producing a nonsignificant effect on normalized strength increment (F = 2.62; p = 0.066). Control exercised animals treated with 50 mg/kg IGF-1 showed increments in forelimb muscle strength totally overlapping that of untreated counterpart, allowing to rule out any aspecific anabolic effect as the basis of IGF-1 effectiveness in exercised mdx animals. In fact, the absolute increment of strength in exercised wild types that were IGF-1-treated was 0.040 ± 0.009 kg (n = 5), whereas the normalized increment strength/body weight value was 0.7 ± 0.3.

**Effect of Drug Treatments on Component Ionic Conductances of Exercised mdx Mice.** The effects of the various drug treatments on gCl and gK of EDL and DIA muscle fibers are illustrated in Figs. 3 and 4. A different effect of the various drugs was observed in the two muscle types. In fact, in the taurine-treated group the value of Rm of EDL muscle fibers was 22.8 ± 2.85 (p = 0.05) for taurine and 22.3 ± 2.8 (p = 0.05) for creatine. Bonferroni’s t test is as follows: Significantly different (p < 0.05) with respect to wild-type (WT) (a), sedentary mdx (Sed mdx) (b), and untreated exercised mdx mice (Exer MDX) (c).

![Fig. 3. Effects of taurine and creatine treatments on gCl of EDL and DIA muscle fibers of exercised mdx mice. Each column is the mean ± S.E.M. from 20 to 88 fibers from 5 to 10 preparations. ANOVA was 22.8 > F > 2.85 (p < 0.05) for taurine and 22.3 > F > 2.8 (p < 0.05) for creatine. Bonferroni t test is as follows: Significantly different (p < 0.05) with respect to wild-type (WT) (a), sedentary mdx (Sed mdx) (b), and untreated exercised mdx mice (Exer MDX) (c).](image-url)
fibers was 396 ± 17 Ω · cm² (n = 6/56), which almost overlapped that typically recorded in the sedentary mdx mice. The ANOVA test between untreated wild-type and mdx and taurine-treated mice was significant (F = 11.9; p < 1 × 10⁻⁶). This was due to the ability of taurine to maintain gCl at the high level typical of regenerating EDL muscle of sedentary mdx mice (Fig. 3). Also the gK value in taurine-treated mice was lower than that of untreated exercised mdx EDL muscle fibers. In contrast, in DIA muscle, the taurine treatment did not significantly change the Rm value that was 573 ± 35 Ω · cm² (n = 6/33), with respect to untreated mdx counterparts, being only able to prevent the 10% decrease of gCl due to exercise (Fig. 3). The treatment with creatine was not effective, being unable to significantly ameliorate Rm, gCl, and gK in both muscle types (Fig. 3).

IGF-1 at 50 μg/kg was able to maintain the Rm value of exercised EDL muscle at 420 ± 15 Ω · cm² (n = 6/70), similar to that typically recorded in wild type. In fact, IGF-1 significantly prevented the drop of gCl induced by exercise, although gCl of IGF-1-treated mdx mice was not as high as that recorded in sedentary mdx mice (Fig. 4). Interestingly, the IGF-1 treatment was highly effective on DIA muscle, the Rm value being 503 ± 28 Ω · cm² (n = 6/50). The gCl value of IGF-1-treated DIA muscle fibers was indeed very close to that recorded in wild-type ones. The increase in dosage did not produce any further increase in the effect. Also, IGF-1 treatment had no significant effect on gK values of both muscle types (Fig. 4). The PDN treatment was highly effective in fully preventing the deleterious effects of exercise on EDL muscle, the value of Rm being 363 ± 10 Ω · cm² (n = 6/63). In fact, PDN was highly effective on gCl of EDL muscle, maintaining it to values similar to those of regenerating sedentary mdx animals. Also PDN was able to bring the Rm value of DIA fibers to 445 ± 14 Ω · cm², a value overlapping that of wild type, being able to fully counteract the decrease of gCl typical of degenerating DIA (Fig. 4).

Specificity of IGF-1 Effects for Dystrophic Muscle. To evaluate the specificity of observed drug effect for dystrophic muscle, we performed a series of experiments with the highly effective IGF-1. First, we evaluated the sensitivity of gCl of EDL and DIA muscles of both untreated and IGF-1-treated exercised mdx mice to the in vitro application of IGF-1. In fact, previous studies showed the ability of IGF-1 to modulate the function of muscle chloride channel through both acute and long-term mechanisms (De Luca et al., 1998, 1999).

The effects of IGF-1 are shown in Table 3. The in vitro application of 3.3 nM IGF-1 to both EDL and DIA muscle of untreated exercised mdx animals was able to produce a significant increase in gCl. According to previous experiments (De Luca et al., 1998), the acute effect of IGF-1 was very rapid, being detectable within a few minutes after drug incubation. The in vitro application of IGF-1 was without effects on the close-to-normal value of gCl of both EDL and DIA of IGF-1-treated animals, suggesting that the in vivo effect of the growth factor is mediated, at least in part, by the restoration of pathways sensitive to the acute effects of IGF-1.

Accordingly, we evaluated the effect of a treatment with 50 μg/kg IGF-1 on exercised wild-type mice. The IGF-1 treatment did not produce any effect on both Rm, gCl, and gK values of both EDL and DIA muscles. Focusing on gCl, this parameter was 2098 ± 76 μS/cm² (n = 5/56) in EDL of IGF-1-treated controls, a value totally overlapping that re-
TABLE 3
Effect of in vitro application of IGF-1 on membrane resistance and resting chloride conductance of EDL and DIA muscle fibers of exercised mdx mice either untreated or IGF-1 treated.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Drugs in Vitro</th>
<th>n</th>
<th>Rm (Ω cm²)</th>
<th>gCl (µS/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exer mdx EDL</td>
<td>IGF-1 3.3 nM</td>
<td>41</td>
<td>567 ± 33</td>
<td>1480 ± 76</td>
</tr>
<tr>
<td>Exer mdx EDL, IGF-1-treated</td>
<td>IGF-1 3.3 nM</td>
<td>28</td>
<td>437 ± 20*</td>
<td>1930 ± 80*</td>
</tr>
<tr>
<td>Exer mdx DIA</td>
<td>IGF-1 3.3 nM</td>
<td>50</td>
<td>423 ± 17</td>
<td>2161 ± 75</td>
</tr>
<tr>
<td>Exer mdx DIA, IGF-1-treated</td>
<td>IGF-1 3.3 nM</td>
<td>50</td>
<td>477 ± 25</td>
<td>1860 ± 77</td>
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<tr>
<td></td>
<td>IGF-1 3.3 nM</td>
<td>16</td>
<td>595 ± 38</td>
<td>1344 ± 57</td>
</tr>
<tr>
<td></td>
<td>IGF-1 3.3 nM</td>
<td>12</td>
<td>497 ± 30</td>
<td>1651 ± 58*</td>
</tr>
<tr>
<td></td>
<td>IGF-1 3.3 nM</td>
<td>16</td>
<td>484 ± 28</td>
<td>1760 ± 62*</td>
</tr>
<tr>
<td></td>
<td>IGF-1 3.3 nM</td>
<td>12</td>
<td>549 ± 65</td>
<td>1652 ± 95*</td>
</tr>
</tbody>
</table>

* Significantly different with respect to the relative control value in the absence of IGF-1 (p < 0.05 or less). Even if not indicated, according to what described in the text, the values of gCl of IGF-1-treated EDL and DIA muscle fibers were significantly higher with respect to those of untreated exercised counterparts.

corded in untreated counterpart (see Table 1 for comparison). Interestingly, the IGF-1 treatment was not effective in preventing the decrease in gCI observed in control DIA fibers upon exercise (Table 1). In fact, in IGF-1-treated DIA gCI was 1574 ± 42 µS cm⁻² (n = 5/40). This latter observation suggests that the effectiveness of IGF-1 on the low gCI of dystrophic fibers relies on the ability of the somatomedin to counteract a specific step of the pathological cascade primarily or secondarily involving chloride channel function.

Effect of Drug Treatments on Mechanical Threshold of EDL Muscle Fibers of Exercised mdx Mice. As shown in Fig. 5, taurine treatment had a significant effect on MT of exercised EDL mdx muscle fibers, shifting the strength-duration curve toward the more positive potentials typical of sedentary wild-type muscles. In fact, the rheobase voltage was −67.9 ± 0.6 mV, about 5 mV more positive than the value recorded in both sedentary and exercised mdx EDL muscle and very close to the value recorded in sedentary EDL fibers.

The taurine treatment was also effective on 1/τ changing it from 0.11 ± 0.01 to 0.13 ± 0.007 s⁻¹, a value similar to that recorded in sedentary wild type. These effects were very similar to those previously observed after in vitro application of taurine on mdx EDL muscle (De Luca et al., 2001). Creatinine had no effect. IGF-1 had a little but significant dose effect at 50 and 500 µg/kg, being the curve shifted toward more positive potentials by 2 and 4 mV, respectively, with respect to the high effectiveness of taurine. IGF-1 treatment was almost as effective as taurine, bringing the rheobase voltage to −74.1 ± 0.4 mV. On the other hand, PDN treatment was effective as taurine.

Discussion

In the present study, we found various compounds that were able to counteract, with different potency and specific-
ity, the in vivo and in vitro signs of the deleterious effect of exercise in mdx mouse, allowing to envisage new drugs for the therapy of DMD.

Apart from the impairment of muscle strength in vivo described previously (Granchelli et al., 2000), the novel finding was that chronic exercise could selectively overwhelm the effect of regeneration in hind limb muscle. Other than by histological analysis, this was detected by the state of gCl, a sensitive index of degeneration and regeneration events occurring in different muscle types of mdx phenotype as well as in other pathophysiological conditions of skeletal muscle (De Luca et al., 1997, 1998). Chronic exercise seriously counteracted the regeneration-induced increase in gCl observed in mdx EDL muscle fibers, whereas it slightly affected the already low gCl typical of degenerating DIA (De Luca et al., 1997, 1999). The drugs used were all effective in preserving muscle strength in vivo, but had different effects on gCl in the two muscle types, suggesting that the lowering of this parameter is triggered by diverse molecular mechanisms of the pathological cascade. A long-term mechanism can ensue in DIA muscle for the continuous mechanical stress caused by the respiratory activity. For an adaptive phenomenon, in mdx DIA an age-dependent higher expression of fatigue-resistant fiber I and IIa versus the fast-glycolytic Iib does occur (Petrof et al., 1993). The phenotype transition can account for the decrease in gCl, because slow fiber types are characterized by lower expression of CIC-1, the muscle chloride channel, and, consequently, by lower gCl values (Klocke et al., 1994; Pierro et al., 2002). In this condition, only drugs such as PDN or IGF-1, able to stimulate regeneration and to act on myogenic programs activating specific transcription factors, may have positive functional effects on gCl by restoring channel expression (De Luca et al., 1999; Semsarian et al., 1999; Anderson et al., 2000). Recent findings support the usefulness of IGF-1 as a long-term therapeutic countermeasure. In fact, muscle-specific overexpression of IGF-1 in mdx mouse significantly enhanced muscle strength, muscle mass, and other signaling pathways associated with muscle regeneration, significantly decreasing fibrosis and necrosis (Barton et al., 2002).

In parallel, an acute effect of exercise can blunt regeneration and account for the marked decrease in gCl in the EDL muscle and for the further lowering of gCl in DIA. The short-term decrease in gCl can represent an early event that, through an increase of membrane excitability (De Luca et al., 1997), contributes to the establishment of a chronic state leading to either necrosis and apoptosis or phenotype transition. In support of this view, in myotonic mouse muscles, in which membrane hyperexcitability is triggered by a loss-of-function mutation of CIC-1 channel, a fast-to-slow transition results from activity and calcium-dependent activation of muscle-specific transcription factors, such as MEF-2 (Wu and Olson, 2002). Interestingly, these pathways, also involved in regeneration and/or apoptosis, are activated by exercise in heart of mdx animals (Nakamura et al., 2002). Thus, the understanding of the molecular mechanisms underlying the acute drop of gCl can help the identification of early therapies. A possible starting disease-related event disclosed by exercise is a functional ischemia, already demonstrated in contracting mdx and DMD muscles, due to a reduced activity of nitric-oxide synthase and nitric oxide production, for the disassembling of the dystrophin-glycoprotein complex (Sander et al., 2000; Hoffman and Dressman, 2001; Blake et al., 2002). The ischemia, along with the increased calcium, can trigger an inflammatory reaction; this latter seems a secondary mechanism playing a key role in the pathogenesis of muscular dystrophy (Porreca et al., 1999; Spencer et al., 2000; Hoffman and Dressman, 2001), and a recent DNA microarray study described a significant overexpression of cytokines, chemokines, and relative receptors in mdx muscle (Porter et al., 2002). The consequent switch-on of inflammation-related transducing signals can acutely modulate various cellular targets, among which muscle chloride channels.

According to the idea of short-term modulation of channel function, the impairment of gCl in untreated exercised EDL muscle was acutely counteracted by in vitro application of IGF-1, an effect that could not be detected in muscles from IGF-1-treated animals. This result, along with the lack of any effect of in vivo IGF-1 treatment in control animals, also supports that the benefit of IGF-1 is addressed, through both short- and long-term actions, to specific steps of the pathological cascade (Pons and Torres-Alemán, 2000). Accordingly, part of the beneficial effect of PDN treatment can also be attributable to its well known anti-inflammatory activity. The effectiveness of taurine on gCl of exercised mdx EDL muscle may be related to its antioxidant activity able to protect from ischemic injury; however, recent data support a specific anti-inflammatory action of the amino acid, mediated by both the reduction of cytokine production and the inhibition of cytokine-dependent activation of nuclear factor-κB (Kontny et al., 1999; Kanayama et al., 2002).

The positive effect of drugs could also be linked to a Ca ++-related mechanism and evaluated on MT. In line with evidence from other laboratories (for review, see Alderton and Steinhardt, 2000; Blake et al., 2002), our preliminary results with Fura-2 imaging are in favor of higher cytosolic Ca ++ level also in adult native muscle fibers of mdx mice (data not shown), and this correlates with the negative rheobase voltage of mdx EDL muscle (De Luca et al., 2001). The modification of MT by exercise cannot be taken by itself as an index of worsening of dystrophic condition, because the function of sarcoplasmic reticulum easily adapts in response to exercise and cytosolic calcium (Booth and Thomason, 1991). In fact, a remarkable negative shift of MT was observed in exercised wild-type mice, in the absence of any sign of degeneration by histological analysis, whereas a minor effect of exercise on MT occurs in mdx muscle, likely in relation to adaptive changes to functionally buffer the excess of cytosolic Ca ++. However, the negative MT is a typical feature of dystrophin-deficient muscle fibers and its modulation by drugs can provide additional information about drug efficacy and mechanism of action. The taurine treatment restored MT of exercised dystrophic muscle, in line its high effectiveness when applied in vitro and likely in relation to a direct stimulation of the Ca ++ ATPase pump of sarcoplasmic reticulum (Huxtable, 1992; De Luca et al., 2001). Conditions of muscle taurine depletion are in fact characterized by negative MT and a difficulty of dystrophic muscle to retain adequate amounts of the amino acid has been documented and can be even worsened by exercise (De Luca et al., 1996, 2001; McIntosh et al., 1998; Matsuzaki et al., 2002). Similarly, PDN was
very effective on MT, corresponding to its ability in vitro to decrease cytosolic Ca2+ levels of dystrophic myotubes (Pasquaun et al., 1998). Interestingly, IGF-1, whose cellular action seems rather to involve an increase in Ca2+ mobilization (Semsarian et al., 1999), was not as effective as taurine and PDN on MT.

Surprisingly, creatine was ineffective on both gC1 of DIA and EDL muscle fibers as well as on MT, in contrast with its high efficacy on muscle force in vivo. Creatine, being converted into phosphocreatine, is widely used as reservoir for quick energy source and clinical trials are currently ongoing in DMD patients and in other neuromuscular disorders (Klivenyi et al., 1999; Tarnopolsky and Beal, 2001). Even by assuming that its positive effect on muscle strength is mediated by the amelioration of nervous system function (Klivenyi et al., 1999), other discrepancies are still present. In vitro creatine supplementation to mdx myotubes counteracts the increase in cytosolic calcium by stimulating the activity of calcium ATPase pump due to the larger availability of ATP (Pulido et al., 1998). Thus, we should have observed an effect of creatine on MT. A possible explanation is that the mice had an overdose of creatine with the drug-enriched food, because creatine toxicity seems to involve muscle and cardiac tissue (Klivenyi et al., 1999; Tarnopolsky and Beal, 2001). In fact, Passaquin et al. (2002), who recently described the ability of creatine to counteract the first degeneration cycle occurring postnatally in mdx mice, performed the creatine feeding to pups with the milk.

In conclusion, we found a wide effectiveness of IGF-1, both on the progressive dystrophic degeneration, likely due to its ability to stimulate muscle regeneration, and on the exercise-induced damage, through an acute mechanism that deserves to be better investigated. Also, the results showed the efficacy of taurine, a natural component of skeletal muscle, already used as food supplement and almost free of side effects (Huxtable, 1992), in contrast with the deleterious effect of exercise. In light of this finding, we propose that taurine could be rapidly considered for clinical trials in DMD alone or in combination with other drugs.

Acknowledgments

We thank Prof. Alfredo Gori for kindly providing IGF-1 and Dr. Loredana Montanari for helpful assistance during some of the in vivo experiments.

References


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