Contractile properties of clenbuterol-treated mdx muscle are enhanced by low-intensity swimming

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Hayes, Alan, and David A. Williams. Contractile properties of clenbuterol-treated mdx muscle are enhanced by low-intensity swimming. J. Appl. Physiol. 82(2): 435–439, 1997.—The β2-agonist clenbuterol has potent anabolic properties in normal and denervated muscle and, as such, may be of use in muscle wasting diseases such as muscular dystrophy. However, potential side effects such as the transformation of the fiber type pool toward increased proportions of fast-twitch fibers must be balanced with the beneficial anabolic properties. In the present report, we clearly show that extensor digitorum longus and soleus muscles from dystrophic mdx mice exposed to a combination of clenbuterol and low-intensity endurance swimming exercise did not undergo the slow- to fast-twitch fiber transformations caused by clenbuterol administration alone, yet increases in the force-generating capacity of the soleus (30–40%) that resulted from the clenbuterol treatment were maintained after the swimming program. The increased sensitivity of dystrophin-deficient dystrophic muscle to clenbuterol and low-intensity exercise that is evident in this study may have therapeutic implications in the treatment of muscle wasting diseases.

β2-agonist; endurance exercise; muscle fiber; muscular dystrophy

The possibility exists that the β2-agonist clenbuterol could be of use in ameliorating some of the progressive muscle wasting in muscular dystrophy due to its potent anabolic effects in both normal (4, 10, 21, 22) and atrophied (1, 3, 27) skeletal muscle. We recently showed that in mdx mice, which are genetically homologous to Duchenne muscular dystrophy sufferers (8) and also lack the protein dystrophin (14), the masses of both fast- and slow-twitch skeletal muscle are increased after clenbuterol treatment, with the slow-twitch soleus also exhibiting increased force-generating capacity (12). In another recent study in irradiated mdx mice (where gamma irradiation was used to destroy mitotically active satellite cells in an attempt to inhibit the pronounced regenerative capacity of mdx muscle; Ref. 23), these anabolic effects have been confirmed in the extensor digitorum longus (EDL) (28). However, chronic clenbuterol treatment also causes significant conversions of slow- to fast-twitch fibers (12, 26). Because fast-twitch fibers are preferentially affected in dystrophin-deficient muscular dystrophy (15, 24), slow- to fast-twitch transformations may render dystrophic muscle at greater risk of damage and degeneration. We report that a low-intensity endurance exercise program is able to prevent the slow- to fast-twitch fiber type conversions that occur with chronic application of clenbuterol, yet the strength-increasing effects of this β2-agonist are retained. Prevention of potentially deleterious fiber type transitions provides further evidence that carefully controlled exercise can have beneficial effects on dystrophic muscles (9, 11, 13).

MATERIALS AND METHODS

Male dystrophic mdx mice were randomly separated into three groups: sedentary (Sed; n = 8), sedentary clenbuterol-treated (Clen; n = 8), and endurance-trained clenbuterol-treated (Exer; n = 7). All procedures were in accordance with The University of Melbourne and National Health and Medical Research Council of Australia guidelines. Clenbuterol treatment (Clen and Exer groups) began at 5 wk of age and followed a treatment protocol we have outlined previously (12) for a duration of 15 wk. Briefly, clenbuterol was administered at a dose of ~2 mg·kg−1·day−1 via drinking water, continuously at 10 mg/l for 1 wk and then following a 2–2, 3-day on-off protocol at 5 mg/l to avoid attenuation of the response to clenbuterol that occurs after 2 wk of continuous administration at this concentration (A. Hayes, personal observations). At 6 wk of age (after 1 wk of clenbuterol treatment), Exer mice began 1-h sessions of low-intensity exercise 5 days/wk for 14 wk. All Exer mice were placed in a large Perspex tank (1.4 × 0.6 × 0.64 m) filled with water (35–36°C) to a depth that was greater than the length of a mouse from nose to tip. Mice initially performed 5 min of swimming; this time was rapidly increased over the next 2 wk until Exer mice performed 1 h of continuous low-intensity (unweighted) swimming, an exercise level that was maintained for the remainder of the experimental period. All mice successfully completed the exercise program. A group of mice exposed to the exercise only was not deemed necessary, since 3 h of daily continuous swimming had little effect on the contractile properties of mdx mice of the same age as used in the present study (A. Hayes, personal observations). Furthermore, to obtain such contractile changes, high-intensity (weighted) swimming is required, as was employed in a previous study where mdx mice performed 2-h daily sessions (5 days/wk) of high-intensity (weighted) swimming (11). The present low-intensity exercise program (1 h/day) alone is not expected to cause alterations to the contractile properties and is more closely related to the type and intensity of program that could realistically be implemented with human sufferers of neuromuscular diseases.

Mice from all groups were killed by cervical dislocation at 20 wk of age, and the EDL and soleus muscles were removed and weighed. Muscles were mounted to a force-recording apparatus and bathed in Krebs-Henseleit mammalian Ringer (maintained between 20 and 22°C), which was aerated with CarboGen (5% CO2-95% O2; Commonwealth and Industrial Gases). Routine isometric contractile properties were measured by stimulating the muscles with supramaximal square-wave pulses (1 ms in duration) delivered via two parallel platinum electrodes. Maximum tetanic tensions were elicited at a frequency of 70 Hz at a pulse duration of 3.2 ms for the soleus and 90 Hz at a duration of 2.75 ms for EDL (2, 12). All measurements were made at the optimal muscle length, the length at which individual maximal twitch tension was achieved. Contractile responses were recorded and analyzed on an eight-channel MacLab analog-to-digital converter (Ana-
logue Digital Instruments) coupled to an Apple Macintosh
Ici computer by using the data-acquisition program chart
3.3.2 (Analogue Digital Instruments). Peak twitch and
tetanic tensions were expressed relative to muscle mass, a
representation previously used in some functional studies of
dystrophic muscle (2, 12, 13, 20), to investigate any alter-
ations in the specific force-generating capability of the muscles.
Muscles were snap frozen in isopentane cooled in liquid
nitrogen. Serial sections were stained for myosin adenosinetri-
phosphatase (ATPase) activity according to a metachromatic
dye-ATPase method (19) and for succinic dehydrogenase
activity. At least 100 fibers were counted from each section,
equal to 10–20% of the total number of muscle fibers. Fiber
proportions were determined from sections stained for myo-
sin ATPase activity, and individual fiber areas were measured
from muscle sections stained for succinic dehydrogenase
activity by using a cursor-driven image analysis program, as
outlined previously (13).

RESULTS AND DISCUSSION
The most remarkable and potentially important func-
tional effects of the combination of clenbuterol and
low-intensity exercise are the abolition of the fiber type
transformations from slow- to fast-twitch and the reduc-
tion of the muscle fiber hypertrophy of the major fiber
types, which normally result from chronic clenbuterol
administration alone. It was essential to determine
whether the program of low-intensity swimming, when
coupled with chronic anabolic treatment, had differen-
tial effects on the functional properties of fast- and
slow-twitch muscle fibers. As such, we have looked at
the properties of two widely investigated muscles: the
EDL, a predominantly fast-twitch muscle, and the
soleus, a mixed muscle not unlike the majority of
human muscles in fiber type composition.
The EDL and soleus of Clen animals exhibited signifi-
cantly higher proportions of type II (A and B) muscle
fibers compared with the same muscles of the Sed
group (Table 1), an observation consistent with the
previously reported capacity of clenbuterol to cause
transitions from slow-to-fast-twitch fibers (12, 26). In
fact, the slow-twitch (type I) content of the Sed EDL
was completely abolished by the clenbuterol treatment.
In addition, all fiber types (types I, IIA, and IIB) in both
muscles were significantly increased in cross-sectional
area by the clenbuterol treatment (Fig. 1). The in-
creased cross-sectional area of the fibers occurs because
of both an increase in protein synthesis and a decrease
in protein degradation (10, 21), whereas the fiber type
transitions are likely to occur in response to altered
gene expression, promoting an increase in the propor-
tion of fast-twitch fibers. The altered gene expression
caused by clenbuterol is likely to be either 1) activity
related (i.e., a modification of recruitment of, or fre-
quency of use of, motor units), an effect that needs a
link between activity and gene expression, or 2) chemi-
cally mediated (i.e., clenbuterol itself, or a product of its
activity, must influence gene expression). Irrespective
of the cause, the alterations in muscle fiber characteris-
tics may render the dystrophic muscles more prone to
damage and degeneration, as it has been clearly shown
that larger caliber fibers, and fast-twitch fibers in

Table 1. Isometric contractile and histochemical properties of EDL and soleus muscles

<table>
<thead>
<tr>
<th></th>
<th>Sed Group</th>
<th>Clen Group</th>
<th>Exer Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass, g</td>
<td>32.2 ± 0.5</td>
<td>33.9 ± 0.3</td>
<td>31.5 ± 0.6</td>
</tr>
<tr>
<td>Muscle mass, mg</td>
<td>19.0 ± 0.7</td>
<td>21.1 ± 0.5</td>
<td>17.2 ± 0.6a</td>
</tr>
<tr>
<td>TTP, ms</td>
<td>45.6 ± 0.3</td>
<td>42.5 ± 0.3</td>
<td>44.3 ± 0.3b</td>
</tr>
<tr>
<td>RTₚ, ms</td>
<td>50.1 ± 4.0</td>
<td>39.1 ± 1.0</td>
<td>42.6 ± 2.3</td>
</tr>
<tr>
<td>P₀, mg</td>
<td>8.7 ± 0.7</td>
<td>8.6 ± 0.5</td>
<td>8.5 ± 0.7</td>
</tr>
<tr>
<td>P₀/MM, g/mg</td>
<td>0.46 ± 0.04</td>
<td>0.41 ± 0.03</td>
<td>0.50 ± 0.04</td>
</tr>
<tr>
<td>P₀, g/MM</td>
<td>39.8 ± 2.5</td>
<td>41.7 ± 2.1</td>
<td>40.2 ± 3.5</td>
</tr>
<tr>
<td>P₂/MM, g/mg</td>
<td>2.10 ± 0.13</td>
<td>1.99 ± 0.13</td>
<td>2.34 ± 0.18</td>
</tr>
<tr>
<td>Type I/IIC, %</td>
<td>19.1 ± 2.5</td>
<td>0.0</td>
<td>26.4 ± 4.2a,†</td>
</tr>
<tr>
<td>Type IIA/IIB, %</td>
<td>80.9 ± 2.5</td>
<td>100.0</td>
<td>73.6 ± 4.2a,†</td>
</tr>
<tr>
<td>Soleus muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle mass, mg</td>
<td>17.8 ± 0.9</td>
<td>20.6 ± 0.8</td>
<td>17.1 ± 0.7</td>
</tr>
<tr>
<td>TTP, ms</td>
<td>107.5 ± 5</td>
<td>80.1 ± 6.3</td>
<td>93.8 ± 3.6</td>
</tr>
<tr>
<td>RTₚ, ms</td>
<td>214.3 ± 19</td>
<td>130.1 ± 10</td>
<td>137.3 ± 22</td>
</tr>
<tr>
<td>P₀, mg</td>
<td>4.3 ± 0.4</td>
<td>6.2 ± 0.4</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td>P₀/MM, g/mg</td>
<td>0.26 ± 0.03</td>
<td>0.30 ± 0.02</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>P₀, g/MM</td>
<td>30.3 ± 2.2</td>
<td>42.7 ± 1.8</td>
<td>40.2 ± 2.6b</td>
</tr>
<tr>
<td>P₂/MM, g/mg</td>
<td>1.74 ± 0.18</td>
<td>2.08 ± 0.08</td>
<td>2.11 ± 0.13a</td>
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<tr>
<td>Type I/IIC, %</td>
<td>59.2 ± 5.6</td>
<td>50.2 ± 2.8</td>
<td>60.9 ± 3.4</td>
</tr>
<tr>
<td>Type IIA/IIB, %</td>
<td>40.8 ± 5.6</td>
<td>49.8 ± 2.8</td>
<td>39.2 ± 3.4</td>
</tr>
</tbody>
</table>

Values are ± SE. EDL, extensor digitorum longus; Sed, sedentary; Clen, sedentary clenbuterol treated; Exer, endurance-
trained clenbuterol treated; MM, muscle mass; TTP, time to peak twitch tension; RTₚ, half-relaxation time; P₀, peak twitch tension; P₂-
peak tetanic tension. Differences were analyzed with one-way analy-
sis of variance, with detected differences compared with Student-
Newman-Keuls post hoc comparison test. Significantly different
compared with Sed at: *P < 0.05; †P < 0.01; ‡P < 0.001. Significantly
different compared with Clen at: ¥P < 0.05; #P < 0.01; ‡P < 0.001.

particular, are preferentially affected in the dystrophic
process because they are more susceptible to damage in
the course of normal muscle contraction (15, 24). Impor-
tantly, these potentially deleterious features were pre-
vented (fiber transformations) or markedly attenuated
(fiber hypertrophy) by the application of low-intensity
activity throughout the clenbuterol administration pe-
riod. The total abolition of type I fibers from EDL and
the significant decrease in the proportion of this fiber
type in the soleus muscle of Clen mice were not evident
in Exer animals because the fiber type proportions
were not significantly different from those of Sed ani-
mals in either muscle type. Evidently, frequent periods
of even low-level activity are sufficient to override the
stimulus for fiber conversion provided by chronic den-
buterol administration. This suggests that although
exercise is not required for the anabolic effects of clen-
buterol to be evident, activity patterns do modify
these effects to a significant extent. However, whether
this activity directly affects gene expression in an
opposing manner to clenbuterol or rather inhibits the
effect of clenbuterol needs to be elucidated.

Fiber type transformations are also expected to con-
tribute to the faster contraction times exhibited by
dystrophic muscles after clenbuterol treatment. The
time-to-peak and half-relaxation times were both signifi-
cantly reduced in the EDL and soleus of Clen mice
(Table 1), a functional characteristic consistent with
the presence of the measured higher proportions of fast-twitch fibers in each of these muscles. As expected from the previously mentioned results, exercise also prevented the alterations to faster contraction times in both muscles, with the exception of the half-relaxation time in the soleus muscle, which remained significantly shorter in Exer mice compared with Sed animals.

These observations strongly suggest that a factor other than the fiber type composition also contributes to the faster contraction times in the soleus muscle of Exer animals because the soleus is not significantly different in fiber type distribution in Sed and Exer animals. Of the potential causal factors, alteration in the Ca\(^{2+}\) handling properties of muscle, which have been suggested in response to \(\beta_2\)-adrenergic stimulation (7), are the most likely to be responsible for this phenomenon. Faster contraction speed would be seen in the Exer soleus if the capacity of the sarcoplasmic reticulum to release and resequester activating Ca\(^{2+}\) was directly enhanced by clenbuterol treatment. This is an extremely interesting possibility given the previous reports of altered Ca\(^{2+}\) handling in dystrophic muscle (5, 6, 25) and is open to further investigation.

Also consistent with our previous results (12) is the observation that the masses of both EDL (11%) and soleus (16%) were increased by chronic clenbuterol administration alone (see Table 1). Although the force-generating capacity of EDL was not significantly affected by this treatment, both the absolute twitch (38%) and tetanic (41%) tensions of the soleus were significantly increased by clenbuterol. These force increases are readily accounted for by the significant increase in mass of the soleus muscle in Clen animals, which is reflected in the pronounced hypertrophy of both types I and II fibers. As a result, the relative force measurements (peak twitch tension/muscle mass and peak tetanic tension/muscle mass) were not different in the Sed and Clen groups. However, when clenbuterol administration was combined with the low-intensity exercise program, the increases in soleus muscle mass were prevented, whereas the increases in muscle absolute twitch and tetanic force were not prevented. Accordingly, the relative tetanic tension (peak tetanic tension/muscle mass) of the soleus muscle from Exer mice was significantly higher (21%) than that of Sed mice. Because the soleus muscle was able to maintain increased force levels in the absence of the changes in muscle size normally caused by clenbuterol, this strongly suggests that there was an increase in the specific tension-generating capacity of this muscle. Although the precise mechanism by which this occurs is presently unknown, the ability of clenbuterol to influence the force output of the slow-twitch soleus is important for its potential as a therapeutic agent for muscular dystrophy.

Treatments that show a specific benefit for slow-twitch muscle fibers, which remain in high proportions in dystrophic muscle, may have functional importance, since it is the larger fast-twitch fibers that are preferentially affected in muscular dystrophy (15, 24).

It is perhaps surprising that both muscle fiber types of dystrophic animals can be influenced by clenbuterol, since the majority of studies on normal muscle indicate that it is the fast-twitch fibers that primarily respond to the anabolic effects of this agent (see Ref. 16). Indeed, it is only the type IIB fibers of the EDL from C57BL/10ScSn mice (the control strain for mdx mice) that exhibit significant hypertrophy in response to the same clenbuterol administration protocol used in the present study (unpublished observations). However, in mdx muscles, the area of all major fiber types from EDL
[types I (145%), II A (115%), and II B (89%)] and the soleus [types I (171%) and II A (139%)] were significantly increased after clenbuterol administration (see Fig. 1). This indicates that both slow- and fast-twitch muscles of mdx mice can respond to clenbuterol. Hence muscular dystrophy appears to increase the sensitivity of skeletal muscles (particularly slow-twitch muscles) to clenbuterol. Although this increased sensitivity may occur due to increased receptor density, alterations in receptor affinity or activity are also likely, given that differences do occur in the response of a muscle to different doses of β2-agonists (17).

Thus this study has clearly shown that clenbuterol is able to increase the mass and force-generating capacity of dystrophic muscle and, in particular, that of the slow-twitch fibers, the joint application of a low-intensity exercise program such as unweighted swimming has the important additional benefits of preventing or reducing the potentially deleterious fiber type conversions and fiber hypertrophy that also result from this anabolic treatment. Although the dose of clenbuterol used in this study is far above the “safe” therapeutic dose in humans (1 mg·kg−1·day−1), dystrophic muscle appears to be more sensitive to clenbuterol than normal muscle, indicating that lower doses may also be effective. In addition, because therapeutic doses of clenbuterol have been shown to be effective in increasing relative muscle strength in orthopedic patients (18), this study supports the use of low-intensity exercise and clenbuterol as a potential form of therapy in muscle-wasting diseases such as muscular dystrophy.

Perspectives. Although the present study investigated the effects of swimming and clenbuterol on the regenerated muscles of mdx mice, the results do have important implications for the treatment of human dystrophic patients. The study has shown that these interventions can influence dystrophin-deficient muscle fibers. Although the regenerated mdx muscle fibers are resistant to further degeneration and hence would not be affected by the fiber type transitions caused by clenbuterol, human dystrophic fibers do undergo repeated episodes of degeneration and regeneration. Thus increases in the proportions of fast-twitch fibers in human dystrophic muscles must be an important consideration. Furthermore, potential treatments must target slow-twitch muscle fibers, since they are less susceptible to damage and thus higher proportions would remain to respond to any interventions. Clenbuterol increased the force-generating capacity of the slow-twitch soleus muscle, and low-intensity exercise maintained the slow-twitch fiber proportions. Because clenbuterol has shown the potential to be a form of therapy for human muscle wasting (18), exercise may be an easy drug-free way of minimizing possible deleterious effects. However, low-intensity exercise (preferably non-weight bearing, such as swimming) needs to be chosen so that human sufferers of the disease would be able to complete the treatment. The combination of clenbuterol and low-intensity exercise may prove to be an important way to delay the progression of muscular dystrophy in human patients and hence improve their quality of life.

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