Long-term effects of clenbuterol on diaphragm morphology and contractile properties in emphysematous hamsters

H. F. M. Van Der Heijden, H. M. Van Der, P. N. R Dekhuijzen, H. Folgering, L. A. Ginsel, and C. L. A. Van Herwaarden

Long-term effects of clenbuterol on diaphragm morphology and contractile properties in emphysematous hamsters. J. Appl. Physiol. 85(1): 215–222, 1998.—The aim of the present study was to investigate the effect of chronic long-term clenbuterol treatment (1 mg/kg subcutaneously twice a day for 12 wk) on diaphragm morphology and function in emphysematous (EH) and normal hamsters (NH). Clenbuterol increased body weight, diaphragm weight, and skeletal muscle weight in both EH and NH to a similar extent. In the diaphragm, clenbuterol significantly increased myosin heavy chain type I, IIA, and IIX muscle fiber cross-sectional areas by ~35–55% in both EH and NH. This response to clenbuterol treatment was not significantly different between EH and NH diaphragm. In EH, twitch force (P_t), maximal tetanic force, and force-frequency curve were significantly reduced compared with NH. In EH, clenbuterol increased P_t by ~10%, restoring P_t to NH level. A similar improvement was observed in the force-frequency characteristics. Clenbuterol did not alter contractile properties in NH. In conclusion, long-term clenbuterol treatment resulted in an increased size of all diaphragm muscle fiber types in both NH and EH. Clenbuterol completely abolished the reduced force generation induced by emphysema.

In contrast to the peripheral skeletal muscles, little is known about the effect of clenbuterol on function and morphology of respiratory muscles. A single dose of clenbuterol (10 and 20 µg/kg iv) increased transdiaphragmatic pressure (Pdi) in dogs in a dose-dependent fashion (19). Preliminary studies reported that administration of clenbuterol resulted in hypertrophy of both type I and type II muscle fibers in normal rat diaphragm after 2 and 10 wk of treatment (10, 22) or of type II muscle fibers after 2 wk of treatment (25). Functionally, however, these morphological changes were accompanied by either a reduction in force (22), or no effects on force generation and fatigue resistance (25), or a better preservation of Pdi after inspiratory resistive loading (10).

Whether clenbuterol can induce morphological and/or functional changes in the diaphragm of emphysematous animals is unknown. Induction of emphysema by intratracheal instillation of elastase in hamsters resembles the alterations found in patients with COPD to some extent. In the hamsters, lung volume and compliance were increased and diaphragm function was impaired (7, 13). We hypothesized that clenbuterol treatment will increase diaphragm muscle mass and cross-sectional area (CSA) and that this may (partly) restore the impairment of diaphragm function found in emphysema.

The aim of the present study was to investigate the effects of chronic clenbuterol treatment on diaphragm morphology in the elastase-induced emphysematous hamster (EH) model and to investigate whether these morphological changes are accompanied by improvement of contractile properties. We also wanted to investigate whether these effects are different from those found in normal, age-matched hamsters (NH). Because the actual presence of clenbuterol in the diaphragm will have a direct positive inotropic effect on diaphragm contractile properties, measurements of contractile properties were performed at least 24 h after the last dose of clenbuterol had been given.

METHODS

Animals, Induction of Emphysema, and Study Design

Adult male outbred golden hamsters (n = 80) were studied. At the age of ~40 wk the hamsters were anesthetized with a mixture of halothane and N_2O, vaporized in air. A polyethylene cannula was inserted into the trachea with the tip located above the carina, and a dose of 25 U/100 g body wt of elastase (type I elastase from porcine pancreas, Sigma Chemical, Bornem, Belgium) was injected intratracheally to induce emphysema (7, 13, 28). Control animals were treated with an equal volume of saline (0.5 ml/100 g body wt). To improve the distribution to the peripheral parts of the lung, 3 ml of room

Dysfunction of the respiratory muscles frequently occurs in patients with severe chronic obstructive pulmonary disease (COPD) (20). Those COPD patients with emphysema in particular are also often malnourished and underweight and have a reduced fat-free body mass, which may indicate muscle wasting (6). This condition is associated with a decrease in skeletal and respiratory muscle function (6) and with an increase in morbidity and mortality (32). Besides dietary supplementation and training, pharmacological interventions such as anabolic steroids, growth hormone therapy, or clenbuterol treatment may be considered to increase respiratory muscle mass and function.

Previous research in healthy animals has indicated that diaphragm contractile properties can be improved pharmacologically by administering β₂-adrenoceptor agonists (1, 19, 29, 30). Within this group of β₂-adrenoceptor agonists, clenbuterol has a special position because it is known to increase muscle cell growth and protein content both in vitro (16) and in vivo (5, 23). This effect was mediated by activation of the β₂-adrenoceptor (3, 26) and was accompanied by increased cAMP levels (17).

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air were injected through the tube. The hamsters were monitored carefully until spontaneous breathing was restored.

The animals were housed under standard conditions and were fed ad libitum. Six months after instillation of elastase or saline, the animals were randomly allocated to treatment groups. Thirty EH and 30 age- and weight-matched NH were treated for 12 wk with either clenbuterol (1 mg/kg subcutaneously twice a day) or saline control. Body weight was measured every 2 wk. Because clenbuterol may have a direct inotropic effect on the diaphragm (19), this could interfere with the assessment of the contractile properties in relation to morphological changes in the diaphragm. There are no data on the exact serum half-life of clenbuterol in hamsters. In other species this pharmacokinetic parameter has been reported to range from 9 h in rats to 30–35 h in rabbits and humans (33). Therefore, the last dose of clenbuterol or saline was administered ≥24 h before the experiment to minimize a potential direct inotropic effect of clenbuterol on diaphragm contractility.

Measurements of the various treatment groups were performed in random order; throughout the experiments the investigator (H. F. M. Van Der Heijden) was blinded with regard to treatment.

This study was approved by the Animal Experiments Committee of the University of Nijmegen and was performed in accordance with the Dutch National Guidelines for Animal Care.

General Procedure and Treatments

The hamsters were anesthetized with pentobarbital sodium (Nembutal, 70 mg/kg i.p.). A tracheotomy was performed, and a polyethylene cannula was inserted. The animals were mechanically ventilated with 100% O2. The diaphragm and adherent lower ribs were quickly excised after combined laparotomy and thoracotomy, and they were immediately submerged in cooled, oxygenated Krebs solution at pH ~7.4. This Krebs solution consisted of 137 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 1 mM KH2PO4, 24 mM NaHCO3, 7 mM glucose, and 25 µM d-tubocurarine chloride (Sigma Chemical). All further preparations (as described Diaphragm Muscle Morphometry) of the diaphragm muscle were performed in Krebs solution.

To evaluate the treatment effects on peripheral skeletal muscle mass, soleus muscle and extensor digitorum longus (EDL) muscle were dissected from the left hindlimb in each hamster. These muscles were blotted dry and weighed.

Diaphragm Muscle Morphometry

Muscle strips dissected from the middle costal part of the left hemidiaphragm were stretched and pinned at approximately optimal length (L o; obtained by dividing the excision length by 0.7 (21, 31)). This procedure does not selectively after the fiber size in the hamster diaphragm muscle; relative sizes of each fiber type remain unaltered (31). Subsequently, these muscle sections were quickly frozen in isopentane cooled in liquid N2 and stored at −80°C until further analysis. Serial cross sections were cut at 7 µm with a cryostat kept at −30°C.

Myosin heavy chain (MHC) antibodies (DSM, Braunsweg, Germany) were used for morphometric examination of serial diaphragm sections. The following antibodies were used: BA-D5 reactive with type I MHC, SC-71 reactive with type IIa MHC, BF-35 reactive with type I, IIa and IIb but not with type IIx MHC, and BF-F3 reactive with type IIb MHC (27). Incubation with these MHC antibodies was performed at room temperature for 1 h. Subsequently these MHC antibodies were incubated with secondary IgG or IgM antibodies labeled with ultrasmall immunogold reagent, followed by silver enhancement (Aurion, Wageningen, The Netherlands). The CSA of at least 250 fibers were examined from each diaphragm by using a Sprynt-based, personal computer-image digital analysis system (Bos, Waddinxveen, The Netherlands).

Measurement of Contractile Properties

From the middle costal region of the right hemidiaphragm, two rectangular strips were dissected parallel to the long axis of the muscle fibers. Silk sutures were tied firmly to both ends. The strips were suspended in two tissue baths containing Krebs solution, maintained at 37°C, and perfused with a 95% O2–5% CO2 mixture. The central tendon end was connected to an isometric force transducer (model 31/1437-10, Sensotec, Columbus, OH) mounted on a micrometer. Two large platinum stimulating electrodes were placed parallel to the bundles. Stimuli were applied with a pulse duration of 0.2 ms and a train duration of 400 ms and were delivered by a stimulator (ID-electronics, University of Nijmegen, Nijmegen, The Netherlands) activated by a personal computer. To ensure supramaximal stimulation, the strips were stimulated at ~20% above the voltage at which maximal forces were obtained (~6 V over stimulating electrodes). Data acquisition and storage of the amplified signal were performed by using a personal computer equipped with a Dash-16 interface (Twist-trigger software, ID-electronics).

Both strips were placed at their L o, defined as the length at which peak twitch force was obtained. The following protocols were performed.

Twitch characteristics. Two twitches were recorded at L o, to determine maximal twitch force (P t), contraction time (CT), and half-relaxation time (RT1/2). Maximal P t and corresponding time characteristics were used for further analysis.

Maximal tetanic force (P o). The diaphragm strips were stimulated twice at 120 Hz to obtain a plateau in force generation. The maximal force was defined as the P o.

Force-frequency characteristics. One muscle strip was stimulated at 2-min intervals at the following frequencies: 25, 50, 80, and 120 Hz.

Fatigue. The second diaphragm muscle strip was used to determine isometric fatigue properties. This strip was stimulated at 2-s intervals at 25 Hz (30-s train duration) for 5 min. Fatigue index (i.e., percentage of initial force) was determined at 2 and 5 min (13).

All these measurements were conducted within 20 min after the thermoequilibration period. Subsequently, the length of each diaphragm strip was measured by using a micrometer (model 560-128, Mitutoyo, Veendoraal, The Netherlands), and the strips were weighed. CSA was calculated by dividing diaphragm strip weight (g) by strip length (cm) times specific density (1.056). Forces were expressed per CSA (N/cm2), and the P o/P o ratio was calculated for each muscle strip.

Verification of Emphysema and Total Diaphragm Weight

In a separate experiment the presence and severity of emphysema was evaluated in 20 animals (NH + saline: n = 5; NH + clenbuterol: n = 5; EH + saline: n = 5; EH + clenbuterol: n = 5). After the hamsters were anesthetized as described in General Procedure and Treatment, the lungs were excised and inflated with 4% Formalin (pH 7.4) to a pressure of 25 cmH2O for 2 h, and subsequently postfixation lung volume was determined by fluid displacement. The lungs were fixated without external pressure in 4% Formalin for at least 5 days.
Subsequently, the left lung was embedded in paraffin, and sagittal sections (6-µm thickness) were cut and stained with hematoxylin-eosin. To determine the extent of emphysema, the changes in the lung, alveolar CSA of at least 200 alveoli was measured by using a Sypn-bright, personal computer-image digital analysis system (Bos). In addition, in this experiment the complete diaphragm was dissected, blotted dry, and weighed to determine the effects of clenbuterol treatment on diaphragm weight. These measurements were performed in separate groups of animals to avoid procedural bias like introducing extra time needed for the careful excision of the lungs and to prevent surgical or handling damage of the diaphragm needed for force measurements.

Data Analysis

Statistical analysis was performed by using a two-way ANOVA. The two experimental factors were disease state (presence or absence of emphysema) and treatment (clenbuterol or saline). In comparing the force-frequency relationship and body weight curves, a repeated-measurements design was used within this two-way ANOVA design. Post hoc analysis (Student-Newman-Keuls test) was used to compare differences in independent groups. The SPSS package (version 6.1.3, Chicago, IL) was used for statistical analysis. Results were considered significant at P < 0.05. All data are expressed as means ± SE.

RESULTS

Verification of Emphysema

Postfixation lung volume, determined by fluid displacement, was increased by ~50%. In saline-treated animals, lung volume increased from 7.8 ± 0.4 ml in NH to 11.6 ± 0.7 ml in EH (P < 0.05). In clenbuterol-treated animals, these values were 7.9 ± 0.5 and 11.9 ± 0.8 ml, respectively. The alveolar CSA was also significantly increased in saline-treated EH (5,147 ± 335 µm² in EH vs. 2,698 ± 91 µm² in NH; P < 0.05). Clenbuterol treatment did not alter the increase in alveolar CSA in EH (5,299 ± 350 µm² in EH + clenbuterol and 2,735 ± 106 µm² in NH + clenbuterol (P < 0.05)).

Effects of Clenbuterol Treatment on Body and Muscle Weight

Body weight and skeletal muscle weight were not significantly different at the start of the protocol. Clenbuterol treatment increased hamster body weight in both EH and NH significantly (P < 0.05; Table 1). After 12 wk of clenbuterol treatment, body weight was increased by ~9 and 12% in EH and NH, respectively. This response to clenbuterol treatment was not significantly different between EH and NH. In Fig. 1 the relative increase in body weight is presented; in both NH and EH clenbuterol treatment significantly increased relative body weight (P < 0.001, repeated-measurements ANOVA).

Soleus and EDL muscle weights were also significantly increased by clenbuterol treatment (P < 0.05; Table 1). When expressed as a percentage of body weight also, these muscle weights were significantly increased. Similar effects were found on diaphragm muscle weight (Table 1). No differences were found between the absence or presence of emphysema, and no interaction was found between the presence of emphysema and treatment.

Table 1. Anabolic effects of clenbuterol treatment on hamster body and peripheral skeletal muscle weight

<table>
<thead>
<tr>
<th></th>
<th>Emphysematous Hamsters</th>
<th>Normal Hamsters</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control (n = 15)</td>
<td>Clenbuterol (n = 15)</td>
</tr>
<tr>
<td>Body weight (start), g</td>
<td>148 ± 2.7</td>
<td>151 ± 3.9</td>
</tr>
<tr>
<td>Body weight (end), g</td>
<td>149 ± 2.7</td>
<td>164 ± 3.7*</td>
</tr>
<tr>
<td>Body weight increase, %</td>
<td>0.2 ± 0.7</td>
<td>8.7 ± 1.6*</td>
</tr>
<tr>
<td>Soleus weight mg</td>
<td>40.1 ± 0.9</td>
<td>48.9 ± 1.4*</td>
</tr>
<tr>
<td>%body wt</td>
<td>0.27 ± 0.00</td>
<td>0.30 ± 0.01*</td>
</tr>
<tr>
<td>EDL weight mg</td>
<td>33.3 ± 0.8</td>
<td>45.1 ± 0.9*</td>
</tr>
<tr>
<td>%body weight</td>
<td>0.22 ± 0.00</td>
<td>0.27 ± 0.01*</td>
</tr>
<tr>
<td>Diaphragm weight, %</td>
<td>2.06 ± 0.04</td>
<td>2.50 ± 0.05*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of hamsters. Diaphragm weight was assessed in a separate experiment (n = 5). EDL, extensor digitorum longus. *Significant increases as compared with saline treatment, P < 0.05.
and treatment for any fiber type or morphological parameter.

Clenbuterol treatment significantly increased CSA of types I and IIa in both EH and NH and IIx muscle fiber CSA in EH (Fig. 2, A–C). No differences in response to clenbuterol treatment were found between NH and EH.

### Table 2. Effects of clenbuterol treatment on diaphragm muscle fiber frequency and contribution to total diaphragm CSA

<table>
<thead>
<tr>
<th>Emphysematous Hamsters</th>
<th>Normal Hamsters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong> (n = 12)</td>
<td><strong>Clenbuterol</strong> (n = 10)</td>
</tr>
<tr>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Type I fiber</td>
<td>27.8 ± 1.2</td>
</tr>
<tr>
<td>Type IIa fiber</td>
<td>43.6 ± 1.7</td>
</tr>
<tr>
<td>Type IIx fiber</td>
<td>28.5 ± 1.8</td>
</tr>
<tr>
<td>%CSA</td>
<td></td>
</tr>
<tr>
<td>Type I fiber</td>
<td>19.3 ± 1.0</td>
</tr>
<tr>
<td>Type IIa fiber</td>
<td>34.6 ± 2.0</td>
</tr>
<tr>
<td>Type IIx fiber</td>
<td>46.0 ± 2.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of hamsters. CSA, cross-sectional area. *Significant increases compared with saline treatment, P < 0.05.

Clenbuterol treatment slightly altered the frequency of muscle fiber types (Table 2). Significant effects of clenbuterol treatment on fiber frequency were found in type IIa and type IIx muscle fibers. The relative contribution of each fiber type to the total CSA (percent CSA) was not altered by clenbuterol treatment compared with saline treatment (Table 2).

Finally, type IIb muscle fibers were very scarcely encountered in the diaphragms of these hamsters. These type IIb fibers were only observed in clenbuterol-treated animals. In 1 of 30 control-treated animals, type IIb fibers were found (in an EH diaphragm). After 12 wk of clenbuterol treatment, type IIb fibers were found in 5 of 15 NH and 4 of 15 EH diaphragm muscles. This may indicate that clenbuterol treatment appears to increase the expression of type IIb MHC. However, calculation of a mean CSA or fiber frequency from such a limited number of fibers does not result in reliable data. Therefore, we have not included these data in our analysis.

### Contractile Properties

Twitch characteristics and  \( P_o \). Emphysema significantly reduced \( P_t \), \( P_o \) and the measured \( L_0 \) of the diaphragm strips compared with NH (\( P < 0.05; \) Table 3). No interactions were found between emphysema and treatment for any parameter of diaphragm function.

### Table 3. Effects of clenbuterol treatment on diaphragm contractile properties

<table>
<thead>
<tr>
<th>Emphysematous Hamsters</th>
<th>Normal Hamsters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong> (n = 15)</td>
<td><strong>Clenbuterol</strong> (n = 15)</td>
</tr>
<tr>
<td>( P_t ), N/cm²</td>
<td>6.4 ± 0.25</td>
</tr>
<tr>
<td>( P_o ), N/cm²</td>
<td>26.9 ± 0.66</td>
</tr>
<tr>
<td>( P_o/P_t ) ratio, %</td>
<td>24.0 ± 0.6</td>
</tr>
<tr>
<td>CT, ms</td>
<td>26.8 ± 0.3</td>
</tr>
<tr>
<td>RT(_{1/2}), ms</td>
<td>26.7 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of hamsters. \( P_t \), maximal twitch force; \( P_o \), maximal tetanic force; CT, contraction time; RT\(_{1/2}\), half-relaxation time. *Significant increases compared with saline treatment, \( P < 0.05. \) §Significantly different from all other groups, \( P < 0.05. \)
tion. $P_t$ and $P_o$ were $\sim 10\%$ lower in the saline-treated EH compared with NH. In these EH, clenbuterol increased $P_t$ by $\sim 10\%$, restoring $P_t$ to the level in the saline-treated NH. In NH, $P_t$ was not affected by clenbuterol. CT was not altered by clenbuterol treatment in either NH or EH. $RT_{1/2}$ was decreased in both NH and EH ($P < 0.05$; Table 3). Again, long-term clenbuterol treatment increased $P_o$ by $\sim 7\%$ in these EH, restoring force production to a level similar to saline-treated NH. In NH, $P_o$ was not affected by clenbuterol. CT was not altered by clenbuterol treatment in either NH or EH. $RT_{1/2}$ was decreased in both NH and EH ($P < 0.05$; Table 3). Again, long-term clenbuterol treatment increased $P_o$ by $\sim 7\%$ in these EH, restoring force production to a level similar to saline-treated NH. In contrast, clenbuterol did not alter $P_o$ in NH. In none of the treatment groups was the $P_t/P_o$ ratio was altered (Table 3).

Force-frequency relationship. The force-frequency relationship was significantly reduced in EH compared with NH ($P < 0.05$, Fig. 3A). Clenbuterol treatment significantly increased the force-frequency relationship in EH, resulting in an upward shift of the force-frequency curve (Fig. 3A). However, a significant interaction was found between treatment and repeated measurements of absolute force. As a consequence, significant increases in force-frequency relationship were found for stimulation frequencies up to 80 Hz but not for higher stimulation frequencies. In EH, clenbuterol restored force production at these lower frequencies to the level similar to the force-generating capacity of NH diaphragm (EH treated with clenbuterol compared with saline-treated EH; $P < 0.05$). In contrast, clenbuterol treatment had no significant effects on the NH force-frequency relationship compared with saline treatment (Fig. 3A).

When the results found in this force-frequency protocol were expressed as a percentage of $P_o$, no differences were found between EH and NH (Fig. 3B). Clenbuterol treatment did not alter these relative force-frequency curves in EH. However, a significant interaction was again found between treatment and repeated force measurements ($P < 0.001$). Compared with saline treatment, clenbuterol caused a small but significant decrease in NH at stimulation frequencies of 80 and 120 Hz but not at the lower frequencies ($P < 0.05$; Fig. 3B).

Isometric fatigue. Fatigue index was not affected by either emphysema or clenbuterol treatment, and no interaction was found between these factors. In saline-treated animals, the fatigue index after 2 min was 58 ± 1% in NH and 59 ± 1% in EH. After clenbuterol treatment values were 61 ± 2% in NH and 59 ± 2% in EH. Also, after 5 min the fatigue index was not altered, with values ranging from 38 to 42%.

DISCUSSION

This study shows that long-term clenbuterol treatment increased body weight, diaphragm weight, and peripheral skeletal muscle weight in both NH and EH. Clenbuterol treatment resulted in hypertrophy of the MHC type I, IIa, and IIx diaphragm muscle fibers in EH and of type I and IIa fibers in NH diaphragm. In EH, this hypertrophy was accompanied by an increase in $P_t$, $P_o$, and force-frequency relationship, restoring force in these animals to the level found in NH. In contrast, long-term clenbuterol treatment had no effect on functional properties in NH diaphragm. Fatigue index was not altered in either EH or NH.

Anabolic Effects of Clenbuterol

In line with the anabolic potency of clenbuterol described in previous studies (3–5, 23, 34), we found a significant increase in hamster body weight, fast- and slow-twitch peripheral skeletal muscle weight (EDL and soleus muscle), and diaphragm fiber CSA. Skeletal muscle weight was increased after 12 wk of clenbuterol treatment, also when expressed as percentage of body weight, which may indicate a specific effect on muscle growth. After $>3$ wk of treatment, the clenbuterol-mediated increase in body weight seemed to attenuate (Fig. 1). In previous studies a similar attenuation of clenbuterol effect on body weight gain in young rats was found within 14 days of treatment (15).
applied to depend on the continuity of clenbuterol treatment (15) and may be explained by a reduction of β₂-adrenoceptor density found after continued exposure to clenbuterol (26).

The anabolic effects of clenbuterol are mediated via β₂-adrenoceptor activation with subsequent cAMP response (17). The precise mechanism of action of the clenbuterol-mediated growth-stimulating effect is not clear but appears not to be mediated by growth hormone or thyroid stimulation (5) or by elevated insulin levels (5). Several studies reported that increased muscle growth was accompanied by an increase in protein and RNA content and increased protein synthesis (indicated by an increased RNA-to-protein ratio) (3, 5). A reduction in protein degradation was suggested in another study (23).

Similar muscle-growth potentiating effects were found for salmeterol, another long-acting β₂-agonist (17). However, these effects were less potent than the clenbuterol effects and depended on the route of administration (17). Because the anabolic effects of short-acting β₂-adrenoceptor agonists like salbutamol, fenoterol or terbutaline were less potent (2, 3, 5, 24), it is likely that a long duration of action is needed to induce these anabolic effects.

Effects of Clenbuterol on Diaphragm Morphology and Function

The increase in specific force production found after long-term clenbuterol treatment in EH may, at least partly, be explained by the changes in diaphragm morphology. In EH the percentage of type IIx muscle fibers was increased, and CSA of every fiber type was increased in both EH and NH. This increase in type IIx/IIb muscle fiber CSA may influence force production because these muscle fibers generate the largest forces and represent the major part (~50%) of the total diaphragm CSA. The reduction in RT₁/₂ observed in the present study may be a reflection of the increase in type IIx muscle fiber CSA, because type IIx muscle fibers not only produce the largest forces but also have short CT and RT₁/₂. A reduction of RT₁/₂ is likely to have contributed to the changes in the shape of the force-frequency curve.

Besides these morphological alterations, other mechanisms may be involved in the clenbuterol-mediated improvements of P₁ and Pₒ. These may include alterations in biochemical processes involved in energy supply and consumption, changes in excitation-contraction coupling-related processes [including alterations in Ca²⁺ release from the sarcoplasmatic reticulum (SR)], or altered Ca²⁺ sensitivity (14). For example, the observation that clenbuterol-treated NH failed to reach 100% of P₁ during the force-frequency protocol (Fig. 3B) is in agreement with earlier observations from studies that used salbutamol in vitro (29, 30). This may be the result of a progressive depletion of the SR Ca²⁺ pool or may indicate that β-agonists have a predominant effect on type II muscle fibers (29, 30). It is possible that the activity of these processes was altered by chronic clenbuterol treatment, which may have influenced force generation in the present study.

Comparison of Effects of Clenbuterol on Peripheral Skeletal Muscle and Diaphragm

Because the diaphragm is a mixed skeletal muscle that is constantly active, it is difficult to compare our results with those found in peripheral skeletal muscles. Moreover, the diaphragm has a specific population of β-adrenoceptors, consisting solely of the β₂-subtype (9), whereas hindlimb muscles contain a mixture of both β₁ (15%) and β₂-adrenoceptors (85%) (26). Nevertheless, effects similar to the present study were found in young rats after long-term clenbuterol administration (1.6 mg·kg⁻¹·day⁻¹ per os for 2–12 wk) (34). In this study, clenbuterol increased type I and II muscle fiber CSA in fast-twitch rat EDL (34) and type II or type IIx fibers in soleus muscle (4, 34). This was accompanied by an increase in P₁ and Pₒ in soleus muscle and an increase in Pₒ in EDL (34).

The results of the present study are in agreement with the effects of clenbuterol on respiratory muscle morphology reported in two preliminary studies (22, 25). However, these morphological changes were accompanied by a reduction in force production and fatigue resistance in female rats after long-term treatment (10 wk) (22). No effects on force generation and fatigue resistance were found in male rats after 10 wk of treatment (22) or in male and female rats after 2 wk of clenbuterol treatment (25). Also, in rabbit diaphragm, a 2-wk clenbuterol treatment induced hypertrophy of type I, II, and IIb muscle fibers without altering the relative contribution to total CSA (10). Again, this hypertrophy was not accompanied by an increase in Pdi in fresh diaphragm, but, after inspiratory resistive loading, tetanic Pdi was better preserved (10).

These morphological findings in diaphragm and skeletal muscles are in agreement with the results found in the present study. However, it is important to note that the aim of the present study was to measure functional effects of clenbuterol treatment on diaphragm morphology and not the direct inotropic effects of clenbuterol. The discrepancies between the clenbuterol effects on contractile properties and fatigue resistance described in these studies (10, 22, 25) and the results found in skeletal muscles (34) can very well be influenced by the actual presence of clenbuterol during the assessment of these contractile properties, because clenbuterol has a clear inotropic effect on diaphragm function (19).

Methodological Considerations

The effects of emphysema on diaphragm force generation are not exactly clear. Previous studies by Farkas and Roussos (7, 8), Kelsen et al. (11), and Supinski and Kelsen (28) did not show a reduction in specific force in emphysematous diaphragm. In contrast, Lewis et al. (12, 13) reported a reduction in specific force of ~25% in emphysematous hamsters, compared with control-treated animals. In these studies, emphysema was induced by installation of elastase at 7–9 wk of age.
Clenbuterol and the Emphysematous Diaphragm

Clinical Relevance

The combination of anabolic effects on skeletal and respiratory muscles (4, 10, 22, 25, 34) and direct inotropic effects on respiratory muscle function (19) and bronchodilatation (18) of clenbuterol may be of clinical importance in the treatment of severe COPD patients. These patients, and particularly those with emphysema, are often malnourished and underweight or have a reduced fat-free body mass despite normal total body weight (6). This condition indicates the presence of muscle wasting, and it is associated with a decrease in function of peripheral skeletal muscles and of respiratory muscles and with an increase in morbidity and mortality (6, 32). These patients are often unable to meet their increased nutritional demands.

Besides dietary supplementation, pharmacological interventions such as anabolic steroids, growth hormone therapy, or clenbuterol treatment may be considered. Clenbuterol has clear anabolic effects, both in normal skeletal muscle and in various models for diseases, including denervation and muscular dystrophy. The present study shows that, in EH diaphragm, long-term clenbuterol treatment increases fiber CSA of types I, IIa, and IIx muscle fibers, but it also increases specific force, restoring it to the level of NH diaphragm. To the best of our knowledge, this is the first study that shows that the reduced function of the emphysematous diaphragm can be restored pharmacologically. In theory, clenbuterol treatment in COPD patients could therefore have a multiple mode of action, but it may be used specifically to increase both respiratory muscle mass and function. Long-term clinical studies are needed to evaluate whether clenbuterol improves respiratory muscle function in patients with COPD.

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Address for reprint requests: P. N. R. Dekhuijzen, Dept. of Pulmonary Diseases, Univ. Hospital Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands (E-mail: P.Dekhuijzen@ong.azn.nl).

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