Effects of β₂-agonist clenbuterol on biochemical and contractile properties of unloaded soleus fibers of rat

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Clenbuterol is a β₂-adrenergic agonist known to produce muscle hypertrophy (6, 16) and to improve functional capacity by increasing muscular strength (8, 33). Dodd et al. (8) showed that this increase in strength was due to hypertrophy of slow- and fast-twitch fibers, whereas others generally demonstrated a preferential fast-twitch fiber hypertrophy (7, 16). Numerous studies have indicated qualitative changes in the phenotypic expression of protein isoforms, i.e., transitions from slow to fast myosin heavy chain (MHC) isoforms, in slow-twitch clenbuterol-treated soleus muscle (7, 18). Such transitions in myosin isoforms are well known to influence the functional characteristics of skeletal muscles (17, 24, 31). Thus, according to the effects of clenbuterol on growth and muscular strength, the study of its action at the myofibrillar protein level appeared to be of particular interest. Moreover, many works have reported the effects of clenbuterol on whole muscles, but its action at cellular and molecular levels remained unknown. Thus a fundamental question would be whether the anabolic effects of the drug themselves can explain the improvement in functional capacity and/or whether a specific effect of the β₂-adrenergic pathway may improve the myofilament responsiveness. For instance, stimulation of β₂-adrenoceptors is known to induce the phosphorylation of many proteins (e.g., membrane proteins and kinases) (32).

In inherited muscle dystrophy (mdx mice) (25) and after denervation (1, 33), clenbuterol has been described as an agent able to reduce muscle atrophy. Another type of atrophy related to muscle disuse is a result of hindlimb unloading (HU). The HU conditions are generally induced in rats by the hindlimb suspension model (22), in which load bearing by the lower extremities is prevented. This experimental model, generally employed to mimic the effects of weightlessness, involves a number of muscular alterations, including 1) losses in mass and force, clearly marked in muscles used in weight bearing, such as the slow-twitch soleus muscle (26, 27, 30), 2) changes in fiber type distribution and protein isoform composition from slow to fast type (30, 31), and 3) corresponding modifications in the functional properties of soleus muscle (20, 26).

Hence, the present study was undertaken with two main objectives. The primary goal was to investigate the method of clenbuterol action at an intracellular level by using single skinned fibers. The skinned fiber preparation has a great advantage over intact fiber, in that it allows free access to contractile proteins and permits study of the Ca²⁺ sensitivity of the myofilament system as the marker molecule in which to assess muscle plasticity because of its abundance in striated muscles and its highly extended range of isoforms.

MATERIALS AND METHODS

Animals and Samples

Adult male Wistar rats (initial body weight ~250 g) were divided randomly into four groups: 1) control (n = 4), 2)
treated with clenbuterol (Sigma Chemical, St. Louis, MO) via their drinking water with an intake of 0.6 mg/day in 20 ml of water for 2 wk (CB, n = 8), 3) HU by the tail suspension model of Morey (22) for 2 wk (HU, n = 4), and 4) treated with clenbuterol during the HU period (HU-CB, n = 9). The suspension apparatus used for the HU and HU-CB groups consisted of an overhead swivel that permitted 360° rotation and allowed the rats to walk freely on their forelimbs and have free access to food and water. Clenbuterol-treated rats received 20 ml of water plus clenbuterol daily for 2 wk. All the rats drank at least this quantity, and water alone was then provided ad libitum. This protocol ensured that each rat received the same dose of clenbuterol during the treatment. This drug concentration (30 mg/l) has been previously shown to be effective in promoting maximal growth of several types of muscles (5, 19, 33). This manner of administering the drug was chosen because it enabled us to avoid manipulation of the HU rats to prevent stress and mobility of the fast myosin isoforms.

The body weight of each rat was measured before and after the different treatments. On the 15th day, the rats were anesthetized with ethyl carbamate (1 mg/kg body wt ip). The soleus muscles from all rats were immediately removed and weighed. Some control extensor digitorum longus muscles were chosen to be effective in promoting maximal growth of several types of muscles (5, 19, 33). This manner of administering the drug was chosen because it enabled us to avoid manipulation of the HU rats to prevent stress and 2) because water absorption has previously been described to be as effective as injections (9, 18). All the rats were individually caged on a 12:12-h light-dark cycle at 23°C room temperature. The animals, as well as the maintenance conditions of the animals, were authorized by the Ministries of Agriculture and Education (Veterinary Service of Health and Animal Protection Authorization 03805).

The body weight of each rat was measured before and after the different treatments. On the 15th day, the rats were anesthetized with ethyl carbamate (1 mg/kg body wt ip). The soleus muscles from all rats were immediately removed and weighed. Some control extensor digitorum longus muscles were taken and used as indicators for the electrophoretic mobility of the fast myosin isoforms.

Electrophoretic Analysis of MHC Isoforms

One soleus muscle of each rat was frozen in liquid N2 and stored at −80°C until analyzed. Fifteen 20-µm-thick muscle sections were removed from three scattered parts of each biopsy, pooled, and treated as described by Carraro and Catani (4). One microgram of protein was then loaded into each electrophoretic well. The MHC isoform content was studied by SDS-PAGE according to the method of Hämaäläinen and Pette (12). The stacking and separating gels consisted of 4.5 and 7.5% polyacrylamide, respectively. Electrophoresis was performed using a vertical chamber (model SE600, Hoefer) at 12°C for 24 h (180 V constant, 13 mA per slab). After the gel run, the gel slabs were silver stained, and a laser scanning densitometer (Quantiscan Microvial Systems, Bio-soft) was used to determine the relative proportion of the different MHC isoforms in each muscle.

Functional Analysis on Single Skinned Fibers

Muscle skinning. The other soleus muscle of each rat was chemically skinned by exposure to an EGTA skinning solution (see Solutions) for 24 h and stored at −20°C in a 50% glycerol-50% skinnning solution (storage solution), as described by Mounier et al. (23). Experiments were carried out on single-fiber segments isolated from the skinned muscles. Experimental procedures and force measurements. Before each experiment, the isolated fiber segment was bathed twice for 7 min in a 2% Brij solution (see Solutions). This procedure irreversibly eliminated the sarcoplasmic reticulum, whereas the actomyosin system remained intact (10). The single-fiber segment was transferred to the experimental chamber containing the relaxing solution (R solution). One end of the fiber was tied by a silk thread to a fixed forceps and the other end to a hook connected to a strain gauge force transducer (model BG10, Kulite; sensitivity 0.70 V/g). The output of the gauge signal was amplified, recorded on an ink recorder (model 6120, Gould), and analyzed with a computer program. The fiber sarcomere length was measured in the R solution with use of the diffraction pattern of an He-Nelaser beam crossing the preparation perpendicularly. It was adjusted to a length of 2.60 µm, which allowed maximal isometric tension (P0) to be elicited, and was readjusted when necessary. The mounted fiber was viewed through high-magnification (>80) binoculars with a micrometer that allowed fiber diameter measurements. All the experiments were conducted at 19 ± 1°C.

At the beginning of each experiment, P0 was elicited by application of a fully activating solution with a pCa of 4.2. The tension-pCa (T-pCa) relationship was then established as described previously (27). Briefly, each amplitude of tension (P) obtained in solutions of various pCa was normalized to P0. The corresponding ratio P/P0 was related to the pCa. The same procedure was used to obtain the Sr2+-contraction activation properties (T-pSr relationship), except the fiber was maximally activated in pSr 3.4 solution. About seven fibers per muscle were analyzed in this way. Fibers on which the pCa protocol could not be applied were rejected (when the fiber broke before the end of the experiment or when a decrease in P0 of 20% was recorded).

The experimental data for the T-pCa relationship were fitted to the Hill equation: P/P0 = ((Ca2+)]/K1)/(1 + ([Ca2+]/K1)]; where P/P0 is the normalized tension, nH is the Hill coefficient, K is the apparent dissociation constant (pK = −logK = pCa50), and [Ca2+] is Ca2+ concentration. Several parameters can be derived from the T-pCa (T-pSr) relationship, including 1) the pCa50 (pSr50) value, which represents the pCa at which the tension reaches 50% of the maximum Ca2+ (Sr2+)-activated tension response (pCa50 and pSr50 are indicators of the affinity of the contractile proteins and, more especially, of troponin C for Ca2+ and Sr2+, respectively); 2) the pCa50[pSr50 threshold value, i.e., the Ca2+ concentration necessary for the fiber to be activated and develop a detectable tension; and 3) the nH value, defined as the slope of the curve that indicates the degree of cooperativity between the proteins of the thin filament.

Physiological Fiber Type Identification: Ca2+- and Sr2+- Affinities

As mentioned above, several characteristics can be derived from the T-pCa and T-pSr relationships, especially pCa50 and pSr50. It is generally assumed that fast-twitch skeletal muscle fibers are less sensitive to Sr2+ than slow-twitch fibers (29). The difference (pCa50 − pSr50), or Δ value, was used to reflect the relative affinity of a fiber to Ca2+ and Sr2+. The statistical test of normality applied to the histogram of Δ distribution frequency revealed that two fiber populations could be discriminated (2 separate Henry's straight lines could be drawn from the relationship between the relative cumulated frequencies and Δ ranges; data not shown). In our study, a fiber exhibiting little difference between Ca2+ and Sr2+- affinities was identified as slow-type fiber (see Table 2). On the contrary, a fast-type fiber could be characterized by Δ > 1.00 (see Table 3). Fast-type fibers could be also distinguished from slow-type fibers by a higher Ca2+ threshold (lower pCa value) and a steeper T-pCa curve (higher nH value).

Solutions

The composition of all solutions was calculated as previously described (23), with a final ionic strength of 200 mM and a pH of 7.00. The EGTA skinning solution was composed of (in mM) 2.5 ATP, 20 MOPS, 170 potassium propionate, 2.5 1.00 D.
magnesium acetate, and 5 K2-EGTA. The following solutions were used for the experimental procedure: a washing solution composed of (in mM) 2.5 ATP, 20 MOPS, 185 potassium propionate, and 2.5 magnesium acetate; an R solution similar to the skinning solution; pCa (pSr)-activating solutions consisting of washing solution plus various concentrations of free Ca2+ (Sr2+) (from CaCO3 or SrCl2, respectively) buffered with EGTA; and a Brij solution composed of R solution plus 2% Brij 58.

To determine whether clenbuterol could act directly on the contractile proteins without requiring a second messenger, we performed a series of experiments on untreated animals (control or HU). The effect of clenbuterol was tested by adding the drug directly to the pCa solutions. Clenbuterol was applied at 50 µM, since this concentration corresponded to the level of the anabolic dose given in vivo (3). For each fiber, T-pCa relationships were established in the absence or presence of clenbuterol.

Statistical Analysis

Values are means ± SE. After one-way ANOVA, Student’s t-test was used as a post hoc test to establish the intergroup comparisons. P < 0.05 was chosen as level of significance.

RESULTS

Morphological Characteristics

After 2 wk of clenbuterol treatment, the body weight (BW) was unaltered, whereas the muscle wet weight (MWW) of the CB group tended to increase (Table 1). Thus the MWW/BW ratio increased significantly (18.5%) for the CB group compared with the control group. Two weeks of HU conditions caused decreases in BW and MWW, leading to a ~40% reduction in the MWW/BW ratio. After 15 days of clenbuterol administration, the MWW/BW ratio became greater in the HU-CB group than in the HU group. However, with regard to the control animals, the HU-CB rats exhibited persistent decreases in BW, MWW, and MWW/BW ratio.

Muscle Biochemical Analysis: SDS-PAGE

Clenbuterol administration to the control animals led to pronounced changes in the MHC isoform pattern (Figs. 1 and 2). MHCI, the predominant isoform in control muscles, decreased by 28% in the CB muscles. The fast MHC isoforms increased, with a fourfold elevation in MHCIIa, reaching a relative concentration of 25%. MHCIId(x) and MHCIIb, normally not detected in soleus muscles, were induced, reaching relative concentrations of 4.3 and 3.5%, respectively. In HU conditions, MHCI decreased, while MHCIId(x) (6.5%) and MHCIIb (4.2%) were induced. The profile of the HU-CB rats resembled that of the HU rats, but MHCIIa was elevated threefold compared with the control group.

Table 1. Comparison of body and muscle weights among the different groups

<table>
<thead>
<tr>
<th></th>
<th>Cont</th>
<th>CB</th>
<th>HU</th>
<th>HU-CB</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>367.5±18.3</td>
<td>344.4±7.8</td>
<td>265.8±11.5*</td>
<td>264.5±6.2*</td>
</tr>
<tr>
<td>MWW, mg</td>
<td>142.4±8.6</td>
<td>156.8±4.8</td>
<td>61.0±2.0*</td>
<td>71.5±4.2*</td>
</tr>
<tr>
<td>MWW/BW, mg/g</td>
<td>0.38±0.01</td>
<td>0.45±0.01*</td>
<td>0.23±0.01*</td>
<td>0.27±0.01†</td>
</tr>
</tbody>
</table>

%Change from Cont: +18.5, -39.5, -28.9

Values are means ± SE of number of animals indicated in parentheses. Mean rat body weight at the beginning of the different treatments was 256 ± 0.63 g (n = 25). Cont, control untreated group; CB, clenbuterol-treated group; HU, hindlimb-unloaded group; HU-CB, hindlimb-unloaded and clenbuterol-treated group. BW, body weight; MWW, muscle wet weight. *Significantly different from Cont; †significantly different from HU.
Table 2. Effects of CB and/or HU on contractile characteristics of slow-twitch fibers from soleus muscles

<table>
<thead>
<tr>
<th></th>
<th>Cont (n = 26)</th>
<th>CB (n = 52)</th>
<th>HU (n = 22)</th>
<th>HU-CB (n = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter, µm</td>
<td>83.3 ± 8.3</td>
<td>93.8 ± 4.7</td>
<td>53.1 ± 2.1*</td>
<td>67.9 ± 3.7†</td>
</tr>
<tr>
<td>P₀, 10⁻¹ N</td>
<td>2.5 ± 0.3</td>
<td>5.1 ± 0.5*</td>
<td>0.8 ± 0.1*</td>
<td>2.1 ± 0.3†</td>
</tr>
<tr>
<td>P₀, kN/m²</td>
<td>49.4 ± 7.2</td>
<td>79.4 ± 7.0*</td>
<td>39.0 ± 3.9</td>
<td>60.3 ± 10.7†</td>
</tr>
<tr>
<td>pCa₅₀</td>
<td>6.17 ± 0.03‡</td>
<td>6.25 ± 0.09*</td>
<td>6.13 ± 0.05*</td>
<td>6.25 ± 0.06†</td>
</tr>
<tr>
<td>pCa₅₀</td>
<td>5.62 ± 0.03</td>
<td>5.75 ± 0.02*</td>
<td>5.66 ± 0.01</td>
<td>5.81 ± 0.03†</td>
</tr>
<tr>
<td>Δ</td>
<td>1.31 ± 0.2‡</td>
<td>1.30 ± 0.08*</td>
<td>1.26 ± 0.07</td>
<td>1.27 ± 0.04†</td>
</tr>
<tr>
<td>n₇</td>
<td>3.85 ± 0.66‡</td>
<td>4.14 ± 0.46*</td>
<td>4.44 ± 0.43</td>
<td>4.63 ± 0.77†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of fibers. *Significantly different from Cont; †significantly different from HU. ‡Significantly different from slow-twitch fibers within an experimental group.

Table 3. Effects of CB and/or HU on contractile characteristics of fast-twitch fibers from soleus muscles

<table>
<thead>
<tr>
<th></th>
<th>Cont (n = 12)</th>
<th>CB (n = 12)</th>
<th>HU (n = 8)</th>
<th>HU-CB (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter, µm</td>
<td>89.4 ± 2.7</td>
<td>89.5 ± 1.9</td>
<td>52.7 ± 2.1*</td>
<td>66.7 ± 2.2†</td>
</tr>
<tr>
<td>P₀, 10⁻¹ N</td>
<td>3.3 ± 0.3</td>
<td>4.6 ± 0.3*</td>
<td>1.0 ± 0.1*</td>
<td>2.0 ± 0.2†</td>
</tr>
<tr>
<td>pCa₅₀</td>
<td>53.2 ± 3.9</td>
<td>74.9 ± 4.1*</td>
<td>47.0 ± 4.0</td>
<td>61.1 ± 5.4†</td>
</tr>
<tr>
<td>Δ</td>
<td>0.27 ± 0.02</td>
<td>0.42 ± 0.03*</td>
<td>0.27 ± 0.03</td>
<td>0.45 ± 0.02†</td>
</tr>
<tr>
<td>n₇</td>
<td>2.37 ± 0.08</td>
<td>2.48 ± 0.09</td>
<td>2.66 ± 0.15</td>
<td>3.05 ± 0.13†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of fibers. *Significantly different from Cont; †significantly different from HU; §significantly different from slow-twitch fibers within an experimental group.

Clenbuterol action on control and atrophied fibers

The present study examined the effects of clenbuterol, a β₂-adrenoceptor agonist, on the biochemical and contractile properties of slow- and fast-twitch fibers from normal and atrophied soleus muscles. By using skinned fiber preparations, we were able to investigate the changes at a subcellular level, i.e., in maximal force and Ca²⁺ sensitivity of the contractile proteins, and thus to distinguish the anabolic effect of clenbuterol from its β₂-adrenoceptor effect.

Hypertrophic Effect of Clenbuterol on Whole Muscle and Single Fibers

The MWW/BW ratio increased by ~18% in normal and atrophied clenbuterol-treated soleus and could be related to the anabolic effect of the drug. This effect appeared more focused on muscle mass than on BW, although the increases in MWW after clenbuterol treatment (CB and HU-CB groups) were not large enough to be statistically significant compared with control and HU groups, respectively. However, the larger MWW/BW ratio after clenbuterol treatment confirmed that clenbuterol induced changes in muscle protein synthesis (14), probably by increasing the ratio of RNA to protein content (6, 19).
At the single-fiber level, the hypertrophic effect of clenbuterol was not obvious, the mean fiber diameters being not significantly larger than those of the control group. Nevertheless, the fast-twitch fibers appeared more responsive than the slow-twitch fibers. The hypertrophic effect became even more evident in atrophied muscles. In fast- and slow-twitch fibers in which diameters were decreased after HU (26, 30), clenbuterol produced 1) a complete diameter recovery of the fast-twitch fibers and 2) a slighter recovery of diameters from slow-twitch fibers. Such fiber-type differences in the hypertrophic effect of β-adrenoceptor drugs have been previously described (16, 33). We can postulate that clenbuterol was able to limit atrophy of the 2-wk unloaded soleus muscles by its specific action on fast-twitch fibers. The fact that clenbuterol can prevent the
loss of α-actin mRNA that normally occurs after 7 days of hindlimb unloading (2) suggested a possible gene regulation by the drug of the expression of slow and fast contractile proteins.

Clenbuterol-Induced Changes in the Expression Pattern of MHC Isoforms

Two weeks of clenbuterol treatment induced a slow-to-fast conversion of normal slow-twitch soleus, as described after 6 wk of clenbuterol treatment. Contrary to our results [increase of MHCIIa and expression of MHCIIId(x) and MHCIIb], no difference in MHCIIa expression was shown, but a large increase was observed in MHCIIId(x). This suggested that, in our conditions, after a 2-wk clenbuterol treatment, the transition from MHCI toward fast isoforms might be limited and that a larger transformation, such as the transition to MHCIIId(x), might occur with longer periods of drug treatment. Hence, our findings support the theory that slow-to-fast clenbuterol-induced transitions occurred in the order MHCI → MHCIIa → MHCIIId(x) → MHCIIb, previously suggested, but in the fast-to-slow order, by Pette and Staron (24) after chronic low-frequency stimulation of extensor digitorum longus muscle. Moreover, after 2 wk of clenbuterol administration, we observed a more pronounced effect on the protein isoform expression than on the anabolic response. Thus the shift from slow to fast fiber type with longer-term β-agonist treatment would become visible when the anabolic response was attenuated (21). Finally, it seemed that clenbuterol treatment of atrophied muscles had no additional effect on the changes in MHC isoform pattern already induced by HU, i.e., an increase in fast MHC isoforms MHCIIa, MHCIIId(x), and MHCIIb and a concomitant decrease in MHCI expressions.

Effect of Clenbuterol on P₀

Clenbuterol induced a reinforcement of absolute and normalized P₀ developed by normal and atrophied single soleus muscle fibers. The increases in normalized P₀ were larger for the fast populations (+60% for the CB group and +72% for the HU-CB group) than for the slow populations (+41% for the CB group and +30% for the HU-CB group). This observation was well correlated to the preferential effect of clenbuterol on the fast-twitch fiber. However, the anabolic effect of clenbuterol estimated by the MWW/BW ratio and the single-fiber diameters was not as obvious as that observed for the tension measurements, especially in normal muscle fibers. Therefore, the stimulating effect of clenbuterol on the maximal force development should be interpreted by one or several additional mechanisms, besides the increase due to protein accretion.

A first assumption may be that each cross bridge becomes able to develop higher strength because of some changes in contractile protein properties (see Effect of Clenbuterol on the Contractile Characteristics of Single Muscle Fibers). A second possibility is that the increase in protein content, especially myofibrillar proteins, contributes to a reduction in the interfilament spacing, promoting subsequently an increase in muscular strength (11, 20).

Effect of Clenbuterol on the Contractile Characteristics of Single Muscle Fibers

Clenbuterol treatment of normal and atrophied muscles induced an increase in the Ca²⁺ sensitivity of the contractile proteins (leftward shift of the tension-pCa relationships), whereas no change was observed for the cooperativity parameter (unchanged nH values). The former effect was opposed to a previous result, which described a rightward shift of the T-pCa curve (18). However, these authors carried out experiments on mice treated with clenbuterol for 15 wk. The decrease in Ca²⁺ sensitivity observed in these conditions might be due to species-related differences, since, in contrast to our results in the rat, clenbuterol has been shown to alter the contractile properties of fast- and slow-twitch fibers of the mouse (13), or it might be due to a toxic effect caused by high dosages resulting from long-term treatment and comparable to that observed on cardiac muscle (15).

Possible Cellular Mechanisms Underlying the Positive Action of Clenbuterol

A fundamental question concerning the mechanisms involved in the anabolic effect of clenbuterol is whether its action can be related to the stimulation of the β₂-adrenoceptor. In the skinned fiber technique, the plasma membrane of muscle fibers is permeabilized and the β₂-receptors are mostly removed. This compromises the possible accumulation of soluble second messengers. Interestingly, it appeared that, in agreement with another work (3), clenbuterol directly added to the activated solutions in the experimental chamber did not induce any change in the maximal force or Ca²⁺ sensitivity. This suggested that the clenbuterol treatment effects on muscle functional properties were due to changes in regulatory mechanisms controlled by the plasma membrane or cytosolic components that were able to modify the contractile proteins. β-Adrenoceptor agonists are able to enhance the responsiveness of the myofilaments to Ca²⁺ through receptor-mediated events (phosphorylation of proteins involved in the excitation-contraction coupling) (32). Among them, a better phosphorylation of the myosin light chain II might be suggested. Indeed, the stimulation of the β₂-adrenoceptor triggers a cascade of kinase phosphorylations (e.g., myosin light chain kinase) and, thereby, sensitizes the myofibrils to Ca²⁺, leading to a leftward shift of the T-pCa curve (28). This hypothesis does not rule out possible phosphorylations of other contractile or regulatory proteins producing increases in Ca²⁺ sensitivity. The leftward shift of the T-pCa curve appeared more evident in the atrophied slow-twitch soleus fibers, which suggests that 1) atrophied muscles are more influenced by clenbuterol than normal muscles and 2) the largest shift in slow-twitch fibers might be related to the higher density of β₂-adrenoceptors in slow-twitch muscles (16).
In conclusion, clenbuterol in normal and atrophied soleus muscles appeared to act as an anabolic drug as well as a β-agonist agent. The stimulating effect of the β-adrenergic pathway appeared to produce more important changes in muscle properties than the process of clenbuterol-induced protein accretion. Because clenbuterol was able to lessen some effects of HU conditions, the potential clinical benefit of treatment by this agonist, for reducing loss of mass and forces in atrophied muscles, should be underlined.

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