Effects of the $\beta_2$-agonist clenbuterol on respiratory and limb muscles of weaning rats

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Pollà, B. V. Cappelli, F. Morello, M. A. Pellegrino, F. Boschi, O. Pastoris, and C. Reggiani. Effects of the $\beta_2$-agonist clenbuterol on respiratory and limb muscles of weaning rats. Am J Physiol Regulatory Integrative Comp Physiol 280: R862–R869, 2001.—The aim of this study was to analyze the effects of chronic administration of the $\beta_2$-agonist clenbuterol (1.5 mg·kg$^{-1}$·day$^{-1}$ for 4 wk in the drinking water) on respiratory (diaphragm and parasternal intercostal) and hindlimb (tibialis and soleus) muscles in young rats during postnatal development (21 to 49 postnatal days). The treatment resulted in very little stimulation of muscle growth. Significant slow-to-fast transitions in the expression of myosin heavy chain isoforms and significant increases in the myofibrillar ATPase activity were found in the diaphragm and soleus, whereas tibialis anterior and intercostal muscles did not show any significant fiber-type alteration. Decrease of oxidative enzyme activities and increase of glycolytic enzyme activities were also observed. It is concluded that whereas the growth stimulation is age dependent and only detectable in adult rats, the fiber-type transformation is also present in weaning rats and particularly evident in the soleus and diaphragm. The fiber-type transformation caused by clenbuterol might lead to an enhancement of contractile performance and also to a reduced resistance to fatigue.

$\beta_2$-agonist clenbuterol is a compound widely used for the treatment of asthma, in relation to its relaxant action on smooth muscle, and to promote muscle growth in humans as well as in farm animals.

Rat skeletal muscles have served as a model for a series of studies that have shown the ability of clenbuterol to increase muscle and body mass (11, 20). Interestingly, two studies on newborn rats could not show any stimulatory effect on muscle growth; on the contrary, a reduction of muscle size and protein content was observed. The first study was based on administration of clenbuterol to the mothers (19), whereas the second study was based on both direct administration to the puppies and indirect administration to the lactating mothers (21). Thus the possibility that clenbuterol did not reach the newborn rats was excluded, but the mechanism of growth suppression remained unexplained. To our knowledge, no study has focused on the early postweaning period that coincides with the fastest increase of rat body weight and muscle mass. In addition to the effect on muscle growth, clenbuterol chronic treatment has been found to produce a fiber-type transition characterized by replacement of slow fibers with fast fibers (34). The effect has not yet been completely characterized in terms of energy-metabolism pathways or in terms of myosin heavy chain (MHC) isoforms, which are considered the best molecular markers of fiber phenotype (27). Also, it is still unknown whether these changes occur during early development when the growth-stimulation effect is not present.

In this study, we aimed to investigate the effects of chronic clenbuterol administration in weaning rats. This age is of particular interest because during this period, the rate of increase of muscle mass attains its peak and because it should be the most suitable age for comparison with clenbuterol treatment in humans. Actually, clenbuterol is mainly used to treat children with asthmatic disease. Because of this therapeutic use of clenbuterol, we thought to study not only the hindlimb muscles, generally considered in previous studies, but also the respiratory muscles. In subjects affected by bronchial asthma, the respiratory muscles meet increased functional demands. It is important to assess whether clenbuterol treatment, which aims for bronchial dilation, can also modify the contractile power or the resistance to fatigue of respiratory muscles. Following this reasoning, in addition to the soleus and tibialis anterior, which represent a typical slow and a typical fast muscle, respectively, we studied the diaphragm, the main inspiratory muscle, and the parasternal and intercostal muscles that are accessory inspiratory muscles.
METHODS

Animal treatment. The study was carried out on Wistar rats born and maintained at the animal facilities of the Institute of Human Physiology, University of Pavia. Young rats were separated from the mothers on the 21st postnatal day and housed separately in individual cages. Two groups were formed: control group and treated group, the latter received clenbuterol in the drinking water for 28 days. Both groups had free access to water and food, and the intake of each animal was recorded every other day. During the entire experimental period, control rats consumed 951 ± 81 ml of water with a daily average of 34 ml and clenbuterol-treated animals consumed 995 ± 124 ml of water with a daily average of 35.5 ml. Clenbuterol concentration in the water was adjusted so that daily consumption was 1.5 mg/kg body wt, similar to that employed in other studies (34).

At the end of the experimental period, the animals were killed by decapitation. The heart and the following muscles were removed and weighed: diaphragm, soleus, tibialis anterior, and parasternal intercostal muscles. The muscles were frozen in fluid nitrogen and stored at −80°C until they were used for various determinations.

Seven groups of control and seven groups of treated rats were studied. Each group was composed of six animals; the muscles of five animals were pooled together and employed for determinations of enzyme activities (see below) and electrophoretic analysis, whereas the muscles of the sixth animal were employed for histological analysis. Five control and treated groups were composed of male rats; the remaining two groups were composed of female rats. No differences in body or muscle weight, myosin isoform composition, and enzyme activities were detectable. The seven groups were therefore used without distinction between males and females.

Determination of myofilament ATPase activity. The muscles dissected from treated and control animals were pooled together to reach approximately the amount of 1 g. They were then cut in little pieces with fine scissors and homogenized for 1 min in cold imidazole buffer (0.3 M sucrose, 10 mM imidazole; pH 7). The resulting homogenate was placed in a centrifuge (J2–21, Beckman, Fuller, CA) for 15 min at 13,000 rpm at 4°C. From this preparation, washed and purified myofibrils were obtained according to a procedure previously described (6). The protein content in the myofibrillar preparation was determined with the Lowry method (16) using bovine serum albumin as standard.

Enzymatic activity of myofibrils was assayed at 27°C in a medium with the following millimolar composition: 50 KCl, 2 MgCl2, 2 NaATP, 20 imidazole, 1.6 EGTA, 1.7 CaCl2 (pH 7; pCa 4.5). This medium ensured maximal Ca activation of the myofibrils. The amount of inorganic phosphate released was determined with the Fiske-Subbarow method and expressed in micromoles per milligram of protein per minute.

MHC isoform composition. MHC isoforms were used as molecular markers to assess the fiber-type composition of each muscle, and their expression was studied with electrophoretic and immunohistochemical methods.

From each myofibrillar preparation, ~1 mg of purified myofibrils was put in 1 ml of Laemmli solution (14) to be then used for SDS-PAGE. Small amounts (2 μl corresponding to 500 ng of myosin) of each sample dissolved in Laemmli solution were applied on 8% polyacrylamide gels prepared according to the method described by Talmadge and Roy (29). Electrophoresis was run for 24 h at 250 V, and gels were silver stained. In the region of MHC isoforms (molecular weight (MW) ~220), four bands were separated (Fig. 1) that corresponded, in order of migration from the fastest to the slowest, to MHC-1 or slow, MHC-2B, MHC-2X, and MHC-2A. The relative proportions of these four MHC isoforms were determined with the use of a computer-assisted densitometer. The areas under the peaks corresponding to the MHC isoforms on the densitometric readings were measured, and the area of each peak was expressed as a fraction of the total area of the four peaks. No correction for MW was calculated. Immunohistochemistry and muscle fiber cross-sectional area.

From muscles of control and treated animals, thin (10 μm) sections were cut in a cryostat and immunostained, as previously described in detail (5), using three monoclonal antibodies against MHC isoforms: BA-F8 against MHC-1, SC-71 against MHC-2A, and G6 against MHC-2B. No antibody was available for fibers containing MHC-2X. Three fiber types (type 1, type 2A, and type 2B) could be identified, and their cross-sectional areas (CSA) were determined and expressed in micrometers squared. Images at ×100 magnification were fed from a video camera fitted to a light microscope (Laborlux, Leitz, Germany) to a personal computer and were analyzed with a specifically designed image-analysis software (Symga, RTI, Monza, Italy). Measurements were repeated in the four selected muscles of control and treated animals. About 100 fibers per muscle were measured, and the results were pooled together to form a control and a treated group.

Glycolytic and oxidative metabolic enzymes. A portion of the muscle samples was washed with a cold solution of sucrose EDTA (sodium salt), quickly freed from the muscle sheath, and weighed. The tissue was then finely minced, homogenized with a Polytron tissue processor (Kinematica Instruments) for 5 s, and subsequently homogenized in 0.25

![Figure 1](http://ajpregu.physiology.org/)

Fig. 1. Examples of the effects of clenbuterol on myosin heavy chain (MHC) isoforms in the soleus (A) and diaphragm (B). The appearance of the fast isoform MHC-2X in the soleus and the altered distribution of MHC isoforms in the diaphragm are visible.
M sucrose and 1 mM EDTA in a precooled Potter-Braun S homogenizer. The homogenate was diluted with 0.25 M sucrose EDTA; 1 g of tissue in 10 ml of sucrose solution. This homogenate was then centrifuged at 800 g for 15 min in a refrigerated centrifuge (Beckman J2–21; rotor JA-20). Part of the supernatant fluid thus obtained was used to determine enzyme activity in the crude extract, and/or for protein evaluation, part of the fluid was stored. The sediment was rehomogenized in 0.25 M sucrose EDTA and centrifuged at 800 g for 15 min. The two supernatants obtained were centrifuged at 14,000 g for 20 min. The mitochondrial sediment was gently resuspended in sucrose solution at a final dilution of 100 mg of sediment in 1 ml of sucrose. An aliquot of this preparation was used to assess the protein content, whereas the remaining portion was used to evaluate enzyme activities.

The maximum rates (V_{max}) of the following two enzyme activities were evaluated in the crude extract and/or in the mitochondrial fraction: phosphofructokinase (PFK) (EC 2.7.1.1) for the anaerobic glycolytic pathway and cytochrome oxidase (COX) (EC 1.9.3.1) for the electron transfer chain. Enzyme activities were recorded graphically for at least 3 min with a double-beam recorder spectrophotometer (Beckman 35), and each value was calculated from two blind determinations on the same sample. Enzyme-specific activities were expressed as nanomoles of substrate transformed per milligram of protein per minute.

**Statistical analysis.** Data are given as means ± SE. The significance of the difference between control and treated groups was assessed by Student's t-test. A probability of 0.05 or less was considered statistically significant.

**RESULTS**

**Effects of clenbuterol on body and muscle growth.** The average values of body weight at the beginning and at the end of the treatment are shown in Table 1. The 4 wk of the treatment period corresponded to fast-rate growth period with an average rate of ~6 g/day. This value was approximately two times greater than the average of 3 g/day of the first 3 wk after birth and was also greater than the average of ~2 g/day of the following (7th to 12th) weeks of postnatal growth. The rate of body growth of clenbuterol-treated rats was slightly above the rate of control rats, but the difference did not reach statistical significance. The muscle weight/body weight ratio of clenbuterol-treated rats was also slightly above the values of control animals.

<table>
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<tr>
<th>Table 1. Body weight at beginning and end of treatment</th>
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<tr>
<td>Control</td>
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<td>Initial body weight, g</td>
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<td>Final body weight, g</td>
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<td>Body weight gain, g</td>
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<td>Heart/body, %</td>
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<td>Soleus/body, %</td>
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<td>Tibialis ant./body, %</td>
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Values are means ± SE. Body weight at the beginning and at the end of the treatment with clenbuterol and ratios of the weights of heart, soleus, tibialis anterior, and diaphragm to body weight at the end of the treatment in controls and clenbuterol-treated animals.

*P < 0.05.

The difference was, however, statistically significant only for tibialis anterior. The heart weight/body weight ratio was also increased by clenbuterol treatment, indicating the development of cardiac hypertrophy (+18%).

**Effects of clenbuterol on MHC isomorf expression and myofibrillar ATPase activity.** Figure 2 shows the MHC isomorph composition of the four selected muscles in control and clenbuterol-treated rats. Although a general trend toward an increase of the proportion of the fast MHC isoforms was present in all muscles, significant differences were found only in the soleus and diaphragm. Examples of the changes in these two muscles are shown in Fig. 1. In the soleus, the reduction of the slow MHC isoform (MHC-1) was accompanied by the appearance of the MHC-2X isoform, which was not expressed in control rats. In the diaphragm, the increase of MHC-2B was associated with a decrease of the three other MHC isoforms.

The values of Ca Mg-dependent myofibrillar ATPase activity are shown in Fig. 3. Significant increases of ATPase activity occurred in the soleus and diaphragm of treated rats, as expected to follow the increased proportion of fast MHC isoforms. The ATPase activity was determined also in myofibrils prepared from ventricular myocardium. The activity of control (0.26 ± 0.01) and in treated (0.27 ± 0.01) rats was not significantly different.

**Effects of clenbuterol on mitochondrial myofibrillar activity.** The values of the enzyme activities of PFK and COX are shown in Fig. 4. As can be seen, the activity of PFK, a key enzyme of the glycolytic pathway, increased significantly only in the diaphragm and intercostal muscles of the treated animals. On the contrary, the treatment produced a significant increase of the enzyme activity of COX, complex IV of the mitochondrial electron transfer chain in all muscles examined.

**Effects of clenbuterol on fiber CSA.** Figure 5 shows some examples of cryosections of soleus immunostained with antibodies specific to MHC-1 (A, C) and MHC-2A (B, D). The increase of the relative number of fast 2A fibers (reactive with SC-71) and the decrease of the proportion of slow fibers (reactive with F8) are detectable by visually comparing the left panels (control rats) with the right panels (clenbuterol-treated rats). Average CSA of fibers recognized with antibodies specific to various MHC isoforms is reported in Fig. 6. The CSA of the fibers reactive with G6 and therefore containing MHC-2B increased significantly in the diaphragm and in the superficial part of tibialis anterior. This would suggest that the increase in MHC-2B concentration (see Fig. 2) was due partly to hypertrophy of the fibers expressing this myosin and partly to fiber transformation. On the other hand, the CSA of both the fibers reactive with F8 (slow fibers) and the fibers reactive with SC-71 (fast 2A fibers) decreased significantly in the soleus muscle. The decrease of MHC-1 content in the soleus of the treated rats (see Fig. 2) was therefore partly due to hypertrophy of slow fibers and partly due to fiber transformation (see Fig. 5). A significant decrease in CSA of fast 2A fibers (reactive with...
SC-71) was also present in the deep region of tibialis anterior of treated rats.

DISCUSSION

In this study, we investigated the changes induced by chronic clenbuterol administration in the skeletal muscles of rats during the phase of rapid postnatal growth. Previous studies (1, 34) had validated the dose (1.5 mg·kg body wt$^{-1}$·day$^{-1}$) and the administration route (via drinking water) as suitable to produce fiber-type transformation and muscle hypertrophy. The results obtained in this study showed that during the postweaning developmental phase, clenbuterol administration induces little or no acceleration of growth but is able to significantly alter muscle fiber phenotype with regard to myofibrillar protein composition and function as well as metabolic enzyme activity.

Effect of clenbuterol on muscle growth. The stimulation of muscle growth triggered by clenbuterol has been demonstrated in a variety of animal species from mice (3) and rats (11) to farm cattle [cow and sheep (8, 33)], and it is very likely present also in humans (24). In contrast to this, two independent studies have shown that clenbuterol administration to lactating mothers or direct intramuscular administration to the pups both produce a loss of body weight in newborn rats (19, 21).

In the postweaning rats considered in this study, the effect of clenbuterol on muscle growth was very modest. Some signs of growth stimulation were observed; for example, tibialis anterior weight increased and the thickness of fast 2B fibers increased in the tibialis anterior and the diaphragm. The thickness of slow and 2A fibers, however, decreased in the soleus and tibialis anterior.
anterior (deep part). Thus the effect of clenbuterol on muscle growth appears to be age dependent, with the effect in the postweaning period (21–50 postnatal days) being intermediate between the negative effect observed in the first postnatal week (19, 21) and the stimulatory effect described in young adult rats (11).

The anabolic effect of clenbuterol is mediated by $\beta_2$-adrenergic receptors (7, 18) and mainly based on stimulation of protein synthesis (18), although inhibition of proteolysis has also been reported (12). The reason for the age dependency of the clenbuterol effect might be related with the development of the responsiveness to $\beta_2$-adrenergic stimulation. The present results, however, do not contribute to an understanding of the underlying mechanism.

Effects of clenbuterol on muscle fiber phenotype. The present results provide evidence that chronic clenbuterol administration induces significant changes of fiber phenotype, even in conditions where little or no anabolic effect is present. The observed changes in fiber phenotype are in agreement with previous observations on rat soleus and extensor digitorum longus (34) and also with observations on the effects of other $\beta$-agonists such as cimaterol (4). Compared with previous studies, however, this work extends the analysis to the electrophoretic separation of all four adult MHC isoforms, their immunohistochemical identification, and the determination of enzyme activity.

In the postweaning period (20–55 postnatal days) examined in this study, muscle maturation is not yet complete in the rat. Fiber maturation occurs asynchronously in different muscles. In the diaphragm and the present results provide evidence that chronic clenbuterol administration induces significant changes of fiber phenotype, even in conditions where little or no anabolic effect is present. The observed changes in fiber phenotype are in agreement with previous observations on rat soleus and extensor digitorum longus (34) and also with observations on the effects of other $\beta$-agonists such as cimaterol (4). Compared with previous studies, however, this work extends the analysis to the electrophoretic separation of all four adult MHC isoforms, their immunohistochemical identification, and the determination of enzyme activity.

In the postweaning period (20–55 postnatal days) examined in this study, muscle maturation is not yet complete in the rat. Fiber maturation occurs asynchronously in different muscles. In the diaphragm and...
intercostal muscles, embryonic and neonatal myosin isoforms are replaced by adult slow and fast isoforms within 20 postnatal days, whereas in fast and slow hindlimb muscles maturation is not complete before 30 postnatal days (9). The asynchronous maturation may offer an explanation for the different responses of muscles to the treatment. A more likely explanation, however, can be found by simply assuming that clenbuterol induces a slow-to-fast transition in MHC isoform expression. The slow-to-fast transition follows the sequence MHC-1 → MHC-2A → MHC-2X → MHC-2B (see Ref. 27 for a review). Depending on the initial muscle isoform composition, the transition can show up as a decrease of type 1 or slow fibers with a concomitant increase of type 2X as in the soleus, a complete transition involving type 1 fibers as well type 2B fibers as in the diaphragm, or virtually no change in muscles composed mainly of fast 2B fibers as in the tibialis anterior and intercostal muscles. In fast 2B fibers and in the “fast” muscle tibialis anterior, hypertrophic growth was stimulated, whereas the slow and 2A fibers became hypotrophic as indicated by the reduction in CSA. The mechanism by which clenbuterol produces the slow-to-fast fiber transformation and the growth of fast fibers is not clear. The density of β2-receptors is greater in slow than in fast fibers (31) and, accordingly, the increase of cAMP or the changes in resting potential is/are more pronounced in slow than in fast fibers (25). A recent study has shown that the clenbuterol-induced increase of cAMP mediates changes in intracellular calcium (23). Oscillations in intracellular calcium might activate signaling pathways that end on transcription regulation and that might differ in slow and fast fibers (22). Importantly, to promote the growth of the fast fibers, clenbuterol treatment must antagonize the contractile activity that stimulates the growth of the most active fibers i.e., the slow and fast 2A fibers of the diaphragm (rhythmic respiratory activity) and soleus (tonic postural activity). An enhancement of muscle contractile activity above physiological level can offset the clenbuterol effect of slow-to-fast transformation (17).

The shift of fiber phenotype from slow to fast found in this study might remind one of that observed in skeletal muscles during chronic heart failure (10, 28). In this study, the rats treated with clenbuterol developed cardiac hypertrophy. However, cardiac hypertrophy induced by clenbuterol in the rat is not associated with any alteration in cardiac function (32), with changes in isomyosin composition (32), or in myofibrillar ATPase activity (this study). Clinical and postmortem examination of the treated rats did not show any sign (as pulmonary or hepatic congestion) that clenbuterol treatment might have caused cardiac failure. It seems, therefore, very unlikely that cardiovascular alterations have played a role in modifying the fiber phenotype.

The MHC isoform transition in the soleus and diaphragm was accompanied by an increase of myofibrillar ATPase activity (this study). Clinical and postmortem examination of the treated rats did not show any sign (as pulmonary or hepatic congestion) that clenbuterol treatment might have caused cardiac failure. It seems, therefore, very unlikely that cardiovascular alterations have played a role in modifying the fiber phenotype.
tivity is proportional to maximum shortening velocity and power output (2, 26). Thus the contractile performance of the soleus and the diaphragm of young rats can be expected to be improved by chronic clenbuterol administration, as recently shown in adult emphysematous hamsters (30). In that study, however, no significant change in fiber-type distribution occurred, and only effects on specific force and fiber thickness were observed (30).

Until now, little attention has been paid to the transformation of fiber metabolic properties induced by clenbuterol administration. In this study, changes in metabolic enzyme were detected in all muscles; the activity of COX, taken as a marker of mitochondrial aerobic metabolism, decreased, and the activity of PFK, considered as a marker of glycolytic anaerobic metabolism, showed a trend to increase. These changes indicate a coordination between myosin isomorph composition and energy-metabolism pathways. In rat muscles, fatigue resistance and mitochondrial enzyme activity vary in direct proportion in different fiber types (13); slow fibers that contain MHC-1 exhibit the highest activity and fatigue resistance, whereas fast 2B fibers containing MHC-2B exhibit the lowest mitochondrial enzyme activity and resistance to fatigue. Fast 2A and 2X fibers have intermediate characteristics (13, 15). If clenbuterol treatment decreases the mitochondrial aerobic metabolism and, at the same time, increases the ATP hydrolysis rate in the myofibrils (replacement of slow with fast myosins), a reduction in the resistance to fatigue is expected. This might present a major drawback of the enhancement of the contractile performance induced by chronic clenbuterol administration.

Perspectives

This study provides the first complete analysis of the changes in MHC isoform expression and metabolic enzyme activity induced by clenbuterol in young postweaning rats. The results not only confirm that the anabolic effect of this compound is age dependent, but they also show that the fiber phenotype changes occur as soon as the fiber-type differentiation is complete. The shift from slow to fast and from aerobic to anaerobic phenotype might be responsible for a decreased resistance to fatigue of the main respiratory muscle, the diaphragm. We believe that it is worth undertaking long-term clinical studies to test if these effects occur during the therapeutic use of clenbuterol for respiratory diseases. The cellular mechanism of the changes induced by clenbuterol is still largely unknown. These results suggest a very interesting dissociation between the stimulation of the growth and the transformation of the fiber phenotype. Further studies focused on signaling pathways might help to clarify the mechanisms of the two effects.

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