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Chronic Clenbuterol Administration Alters Myosin Heavy Chain Composition in Standardbred Mares

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SUMMARY

The purpose of this study was to examine changes in myosin heavy chain (MHC) composition due to chronic clenbuterol administration with or without exercise in mares. Unfit Standardbred mares (aged 10 ± 3 years) were divided into four groups: clenbuterol (2.4 μ/kg BW twice daily) plus exercise (3 days/week for 20 min at 50% $VO_{2\,max}$; CLENEX; $n = 6$), clenbuterol only (CLEN; $n = 6$), exercise only (EX; $n = 5$), and control (CON; $n = 6$). Muscle biopsies were obtained from *gluteus medius* muscle before and after the eight-week training/administration period. MHC composition was determined *via* SDS gel electrophoresis and quantified using a scanning and densometric system.

CLENEX and CLEN exhibited significant ($P < 0.05$) MHC changes while EX and CON did not. MHC type IIA decreased (29.8 ± 6.1 to 19.3 ± 4.0%, CLENEX; and 36.8 ± 12.4 to 26.4 ± 7.9%, CLEN) and MHC type IIX increased (59.4 ± 7.2 to 71.8 ± 5.8%, CLENEX; and 50.5 ± 12.5 to 62.0 ± 9.3%, CLEN). Chronic clenbuterol administration with and without exercise resulted in a significant shift in MHC profile in Standardbred mares.

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KEYWORDS: Aerobic; horse; fiber type; performance; bronchodilator.

INTRODUCTION

The β2 sympathomimetic agent clenbuterol has been used primarily as a bronchodilator in horses (Sasse & Hajer, 1978). However, clenbuterol has been known to produce changes in skeletal muscle fiber type (determined histochemically) and myosin heavy chain (MHC) composition (Dodd *et al.*, 1996). Clenbuterol causes a change from the presumed ''slow-twitch'' fibers to the presumed ''fast-twitch'' fibers in rats (Zeman *et al.*, 1988; Criswell *et al.*, 1996; Dodd *et al.*, 1996). The change in fiber type produces an increase in absolute tension but not specific tension (Dodd *et al.*, 1996; Lynch *et al.*, 1996; Lynch *et al.*, 1999) and increases the muscle's shortening velocity (Zeman *et al.*, 1988; Dodd *et al.*, 1996). These changes occur with a corresponding decrease in the Ca^{2+}-activated contractile sensitivity of rat muscles (Lynch *et al.*, 1996). Furthermore, clenbuterol reduces the energetic potential of muscle by decreasing the maximal enzyme activities of citric synthase and phosphofructokinase in rats (Dodd *et al.*, 1996; Kim *et al.*, 2000) and thereby increasing muscle fatigability. Taken together, these data suggest that clenbuterol detrimentally affects peripheral factors that potentially could limit aerobic capacity.

Data from whole animal aerobic performance experiments support this idea as clenbuterol has been shown to adversely affect aerobic performance in mice (Ingalls *et al.*, 1996) and rats (Duncan *et al.*, 2000). After eight weeks, clenbuterol-treated mice demonstrated a significantly lower capacity for exercise performance than untreated animals (Ingalls *et al.*, 1996). Similarly, clenbuterol-treated rats ran 57% less total distance than untreated animals and tail weights had to be removed from the clenbuterol-treated rats in order for them to complete their exercise protocols (Duncan *et al.*, 2000). In

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both studies, the effects of exercise were antagonistic to the effects of clenbuterol. In other words, exercise was able to ameliorate the deleterious effects of clenbuterol.

While ample data has been published using rats, no data exist concerning chronic administration of clenbuterol and its effects on MHC composition in horses. Additionally, the dosage (based on body weight) of clenbuterol used in the rat and mouse studies was greater and given for shorter periods of time than commonly prescribed therapeutic dosages for horses. However, horses have been shown to be highly sensitive to sympathomimetic drugs (McKeever, 1993). It is thus reasonable to assume that lower doses of clenbuterol (similar to those commonly prescribed for its bronchodilator effects) over a long period of time may be able to alter horse MHC composition. Therefore, the purpose of this study was to examine changes in MHC composition in horse skeletal muscle after chronic clenbuterol administration in a dosage commonly prescribed, with and without aerobic exercise.

MATERIALS AND METHODS

Animals and drug administration

Twenty-three healthy, untrained Standardbred mares (age: 10 ± 3 years; body weight: 514.3 ± 12.3 kg) were evaluated. Mares were unfit, but accustomed to the laboratory and running on the treadmill prior to the start of the experiment. During the trial the horses were housed as a group on pasture. Each mare was fed approximately 6 kg/day of alfalfa and grass hay and approximately 3 kg/day of a commercially available grain ration (split into two feedings). Water was provided *ad libitum*. The Rutgers University Institutional Animal Care Review Board approved all methods and procedures used in this experiment.

Horses were divided into four experimental groups: clenbuterol and exercise (CLENEX; $n = 6$) and clenbuterol only (CLEN; $n = 6$) were both orally administered 2.4 μg/kg body weight of clenbuterol (Boehringer Inglelheim, UK) twice daily as a syrup (for an average volume of 20 mL) on a schedule of five days on and two days off for the duration of the study. CLENEX also aerobically trained for 3 days/week. Exercise (EX; $n = 5$) were used as the training group and aerobically trained for 3 days/week. Control (CON; $n = 6$) were used as the control group. Both EX and CON were administered molasses (20 mL) twice daily on a five days on and two days off schedule.

Training program

The exercise program consisted of continuous treadmill running 3 days/week for eight weeks. The horses ran initially for 15 min/day at a work rate set at an intensity of 50% $VO_{2\,max}$. After one week, the duration of run time was increased to 20 min/day and was held at this duration for the remainder of the study. During the exercise training, the treadmill was set at a fixed 6% grade.

Muscle biopsy

Muscle biopsies were obtained from the *gluteus medialis* of each horse at a depth of 2 cm using a Bergstrom needle (Snow & Guy, 1976). All biopsies were taken by the same investigator in order to minimize sampling error. The position of each biopsy was standardized as described previously (Valette *et al.*, 1999). Briefly, biopsy location was determined as being one-third of the distance from the tuber coxae to the caudal Cd1–Cd2 intervertebral joint. A sampling depth of 2 cm was chosen as it is the most commonly used depth reported in the literature. Biopsies were used for myosin heavy chain identification.

Myofibrillar isolation technique

Myofibrillar protein was isolated by the methods of LaFramBoise *et al.* (1992). Muscle was removed from the −80 °C freezer and powdered under liquid nitrogen. Powder was removed to glass-on-glass homogenization tubes (Kontes). Muscle powder was homogenized in 4 volumes homogenization buffer (300 mM NaCl, 100 mM NaH_2PO_4, 50 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM $Na4P_2O_2$, 10 mM EDTA). Extracts were then centrifuged at 13,000*g* for 30 min at 4 °C. The supernatant was recovered and diluted in 9 volumes precipitation buffer (1 mM EDTA and 0.1% Beta-mercaptoethanol). Diluted extracts were stored overnight at 4 °C to allow precipitation of myosin filaments. The solution was subsequently centrifuged at 13,000*g* for 30 min at 4 °C to form a pellet. The pellet was resuspended (1:1) in 0.5 M NaCl and 10 mM NaH_2PO_2 buffer and then diluted 1:100 in SDS buffer (62.5 mM Tris–HCl, 2% SDS, 10% glycerol, 5% Beta-mercaptoethanol, and 0.001% bromophenol blue at pH 6.8). The samples were boiled for 3 min and stored at −80 °C.

Myosin heavy chains

Myosin heavy chain (MHC) composition in the *gluteus medialis* was determined using one-dimensional sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Talmadge

and Roy (1993). One to three micrograms of protein was loaded onto 20 cm long vertical gels (Bio-Rad) and electrophoresed for 24 h at ~4 °C using SDS–PAGE (14% bis-acrylamide stacking, 8% bis-acrylamide separating gel) (Talmadge & Roy, 1993). Gels were stained with Coomassie Blue R-250 and destained with a 10%/10% methanol and acetic acid solution. This protocol has been previously shown to result in three MHC isoforms (I, IIA, IIX) in horse skeletal muscle (Rivero *et al.*, 1997). The relative concentration of MHC was determined by scanning the gels using a National Institutes of Health (NIH) computerized image analysis system (Scion Image for Windows, Scion Corporation). The coefficient of variation for this measure in our laboratory was < 5%.

Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). For comparison by group and time a two-way ANOVA for repeated measures was used with the a priori level of statisticalsignificance set a $P < 0.05$. Post-hoc differences were determined with the Tukey test (Sigma Stat 2.0).

RESULTS

Myosin heavy chain

The relative contribution of each MHC isoform to the total myosin pool (expressed as percentage), as determined by SDS–PAGE analysis, is presented in Fig. 1. CLENEX and CLEN exhibited significant ($P < 0.05$) changes in MHC type IIA and IIX but not type I during the training/administration period. MHC type IIA decreased (29.8 ± 6.1 to 19.3 ± 4.0%, CLENEX; and 36.8 ± 12.4 to 26.4 ± 7.9%, CLEN) and MHC type IIX increased (59.4 ± 7.2 to 71.8 ± 5.8%, CLENEX; and 50.5 ± 12.5 to 62.0 ± 9.3%, CLEN). EX and CON did not exhibit any significant MHC composition shifts during the training/administration period. Fig. 2 shows an example of myosin isoform banding pattern pre- and post-treatment from SDS–PAGE for representatives of each of the four groups.

DISCUSSION

This study is the first to demonstrate that clenbuterol administration, in commonly used therapeutical doses, results in substantial changes in skeletal muscle MHC composition in the horse that are possibly det-

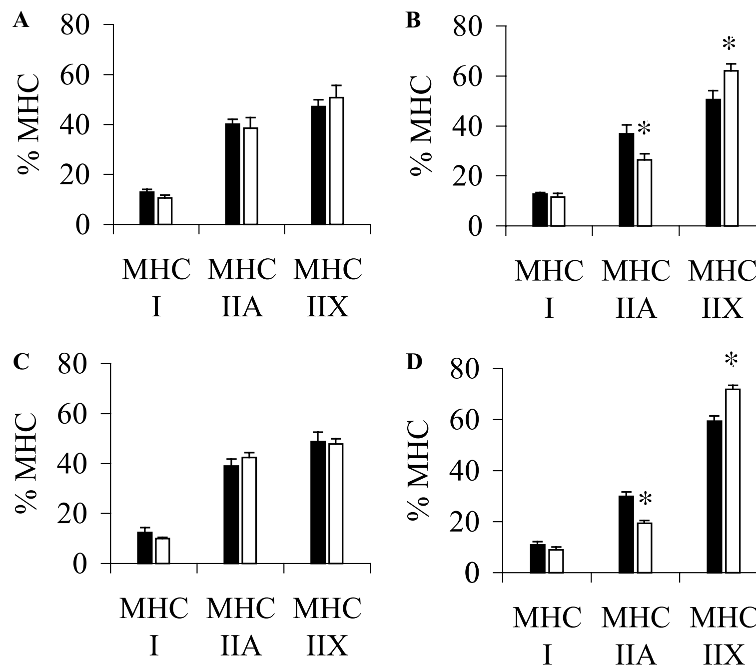

Fig. 1. Pre (filled boxes) and post (unfilled boxes) myosin heavy chain (MHC) composition (%, mean ± SE), based on electrophoresis analysis, in biopsy samples removed from the *gluteus medius* muscle of: (A) Control horses, (B) clenbuterol-treated horses, (C) exercised horses, and (D) clenbuterol-treated and exercised horses. *$P < 0.05$.

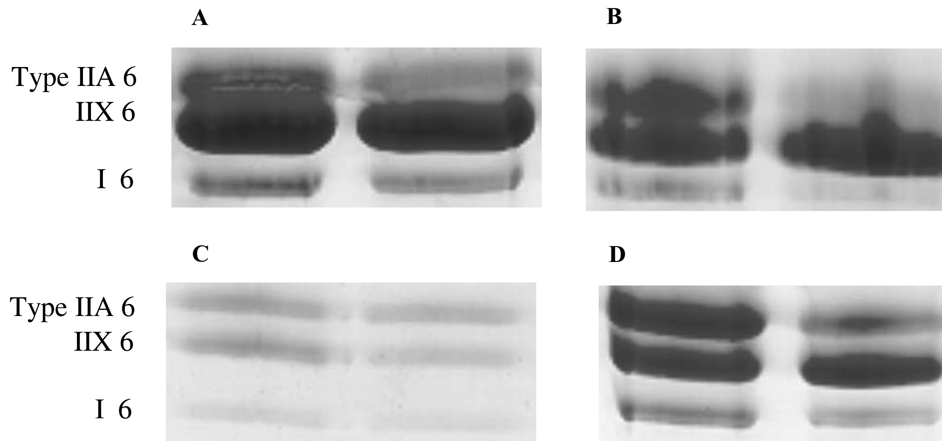

Fig. 2. Representative sodium dodecyl sulfate–polyacrylamide gels of horse *gluteus medius* muscle samples showing separation of myosin heavy chain isoforms (labeled at left) taken from: (A) Control horses, (B) clenbuterol-treated horses, (C) exercised horses, and (D) clenbuterol-treated and exercised horses. For each pair of gels, the left gel is pre-treatment, the right gel is post-treatment.

rimental to aerobic performance. The MHC changes are in the direction of IIA → IIX, which are opposite of that typically seen during aerobic training in animals (IIX → IIA → I). This study also suggested that aerobic exercise may not be able to reverse these effects.

Aerobic training did not result in changes in MHC content in the present study. However, recent evidence suggests that aerobic training in horses can result in MHC changes (Serrano *et al.*, 2000). For instance, three months of training (45 min trotting, 60 min treadmill running, 5 days/week) in stallions resulted in significant increases in MHC IIA (11%) and significant decreases in MHC IIX (12%) (Serrano *et al.*, 2000). A reason we did not see changes in MHC composition with aerobic training could be that we exercised our horses for only 60 min/week, *vs.* the 525 min/week used in the Serrano study. Further, the duration of our study was two months vs. the three months in the Serrano study. Finally, Serrano *et al.* (2000) took muscle biopsies at depths of 2, 4, and 6 cm while we only took biopsies at a depth of 2 cm. Serrano *et al.* (2000) noted that the changes in MHC composition due to aerobic training were much more evident in the deep biopsies. Thus, it appears that changes in MHC composition due to aerobic training in horses are dependent upon exercise dose and depth of biopsy sampling.

We noted that exercise training did not prevent the clenbuterol-induced change in MHC (Fig. 1D). Likely this was because we found no change in MHC distribution after training alone, as discussed above. It was previously found in mice that exercise training prevented the ~25% decrease in total work due to clenbuterol treatment alone (Ingalls *et al.*, 1996). In rats it was found that exercise training could not prevent decreases in exercise performance or hypertrophy of the heart due to clenbuterol treatment alone, but exercise did prevent the collagen infiltration of the heart due to clenbuterol treatment alone (Duncan *et al.*, 2000). It is likely that the differing results are due to differences in exercise prescription and clenbuterol dose, as well as the different animal models used.

Did these MHC shifts lead to altered aerobic performance? These same horses, in data published elsewhere (Kearns & McKeever, 2002), showed reductions in time to exhaustion on a treadmill when administered clenbuterol (~20% in CLENEX and CLEN groups). CLENEX also showed reductions in $VO_{2\,max}$ by ~6%, although CLEN did not. EX showed an increased time to exhaustion (~32%) and an increased $VO_{2\,max}$ (~7%); CON did not change in either parameter. This suggests that the changes in MHC due to clenbuterol administration noted in this study could lead to reductions in aerobic performance. However, one should note that only one sample depth was used in this study and we cannot rule out variations in response at different muscle depths.

Endurance-type training has been reported to result in skeletal muscle MHC composition shifts in humans (O'Neill *et al.*, 1999), horses (Serrano *et al.*, 2000) and other animals (reviewed in Pette & Staron, 1997). The MHC shifts are of the direction IIX → IIA → I. These shifts in MHC profile may be advantageous to aerobic performance (Pette &

Staron, 1997; Pette *et al.*, 1999). Supporting this suggestion is the observation that these MHC shifts parallel increases in aerobic enzymes and intramuscular glycogen and triglyceride stores that are due to aerobic training. Conversely, a recent study of aerobically trained horses showed shifts from MHC IIA to MHC IIX during detraining of the horses (Serrano *et al.*, 2000). All of this evidence suggests that the MHC shift of IIX → IIA typically noted in aerobic training studies appears advantageous and a MHC shift of IIA → IIX is likely deleterious to extended muscular performance. Thus, data from the present study showing that clenbuterol administration results in a MHC shift (IIA → IIX) which could compromise aerobic performance of the horse agree with previous data.

However, recent evidence suggests that fast MHC types in horse *gluteus medius* and *biceps femoris* correlate positively with a ''performance index'' indicative of performance in gallop racing, steeplechasing, show jumping, and three-day eventing competitions (Barrey *et al.*, 1999). Unfortunately, the authors correlated the performance index with both fast type MHCs (type IIA and IIX, identified as IIB). Thus, one could not determine which fast MHC correlated with exercise performance. Additionally, the correlation coefficient in the best case (*gluteus medius*) was 0.47, indicating that both fast MHC types explained only ~24% of the variance in performance. The fast MHC composition of the *biceps femoris* explained even less of the variance in performance. Thus, although their evidence, evidence from the present study, and evidence in other species (as discussed above) suggests MHC type correlates with performance, it remains to be definitively tested whether a particular MHC correlates with exercise performance in the horse.

A decrement in aerobic performance due to clenbuterol administration has been noted in other species (Ingalls *et al.*, 1996; Duncan *et al.*, 2000). In rats, exercise performance during treadmill sprinting, endurance swimming, and voluntary running were all reduced due to clenbuterol treatment (Duncan *et al.*, 2000). In mice, run-to-exhaustion time was decreased in clenbuterol-treated animals, and a potential improvement in aerobic performance due to run training was negated by clenbuterol administration (Ingalls *et al.*, 1996). The authors concluded that the negative effects of clenbuterol on exercise performance were due to unknown reasons (Ingalls *et al.*, 1996) or due to deleterious alterations in cardiac function secondary to clenbuterol treatment (Duncan *et al.*, 2000). Interestingly, a recent paper from our lab has shown that therapeutic doses of clenbuterol and exercise result in decreased $VO_{2\,max}$, time to exhaustion, plasma volume, and a higher recovery heart rate in standardbred mares (Kearns & McKeever, 2002). Additionally, it has also been shown that cardiac remodeling and detrimentally altered cardiac function leads to decreased aerobic capacity in standardbred mares receiving therapeutic doses of clenbuterol (Sleeper *et al.*, 2002). Thus, clenbuterol administration could reduce aerobic exercise performance in several possible ways: detrimentally altered cardiac function (Duncan *et al.*, 2000; Kearns & McKeever, 2002), decreases in plasma volume (Sleeper *et al.*, 2002) and detrimental changes in skeletal muscle MHC composition (present study).

The evidence is clear that clenbuterol administration in mammals results in skeletal muscle MHC shifts from I → IIA → IIX (Criswell *et al.*, 1996, Dodd *et al.*, 1996, Zeman *et al.*, 1988). Our results agree with previous data in this regard. We show a significant reduction in type IIA with a corresponding increase in type IIX MHC percentage in horses administered clenbuterol. However, the expected reduction in the type I MHC was not seen in the present study, although a mild (non-significant) decrease was observed (see Figures). This may be due to the fact that all horses were severely untrained at the outset of the study and therefore there was only a modest amount (~10%) of type I MHC present in all horses studied.

The exact mechanism(s) by which clenbuterol alters muscle fiber type has yet to be fully ascertained. Clenbuterol treatment increases the cross-sectional area of type II, but not type I fibers (Kim *et al.*, 1992; Maltin *et al.*, 1986; Zeman *et al.*, 1988). It has been suggested that the clenbuterol-mediated fiber type switching may be related to an increase in IIa, IIx, and IIb MHC protein content in rats (Dodd *et al.*, 1996). These changes may be due more to stimulation of β3-adrenocepters than β2-adrenocepters (Rajab *et al.*, 2000), as of β3-adrenocepters have been shown to be less susceptible to downregulation (Cartana & Stock, 1995) than of β2-adrenocepters (Kim *et al.*, 1992). Recently, it has been shown that increases in IIa, IIx protein content are accompanied by an increase in total adenine nucleotide poos (Rajab *et al.*, 2000). These data indicate that fiber type switching is also associated with concomitant changes in cellular energy metabolism (Rajab *et al.*, 2000).

In conclusion, clenbuterol administration at common therapeutic doses results in significant shifts in MHC composition (IIA → IIX) in Standardbred mares. These shifts cannot be prevented

by a modest endurance exercise training program (eight weeks, three days/week for 20 min at 50% $VO_{2\,max}$). It is likely that these MHC shifts are detrimental to prolonged exercise performance in horses running races comparable to Standardbreds.

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