Skeletal muscle myosin heavy chain isoforms and energy metabolism after clenbuterol treatment in the rat

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Clenbuterol is a synthetic β2-adrenoceptor agonist that has been used clinically to induce bronchial dilation after inhalation (typical dose 0.60–0.86 μg daily). Its primary advantage over other bronchodilators is its much longer half-life (29). It has also been utilized in the livestock industry at higher doses (2–5 mg·kg body mass⁻¹·day⁻¹) to increase lean muscle mass and reduce fat content, a phenomenon that is known as repartitioning. Typically, daily treatment with clenbuterol at this higher dose has been shown to induce an increase in rat skeletal muscle mass of 10–20% (13, 26, 33). This is particularly significant, because the increase in mass occurs independently of any contraction-induced hypertrophy (2, 13, 16). Muscle growth under these conditions is characterized by an increase in the cross-sectional area of fast-contracting fibers, as determined by use of histochemical methods (20, 26, 38). Evidence for slow-fiber hypertrophy is equivocal (25, 38). However, prolonged treatment for a period of 6–8 wk has been shown to result in the conversion of slow-contracting fibers to fast-contracting fibers in the soleus, and to a lesser extent in the extensor digitorum longus (EDL) (7, 9, 38).

Despite the generally accepted observation that prolonged clenbuterol treatment causes a slow-to-fast fiber type conversion in rat skeletal muscle (38), we are unaware of any data relating to the effects of clenbuterol on muscle energy metabolism. This is surprising, given the primary link between changes in cellular energy charge and the initiation of skeletal muscle growth (3). Indeed, fast-contracting muscle is known to have a higher adenine nucleotide content and energy charge than slow-contracting muscle (12).

The anabolic effect of clenbuterol has raised the possibility that it may be useful as a treatment for loss of skeletal muscle mass due to limb immobilization (24). Previously, an investigation of the effects of 4 wk of daily clenbuterol treatment (−0.26 μg·kg body mass⁻¹·day⁻¹) on knee extensor strength in humans after meniscectomy failed to demonstrate a significant increase in absolute strength. However, a significantly more rapid rehabilitation of strength was observed in the clenbuterol-treated patients compared with controls (27). Importantly, in the same trial, clenbuterol treatment did not ameliorate the reduction in muscle cross-sectional area due to injury. Therefore, we investigated the effects of 8 wk of clenbuterol treatment on slow- and fast-contracting muscle of the rat at a higher dose (250 μg·kg body mass⁻¹·day⁻¹), which is within the range known to induce hypertrophy (33). Our aims were, first, to characterize whether this dose would induce slow-to-fast changes in myosin heavy chain...
(MyHC) protein isoform content similar to those reported for prolonged higher-dose treatment, and, second, to investigate whether any such changes were accompanied by alterations in cellular energy metabolism, a point that has not been investigated to date.

METHODS

Study Protocol

The study involved two groups of male Wistar rats (control, n = 6; treatment, n = 8). The starting body mass was 330–350 g, and the animals were 11–12 wk of age (Charles River, Margate, UK). The treatment and control groups were administered 250 μg·kg body mass · day⁻¹ of clenbuterol and an isovolume of saline, respectively, by subcutaneous bolus injection for 8 wk. This length of treatment was chosen because it has been previously reported to result in a change in fiber-type composition of rat skeletal muscle (38). All animals were weighed at least weekly, and food and water consumption was determined daily. Clenbuterol was not given on the final day, and the animals were killed in a randomized order. The soleus and the EDL muscles of the left limb were freeze-clamped in situ with precooled tongs, stored in liquid nitrogen, and then freeze-dried at a later date. The soleus and the EDL were selected because they are ideal for comparison of predominantly slow-contracting (soleus, 85% slow-twitch fibers) and fast-contracting (EDL, 96% fast-twitch fibers) muscles (8). After this, the corresponding muscles were dissected from the contralateral limb and were weighed.

SDS-PAGE

Frozen muscle samples, each from individual animals, were used to determine MyHC isoform expression. Approximately 10 μg of muscle tissue were homogenized in 100 μl of sample buffer, containing 62.5 mM Tris·HCl, 2.3% SDS (wt/vol), 10% glycerol (vol/vol), 5% 2-mercaptoethanol (vol/vol), and 0.001% bromophenol blue (wt/vol). After homogenization, samples were prepared for SDS-PAGE by heating to 60°C for 10 min in the sample buffer, followed by centrifugation at 12,000 g for 5 min at 4°C. Samples were then further diluted by a factor of 10 in sample buffer.

MyHC composition of the control and treated samples was determined using one-dimensional gel electrophoresis (SDS-PAGE) according to the method of Talmadge and Roy (35). Approximately 1–2 μg of protein were loaded onto 8-cm-long slab gels (SE245, Hoefer Scientific, San Francisco, CA) and electrophoresed for 24–28 h at 4°C by use of discontinuous SDS-PAGE (4:1% stacking and 8:1% resolving gel). After electrophoresis, the gels were fixed for 1 h in 5% acetic acid and 50% ethanol and were then rinsed overnight in 5% acetic acid and 5% ethanol. The gels were then silver-stained using a modified method of Oakley et al. (30), and quantitative measurements were made by laser densitometry. Each optical density obtained was then expressed as a percentage of total MyHC content for the corresponding gel.

Muscle Metabolite Analysis

A portion of freeze-dried muscle was dissected free of visible connective tissue and blood and was powdered, and an aliquot (~10 mg dry weight) was extracted in 0.5 M perchloric acid containing 1 mM EDTA. The samples were then centrifuged, and the supernatant was neutralized with 2.2 M KHCO₃ and stored frozen at −80°C. Spectrophotometric analysis of ATP, ADP, AMP, phosphocreatine (PCr), and creatine concentrations was performed at a later date (17). Total creatine (TCr) was calculated to correct the adenine nucleotide concentrations for contamination with nonmuscle constituents (TCr = PCr + creatine).

Calculations and Statistics

All data are reported as means ± SE. Total adenine nucleotides (TAN) = [ATP] + [ADP] + [AMP], and energy charge (E-C) = [ATP] + 0.5 [ADP]/[ATP] + [ADP] + [AMP] were calculated. Comparisons between groups were made using one-way ANOVA, and when a significant F value was found (P ≤ 0.05), a Fisher’s post hoc test was used to identify any significant differences. Where appropriate, a comparison of differences in the percentage of type I and type IIa, IIx, and IIb MyHC protein contents between control and clenbuterol groups was made using an unpaired Student’s t-test.

RESULTS

MyHC Content

Control animals. In the soleus (n = 5), the MyHC protein content was of the slow-contracting type I isoform (99.5 ± 0.5%), and the remainder was type IIa (0.5 ± 0.5%); Fig. 1. In the EDL (n = 6), the MyHC content was of the fast-contracting type II isoforms (97.3 ± 1.8%), and the remainder of the slowly con-
tracting type I isoform (2.8 ± 1.8%). Further analysis of the gels for EDL demonstrated that the type II isoforms were IIa (17.7 ± 6.11.6%), IIx (37.5 ± 67.1%), and IIb (42.1 ± 610.3%).

Clenbuterol-treated animals. In the soleus (n = 5), the type IIa MyHC protein isoform became apparent in all samples after clenbuterol treatment (18.2 ± 65.2%; P < 0.05 compared with respective controls); the remainder were type I (81.8 ± 65.2%) (Fig. 1). In the EDL (n = 8), there was no type I MyHC protein detected in any of the samples after treatment (P < 0.05 compared with controls). Statistical comparison between IIa and IIx bands of EDL in clenbuterol-treated (7.5 ± 65.4% and 43.1 ± 68.8%, respectively) vs. control animals was not performed because of the overlap of these bands. The percentage of IIb MyHC content (49.5 ± 69.2%) was not different from respective EDL controls.

Muscle Metabolites

Control animals. The ATP concentration of the EDL was higher (64%, P < 0.05) than that of the soleus (Table 1), but there was no significant difference in the concentration of ADP between the muscles. The AMP concentration of the soleus was higher (50%, P < 0.05) than that of the EDL. These differences in adenine nucleotides resulted in a higher TAN (53%, P < 0.05) in the EDL compared with the soleus (Fig. 2). The E-C of the soleus was lower (4.4%, P < 0.05) than that of the EDL (Fig. 3). Finally, TCr was higher (44%, P < 0.05) in the EDL (144.0 ± 65.9 mmol/kg dry mass (dm)) compared with the soleus (100.0 ± 61.5 mmol/kg dm).

Clenbuterol-treated animals. There was an increase in ATP (29%) and reductions in ADP (26%) and AMP (33%) concentrations in the soleus after clenbuterol treatment compared with the control animals (Table 1). As a result of these changes, the TAN of the soleus increased by 19%. However, it remained 28% (P < 0.05) and 30% (P < 0.05) lower than the TAN of the EDL in the control and clenbuterol-treated animals, respectively (Fig. 2). The E-C of the soleus also increased after clenbuterol treatment, such that it was no different from the value in EDL of control and clenbuterol-treated animals (Fig. 3). There was no change in adenine nucleotide content of the EDL after clenbuterol treatment. Similarly, TCr was not different between the control and treatment groups for either the soleus or the EDL.

Muscle Mass

There was no significant difference in soleus mass between the control and clenbuterol-treated groups (Fig. 4). However, EDL mass was significantly greater (20%) in the treatment group compared with the control group (Fig. 4).

| Table 1. Skeletal muscle metabolites after control and clenbuterol treatment |
|---|---|---|---|---|
| | Soleus | EDL | | |
| Control | Clenbuterol | Control | Clenbuterol | |
| ATP (nmol/kg dm) | 19.5 ± 60.4 | 25.1 ± 61.3* | 32.1 ± 60.9 | 32.7 ± 61.0 |
| ADP (nmol/kg dm) | 3.5 ± 60.1 | 2.6 ± 60.1* | 3.5 ± 60.4 | 3.4 ± 60.3 |
| AMP (nmol/kg dm) | 0.6 ± 60.0 | 0.4 ± 60.1* | 0.4 ± 60.0 | 0.4 ± 60.1 |
| TCr (nmol/kg dm) | 100.0 ± 61.5 | 102.6 ± 64.3 | 144.0 ± 65.9 | 140.2 ± 63.1 |

Data are means ± SE expressed as mmol/kg dry mass. EDL, extensor digitorum longus; TCr, total creatine; total adenine nucleotide pool (TAN) = ATP + ADP + AMP. *Significantly different from corresponding control value (ANOVA and Fisher’s post hoc test, P ≤ 0.05).
after treatment, we demonstrated an increase in the cellular E-C. An increase in resting ATP/ADP ratio or E-C is one feature associated with an increase in mitochondrial content (10). This suggests, therefore, that the increased E-C after clenbuterol treatment in the present study may have been related to an increase in mitochondrial content. In support of this suggestion, it has been reported that 7–8 wk of clenbuterol administration can increase soleus muscle citrate synthase activity in obese female Zucker rats (36).

It has previously been reported that prolonged clenbuterol treatment, albeit at a higher doses (~1–2 mg·kg body mass⁻¹·day⁻¹) than that used in the present study, can increase the ratio of fast-twitch to slow-twitch fibers in the EDL (7, 9, 38). In support of this observation, there was no expression of type I MyHC protein in the EDL after clenbuterol treatment (Fig. 1). Given that type I fibers represent a very small proportion of the total fiber pool in EDL, it is not surprising that TAN and E-C were unchanged after clenbuterol treatment.

We were also able to demonstrate that 8 wk of low-dose clenbuterol treatment resulted in an increase in EDL mass compared with the control group. This finding is consistent with previous reports that treatment with 1–2 mg·kg body mass⁻¹·day⁻¹ of clenbuterol increases the cross-sectional area of type II, but not type I, fibers (20, 26, 38).

In the present study, clenbuterol treatment increased the content of type IIa MyHC proteins in the soleus and reduced type I MyHC proteins in the EDL muscles. These observations are consistent with a previous report of an ~8% greater type II fiber content in the soleus and a 2% lower type I fiber content in the EDL (38) after prolonged clenbuterol treatment. Recently it has been shown that this fiber-type switching in soleus is due to an increase in IIa, IIX, and IIB MyHC protein contents (9). It seems unlikely that the above changes in MyHC protein isoform content of the soleus and the EDL muscles were due to stimulation of $\beta_2$-adrenoceptors, because these receptors are downregulated by 50% within 2–3 wk of daily $\beta$-agonist administration (e.g., 21, 34), and fiber-type changes have only been reported after 6–8 wk of treatment. It is possible that these alterations in protein isoform content were due to stimulation of $\beta_2$-adrenoceptors, which are known to be less susceptible to downregulation (5, 28) and which have been reported to be present in the soleus but not in the EDL. Alternatively, it is possible that prolonged clenbuterol treatment may affect hormone- or growth-factor receptor population densities.

In summary, we have demonstrated that prolonged low-dose clenbuterol treatment increased the content of type IIa MyHC protein in the soleus. Concomitant with this change was an increase in the TAN and the E-C. These findings are in accordance with previously reported changes in muscle composition after clenbuterol administration at higher doses. In the EDL, a reduction in the less prevalent type I MyHC protein isoform occurred without any alterations in the TAN pool and E-C of this muscle. This may have been due to the relatively small fiber-type shift being masked by
the high fast fiber population of the EDL (~97% fast muscle fibers). Finally, we have confirmed that a dose of 250 μg·kg body mass⁻¹·day⁻¹ is sufficient to induce hypertrophy of EDL.

Perspectives

We have investigated the effects of the anabolic agent clenbuterol on skeletal muscle adenine nucleotide metabolism because of the proposed central role of the cellular E-C in metabolic control (1). For example, a number of studies have shown that catabolic states such as sepsis and trauma are associated with marked reductions in the TAN and cellular E-C (6, 37). We have demonstrated that clenbuterol increased the type IIa MyHC isoform content, the TAN pool, and the cell E-C of soleus but not of EDL. The mechanism by which these changes occur, however, remains unclear. Future work would add to our knowledge by elucidating whether the increase in type IIa MyHC protein expression of soleus observed in the present study is accompanied by an increase in mitochondrial content and/or function. In addition, a time-course study might establish whether changes in adenine nucleotide metabolism are linked to changes in MyHC protein isoform expression.

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