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Attenuating the decline in ATP arrests the exercise training-induced increases in muscle GLUT4 protein and citrate synthase activity

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ABSTRACT

Thirty-two female Sprague–Dawley rats were assigned to one of four groups: control (CON); exercise training (TR); exercise training + clenbuterol treatment (0.8 mg kg body wt^{-1} d^{-1}) (TR + CL) or exercise training + clenbuterol treatment + 2% β-guanidinoproprionic acid diet (TR + CL + β) to examine whether alterations in the high energy phosphate state of the muscle mediates exercise training-induced increases in skeletal muscle GLUT4 protein concentration and citrate synthase activity. Exercise training consisted of running the rats 5 d $week^{-1}$ for 8 weeks on a motor-driven treadmill (32 m min^{-1}, 15% grade). Gastrocnemius GLUT4 protein concentration and citrate synthase activity were significantly elevated in the TR animals, but these adaptations were attenuated in the TR + CL animals. Providing β-GPA in combination with clenbuterol enabled training to elevate GLUT4 protein concentration and citrate synthase activity, with the increase in GLUT4 being greater than that observed for the TR animals. Skeletal muscle ATP levels were reduced in the TR + CL + β animals while ATP levels in the TR + CL animals were significantly elevated compared with CON. An acute 40-min bout of electrical stimulation of the sciatic nerve was found to lower skeletal muscle ATP levels by ≈50% and elevate cAMP levels in all groups. No difference in post-contraction cAMP levels were observed among groups. However, post-contraction ATP levels in the TR + CL animals were significantly greater than the other groups. Collectively, these findings suggest that exercise training-induced increases in skeletal muscle GLUT4 protein concentration and citrate synthase activity are initiated in response to a reduction in the skeletal muscle ATP concentration.

Keywords acute electrical stimulation, adenosine 3′,5′-cyclic monophosphate, adenylate cyclase, β-guanadinoproprionic acid, clenbuterol.

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Currently, it is unclear how skeletal muscle contractile activity translates into alterations in the physiological and metabolic profile of the activated muscle. Two potential pathways that have been suggested as mediating the contraction signal are alterations in the high energy phosphate state of the skeletal muscle (Shoubridge *et al.* 1985, Lai & Booth 1990, Green *et al.* 1992, Ren *et al.* 1993) and the β-adrenergic receptor–adenylate cyclase–cAMP pathway (Williams *et al.* 1984, Kraus *et al.* 1992, Yaspelkis *et al.* 1997). We (Yaspelkis *et al.* 1997, 1998) recently investigated the relationship of these signals to elevations in skeletal muscle GLUT4 protein concentration in response to chronic low frequency electrical stimulation both in the presence and absence of β-guanidinoproprionic acid (β-GPA), a structural analogue of creatine that reduces creatine phosphate and ATP levels (Fitch & Shields 1966, Fitch *et al.* 1975). Our findings suggested that the primary component of the contraction signal arose from alterations in the high energy phosphate state of the muscle (Yaspelkis *et al.* 1998), and that activation of the β-adrenergic receptor signalling pathway may potentiate, but does not appear to initiate, these skeletal muscle adaptations in response to exercise training.

In the present study, we sought to further evaluate the relationships among the high energy phosphates and β-adrenergic signalling pathway, and increases in skeletal muscle GLUT4 protein concentration and oxidative capacity in response to a normal exercise training protocol. Sprague–Dawley rats were subjected

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to 8 weeks of treadmill running while being provided the β_2-adrenergic agonist clenbuterol. Clenbuterol has been shown to inhibit exercise training-induced increases in skeletal muscle GLUT4 protein and oxidative capacity (Torgan *et al.* 1993a, b, 1995, Kuo *et al.* 1996). The mechanism is unclear, but it may be caused by a down-regulation of the β-adrenergic receptors (Torgan *et al.* 1993a). Reducing the high energy phosphate state of the muscle, however, appears to initiate an increase in skeletal muscle GLUT4 protein concentration and oxidative capacity (Shoubridge *et al.* 1985, Lai & Booth 1990, Ren *et al.* 1993, Yaspelkis *et al.* 1998). Therefore, we also investigated the effects of chronic clenbuterol treatment on the high energy phosphate state of the muscle. Specifically, we determined if clenbuterol would prevent the exercise-induced increase in skeletal muscle GLUT4 protein concentration and oxidative capacity when the high energy phosphate state of the muscle was chronically lowered by exercise training and a diet containing 2% β-GPA. Finally, to further characterize the potential role of these pathways on initiating the contraction signal, we determined if skeletal muscle high energy phosphate levels and/or cAMP concentration differed among treatment groups in response to 40 min of muscle contractile activity elicited by low-frequency electrical stimulation.

MATERIALS AND METHODS

Experimental animals

Thirty-two female Sprague–Dawley rats ≈6 weeks of age were obtained from the Animal Resource Center at the University of Texas at Austin and randomly assigned to one of four groups: (1) control ($n = 8$), (2) exercise training ($n = 8$), (3) exercise training + clenbuterol ($n = 8$) or (4) exercise training + clenbuterol + β-GPA diet ($n = 8$). The control, exercise training and exercise training + clenbuterol groups were provided standard laboratory chow throughout the investigation. The exercise training + clenbuterol + β-GPA diet group received chow containing 2% β-GPA beginning 3 weeks prior to the onset of training and remained on this diet for the duration of the training. Thirty minutes prior to each training session the groups receiving clenbuterol were intubated with 0.8 mg clenbuterol kg body wt^{-1} d^{-1} dissolved in water while the control and exercise-trained groups were intubated with a similar volume of vehicle only (deionized water). Rats were housed three to a cage and provided chow and water *ad libitum*. The temperature of the animal room was maintained at 21 °C and an artificial reversed 12 : 12 h light–dark cycle was set.

Exercise training consisted of having the rats run during their dark cycle on a motor-driven treadmill up a 15% grade, 5 d $week^{-1}$ for 8 weeks in a room maintained at 18 °C. The speed and duration of the treadmill running were rapidly increased over the first 4 weeks until the rats were running continuously for 2 h at 32 m min^{-1}. This exercise intensity was maintained for the remainder of the training period.

Procedure for acute electrical stimulation

Following the 8-week period of training, all animals were instrumented for and subjected to an acute bout of low-frequency electrical stimulation of the triceps surae group. This procedure provided a standardized and repeatable level of activation in the triceps surae group and enabled us to determine if high energy phosphate levels and/or cAMP concentration differed within the muscles among the treatment groups following contraction. In addition, by stimulating only the right triceps surae group, the contralateral muscles of each animal were able to serve as non-stimulated controls.

The acute stimulation procedure was similar to that which was described by Hood & Parent (1991). Animals were anaesthetized with an intraperitoneal injection of pentobarbital sodium (6.5 mg 100 g body wt^{-1}). The groups receiving clenbuterol were intubated with 0.8 mg clenbuterol kg body wt^{-1} d^{-1} dissolved in water 30 min prior to being anaesthetized while the control and exercise-trained groups were intubated with vehicle only (deionized water). The right triceps surae group was exposed which consisted of reflecting the skin from the leg and removal of the hamstrings. A section of the calcaneus, with the Achilles tendon still attached, was clipped from the foot. The triceps surae group was then reflected from the tibia and a jewelers chain was clipped to the calcaneus-Achilles tendon interface. The muscle was wrapped in saline dampened tissue paper to prevent tissue dehydration. The right sciatic nerve was then surgically exposed and a miniature electrode was attached. Next, the right hind limb of the rat was immobilized in a specially designed acrylic cradle and the free end of the jewelers chain was attached to a force transducer (Harvard Apparatus, Millis, MA, USA). The length of the muscle was adjusted to provide a maximal isometric twitch tension which corresponded to a resting length passive force of ≈150 g. A low frequency (10 Hz) electrical stimulation period of 40 min was then initiated using a Grass stimulator (Quincy, MA, USA) set to generate 0.1 ms square-wave pulses at a supramaximal voltage (8 V).

Tissue collection

During the last 10 s of the acute electrical stimulation, the gastrocnemius of the activated leg was isolated and

freeze-clamped *in situ* while contracting with tongs cooled in liquid N_2. The gastrocnemius from the contralateral limb was then freeze-clamped *in situ* with tongs cooled in liquid N_2. Muscle from the activated right leg was used for analysis of cAMP and high energy phosphate concentrations while muscle from the non-stimulated left leg was used for determination of GLUT4 protein concentration, citrate synthase activity, adenylate cyclase activity and levels of the high energy phosphate compounds. All muscles were stored at −80 °C until analysed.

Determination of skeletal muscle GLUT4 protein concentration

Gastrocnemius GLUT4 glucose transporter content was determined by Western blotting as described previously (Yaspelkis *et al.* 1997), by using the affinity purified rabbit polyclonal GLUT4 antibody R1184A (donation of Dr Mike Gibbs, Pfizer Central Research, Groton, CN) followed by incubation with HRP-labelled Donkey Anti-Rabbit IgG (Amersham Life Science, Arlington Heights, IL, USA). Antibody binding was visualized using enhanced chemiluminescence autoradiography in accordance with the manufacturer's instructions. Labelled bands were quantified by capturing images of the autoradiographs in a Macintosh IIsi computer (Apple Computer, Cupertino, CA, USA) which were produced by an image scanner (600 Plus Scanner, Mirror Technologies, St. Paul, MN, USA) equipped with a transparency module. The captured images were digitized and imported into imaging analysis software (NIH Image 1.55, Bethesda, MD, USA) and the density of the labelled bands were calculated. Each band was corrected for background activity and expressed as a percentage of a standard (30 μg of heart homogenate protein) run on each gel.

Measurement of tissue high energy phosphate compounds and cAMP concentration

Portions of the muscles were pulverized in the frozen state under liquid N_2 and extracted with perchloric acid (PCA) according to the procedure described by Williamson & Corkey (1969). Aliquots of the PCA extracts were stored at −80 °C until measured for the high energy phosphate compounds, ATP (Lamprecht & Trautschold 1974), creatine phosphate (Lamprecht *et al.* 1974), creatine (Bernt *et al.* 1974), and inorganic phosphate (Sigma Diagnostics, Kit #670-C, St. Louis, MO, USA).

For tissue cAMP determination, 400 μL of the PCA extracts were lyophilized and resuspended in 150 μL of DPC assay buffer (Diagnostic Products, Los Angeles, CA, USA). Fifty microlitres of the resuspended solution were then assayed for cAMP content using a commercially available radioimmunoassay kit (Diagnostic Products, Los Angeles, CA, USA).

Measurement of adenylate cyclase activity

Muscles were thawed over ice and finely minced in 4 mL of homogenization buffer (50 mM Tris, 5 mM EDTA and 90 mM NaCl, pH 7.5.) at 4 °C. The tissue was then homogenized, filtered through a layer of silk screen mesh to remove large pieces of connective tissue, and then the filtrate was centrifuged at 40 000 × *g* for 30 min at 4 °C. The supernatant was decanted and the remaining pellet was resuspended in 4 mL of resuspension buffer (50 mM Tris, 90 mM NaCl, 1.5 mM MgCl and 1.0 mM EDTA, pH 7.5). This suspension was then centrifuged at 40 000 × *g* for 30 min at 4 °C. The pellet, which was assumed to represent the crude sarcolemmal membrane fraction, was resuspended (≈10–50 mg protein mL^{-1}) in 1.5 mL of resuspension buffer. The final suspension was aliquoted and stored at −80 °C until analysed for adenylate cyclase activity and protein concentration (Bradford 1976).

For determination of adenylate cyclase activity, a 100 μL aliquot of the membrane fraction was diluted to a final concentration of 0.30 mg protein mL^{-1} in resuspension buffer and added to a reaction mixture containing 0.2 mM ATP, 100 μM GTP, 10 mM Tris–HCl (pH 7.5), 1 mM $MgSO_4$, 1 mM 3-isobutyl-1-methylxanthine, 0.1% BSA, 25 U mL^{-1} creatine phosphokinase, 20 U mL^{-1} adenosine deaminase and 10 mM phosphocreatine with one of the following stimulatory compounds: 100 μM forskolin, 5 mM NaF or 10 μM isoproterenol. These compounds were utilized to assess possible differences in activation of adenylate cyclase activity that may have existed among groups by directly stimulating the enzyme with forskolin, and indirectly stimulating the enzyme through guanine-nucleotide regulatory G-proteins (G_s) and β-receptors with NaF and isoproterenol, respectively. The assay reaction was allowed to run for 15 min at 30 °C and then stopped by addition of 100 μL of 10% PCA. The samples were incubated on ice for 30 min, titrated (pH 5.5–6.5) with 30% (wt wt^{-1}) potassium bicarbonate and centrifuged for 15 min at 4 °C (2000 × *g*). Duplicate 50 μL aliquots of the supernatant were removed for cAMP determination using a commercially available radioimmunoassay kit (Diagnostic Products, Los Angeles, CA, USA). Adenylate cyclase activity is expressed as pmol cAMP mg $protein^{-1}$ min^{-1}.

Determination of citrate synthase activity

Muscle samples that were homogenized 1 : 20 in HES buffer were diluted 1 : 10 in 0.1 M Tris–HCl + 0.4% Triton X-100 (pH 8.1) and allowed to incubate at room

temperature for 10 min. Samples were centrifuged for 15 min and the supernatant was used for determination of citrate synthase activity according to the procedure of Srere (1969).

Statistical analysis

The data were analysed using a one-way analysis of variance (ANOVA). Differences between means were determined using Fisher's least-significant differences post-hoc test. Differences were considered significant if *P*-values less than 0.05 were obtained.

RESULTS

Body weight, muscle weight and total muscle protein

Body weights of the control (CON), trained (TR) and trained + clenbuterol + β-GPA (TR + CL + β) animals were not significantly different among groups (Table 1). However, the trained + clenbuterol (TR + CL) animals were significantly heavier compared with the CON and TR + CL + β groups.

Gastrocnemius weight was not different between the CON and TR + CL + β groups (Table 1). The gastrocnemius weight of the TR and TR + Cl groups were not significantly different from each other, but both of these groups had muscles that were significantly heavier than those of the CON and TR + CL + β groups. No difference in muscle protein concentration existed among groups (Table 1).

Skeletal muscle GLUT4 protein concentration

Exercise training increased skeletal muscle GLUT4 protein concentration ≈85% in the TR animals when compared with the CON group (Fig. 1). Clenbuterol treatment attenuated the exercise-induced rise in muscle GLUT4 protein concentration as evidenced by the CON and TR + CL animals having similar levels of the glucose transporter protein. The TR + CL + β animals had a skeletal muscle GLUT4 protein concentration that was significantly increased by 179, 51 and 155% when compared with the CON, TR and TR + CL animals, respectively.

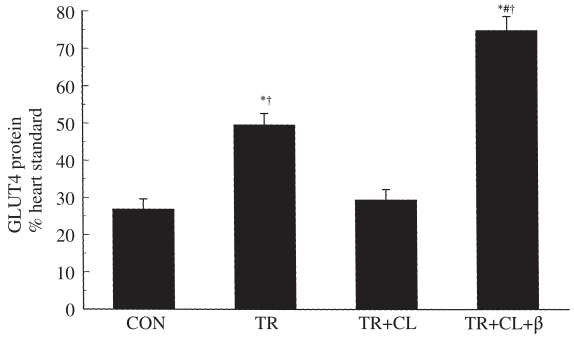

Figure 1 GLUT4 protein concentration, expressed as a percentage of a heart standard, in gastrocnemius muscles from female Sprague–Dawley rats that were divided among one of four groups: control (CON); exercise training (TR); exercise training + clenbuterol treatment (TR + CL) or exercise training + clenbuterol treatment + 2% β-guanadinoproprionic acid diet (TR + CL + β). Values are mean ± SE. *, significantly different from CON ($P < 0.05$). #, significantly different from TR ($P < 0.05$). †, significantly different from TR + CL ($P < 0.05$).

Skeletal muscle citrate synthase activity

Gastrocnemius citrate synthase activity was found to be elevated 71% in the TR animals when compared with the CON animals (Fig. 2). No difference in citrate synthase activity existed between the CON and TR + CL animals. Citrate synthase activity of the TR + CL + β animals was significantly greater than both the CON and TR + CL animals, but was not different from that of the TR group.

Skeletal muscle high energy phosphate levels

Resting skeletal muscle ATP, ADP_f and ATP/ADP were not significantly different between CON and TR animals (Table 2). However, TR + CL animals had a resting ATP concentration in the gastrocnemius muscle that was ≈37% greater compared with the CON and TR groups and ADP_f levels that were elevated above the CON animals. No difference in resting creatine

Table 1 Body weight, gastrocnemius weight and total muscle protein of female Sprague–Dawley rats

	CON	TR	TR + CL	TR + CL + β
Body weight, g	244 ± 5	265 ± 7	283 ± 12*	246 ± 4†
Muscle weight, g	1.37 ± 0.06	1.65 ± 0.08*	1.75 ± 0.05*	1.29 ± 0.04#†
Muscle protein, mg g^{-1}	190 ± 6	200 ± 5	193 ± 10	176 ± 9

Values are mean ± SE. CON, control; TR, exercise trained; TR + CL, exercise trained in the presence of clenbuterol; TR + CL + β, exercise trained in the presence of clenbuterol and 2% β-guanadinoproprionic acid diet. *, significantly different from CON ($P < 0.05$). #, significantly different from TR ($P < 0.05$). †, significantly different from TR + CL ($P < 0.05$).

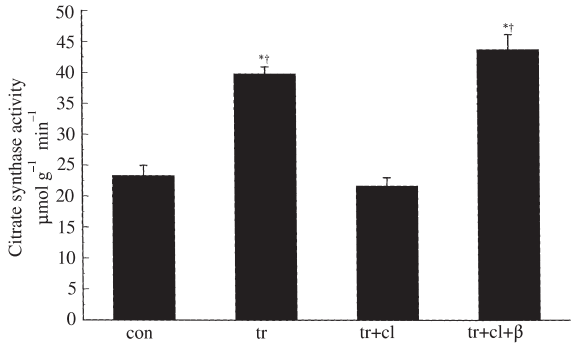

Figure 2 Citrate synthase activity in gastrocnemius muscles from female Sprague–Dawley rats that were divided among one of four groups: control (CON); exercise training (TR); exercise training + clenbuterol treatment (TR + CL) or exercise training + clenbuterol treatment + 2% β-guanadinoproprionic acid diet (TR + CL + β). Values are mean ± SE. *, significantly different from CON ($P < 0.05$). †, significantly different from TR + CL ($P < 0.05$).

phosphate or inorganic phosphate levels were observed among CON, TR and TR + CL. Resting creatine levels were not significantly different between the CON and TR + CL animals or the TR and TR + CL animals. Resting creatine concentration in the skeletal muscle of the TR animals was reduced when compared with CON animals. The TR + CL + β animals exhibited substantially reduced resting ATP, creatine phosphate, creatine, inorganic phosphate, ATP/ADP and elevated ADP_f levels when compared with the other three groups.

Adenylate cyclase activity

No difference in adenylate cyclase activity existed between the TR and CON animals under any of the biochemical stimulatory reagents utilized to assess activity (Fig. 3). In the TR + CL animals, GTP-, NaF- and isoproterenol-stimulated adenylate cyclase activity was reduced below that of the CON animals, while forskolin-, NaF- and isoproterenol-stimulated adenylate cyclase activity was significantly less than that of the TR animals. Basal adenylate cyclase activity in the TR + CL + β animals was elevated above that of the TR and TR + CL groups. The TR + CL + β animals also exhibited a forskolin-stimulated adenylate cyclase activity that was greater than that of the CON and TR + CL animals and a NaF-stimulated activity that was greater than the TR + CL group.

Low-frequency electrical stimulation

Peak twitch tension occurred within 10 s following the initiation of the acute stimulation and was not significantly different among the CON (880.0 ± 11.3 g), TR (923.8 ± 17.2 g) and TR + CL (942.5 ± 30.0 g) ani-

Table 2 Adenosine triphosphate (ATP), creatine phosphate (CP), creatine, inorganic phosphate (P_i), calculated free adenosine diphosphate (ADP_f) and ATP/ADP in Sprague–Dawley rat gastrocnemius muscles that were electrically stimulated (10 Hz) for 40 min or served as non-stimulated controls

	CON		TR		TR + CL		TR + CL + β	
	Control	Stimulated	Control	Stimulated	Control	Stimulated	Control	Stimulated
ATP, μmol g wet wt^{-1}	5.7 ± 0.1	2.9 ± 0.3	5.4 ± 0.2	2.3 ± 0.3	7.6 ± 0.3*#	3.6 ± 0.2*#	3.5 ± 0.4*#†	1.9 ± 0.2*†
CP, μmol g wet wt^{-1}	21.6 ± 0.7	5.5 ± 0.2	20.6 ± 1.3	5.0 ± 0.6	21.5 ± 1.3	4.0 ± 0.5*	2.0 ± 0.3*#†	0.5 ± 0.1*#†
Creatine, μmol g wet wt^{-1}	9.1 ± 0.7	13.4 ± 1.3	6.8 ± 0.6*	13.4 ± 1.3	8.8 ± 1.1	15.7 ± 1.6	2.4 ± 0.3*#†	4.1 ± 0.80*#†
P_i, μmol g wet wt^{-1}	5.5 ± 0.1	5.7 ± 0.2	5.3 ± 0.3	5.0 ± 0.3	5.3 ± 0.1	5.4 ± 0.2	1.4 ± 0.1*#†	3.5 ± 0.3*#†
ADP_f, μmol g wet wt^{-1}	0.2 ± 0.1	0.5 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	0.2 ± 0.03	1.1 ± 0.2*#	0.3 ± 0.1*#†	1.1 ± 0.2*#
ATP/ADP	37.4 ± 3.4	6.5 ± 0.6	48.5 ± 6.2	5.8 ± 0.9	40.4 ± 4.9	4.4 ± 0.9*	12.2 ± 1.2*#†	1.9 ± 0.3*#†

Values are mean ± SE. CON, control; TR, exercise trained; TR + CL, exercise trained in the presence of clenbuterol; TR + CL + β, exercise trained in the presence of clenbuterol and 2% β-guanadinoproprionic acid diet. *, significantly different from CON ($P < 0.05$). #, significantly different from TR ($P < 0.05$). †, significantly different from TR+CL ($P < 0.05$).

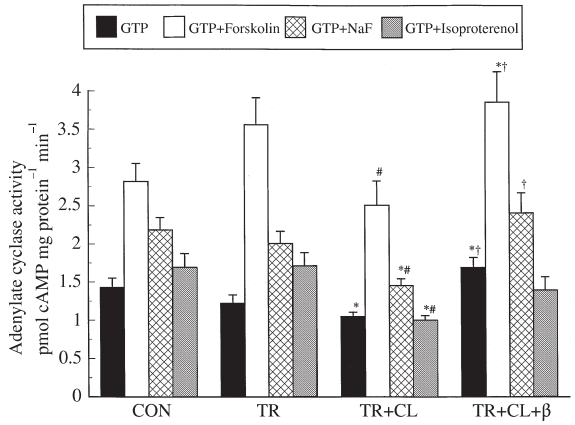

Figure 3 Adenylate cyclase activity in gastrocnemius muscles from female Sprague–Dawley rats that were divided among one of four groups: control (CON); exercise training (TR); exercise training + clenbuterol treatment (TR + CL) or exercise training + clenbuterol treatment + 2% β-guanadinoproprionic acid diet (TR + CL + β). Values are mean ± SE. *, significantly different from CON ($P < 0.05$). #, significantly different from TR ($P < 0.05$). †, significantly different from TR + CL ($P < 0.05$).

mals, but TR + CL + β animals had a peak twitch tension that was significantly less compared to the other groups (760.0 ± 21.8 g). However, when peak twitch tension was expressed relative to muscle weight no differences existed among groups ($P = 0.18$). A steady-state twitch tension was achieved by 5 min of stimulation and then remained at this level for the duration of the 40 min stimulation period. The percentage of peak twitch tension maintained during stimulation was not significantly different among the CON (33.5 ± 2.6%), TR (35.1 ± 0.9%) and TR + CL (33.4 ± 1.7%), but was significantly greater in the TR + CL + β (41.2 ± 2.6%) animals. With the exception of the TR + CL + β group, these findings are in excellent agreement with those of Hood & Parent (1991), who under similar experimental conditions reported a peak twitch tension of 883 ± 36 g and a steady-state twitch tension of ≈36%.

By the end of stimulation, ATP levels had fallen ≈50% in all groups (Table 2). Although the ATP concentration declined 53% in the TR + CL group, the absolute level of ATP in the gastrocnemius was significantly greater than that of the CON and TR animals. ADP_f was not significantly different between the CON and TR animals or TR + CL and TR + CL + β animals. ATP/ADP, an index of the energy charge of the muscle, was not significantly different between the CON and TR animals, but the TR + CL group had a reduced ATP/ADP compared with CON animals. No difference in creatine phosphate, creatine or inorganic phosphate existed among the CON, TR or TR + CL groups. ATP, creatine phosphate, creatine, inorganic phosphate and ATP/ADP were significantly lower in the muscles of the TR + CL + β animals following 40 min of electrical stimulation when compared with the CON, TR and TR + CL animals.

cAMP levels of the gastrocnemius were not significantly different among the CON (362.60 ± 61.67 pmol g wet wt^{-1}), TR (259.76 ± 31.51 pmol g wet wt^{-1}), TR + CL (321.77 ± 24.32 pmol g wet wt^{-1}) and TR + CL + β (352.55 ± 41.72 pmol g wet wt^{-1}) animals following stimulation.

DISCUSSION

In the present investigation we found exercise training to increase both skeletal muscle GLUT4 protein concentration and citrate synthase activity, which is consistent with previous reports (Banks *et al.* 1992, Rodnick *et al.* 1992, Brozinick *et al.* 1993, Etgen *et al.* 1993). Also in agreement with previous investigations was our finding that clenbuterol administration attenuated the exercise training-induced elevations in skeletal muscle GLUT4 protein concentration and oxidative capacity (Torgan *et al.* 1993a, b, Kuo *et al.* 1996). Of particular interest, however, was that clenbuterol did not attenuate the exercise training-induced rise in either GLUT4 protein concentration or citrate synthase activity in animals fed β-GPA, and GLUT4 protein concentration was significantly greater in these animals compared with the TR animals. Although the present investigation did not provide clenbuterol to rats in the absence of exercise training, we have consistently shown clenbuterol to reduce citrate synthase activity in skeletal muscle of non-exercise trained rats (Torgan *et al.* 1993a, b, 1995, Kuo *et al.* 1996)

It has been proposed that contractile activity induces increases in skeletal muscle oxidative capacity and GLUT4 protein concentration by altering the high energy phosphate state of the muscle (Green *et al.* 1992, Wiesner 1997, Yaspelkis *et al.* 1997, Yaspelkis *et al.* in press). This hypothesis is supported by the observation that providing a 1–2% β-GPA diet to rats for 8–10 weeks decreases the skeletal muscle high energy phosphates while increasing oxidative capacity and GLUT4 protein concentration (Shoubridge *et al.* 1985, Lai & Booth 1990, Ren *et al.* 1993, Yaspelkis *et al.* 1998). Furthermore, a significant correlation between the decline in muscle ATP and increase in GLUT4 protein concentrations induced by chronic electrical stimulation and β-GPA diet has been demonstrated (Yaspelkis *et al.* 1998). In the light of this evidence, it is also conceivable that elevating skeletal muscle ATP may reduce muscle oxidative capacity and GLUT4 protein concentration or possibly attenuate their increase with training. Thus, we propose that changes in the oxidative

capacity and GLUT4 protein concentration of muscle are reciprocal with changes in ATP concentration.

In this regard, we found ATP levels to be significantly elevated in TR + CL animals which may partially account for our previous observation of clenbuterol reducing citrate synthase activity in non-exercise trained muscle (Torgan *et al.* 1993a, 1995, Kuo *et al.* 1996). Furthermore, training adaptations in skeletal muscle of TR + CL animals may have been attenuated as a result of clenbuterol elevating ATP levels. When the gastrocnemius was subjected to 40 min of low frequency electrical stimulation ATP levels were reduced by ≈50% in all experimental groups. However, skeletal muscle ATP levels of TR + CL animals were not lowered to the same absolute concentration compared with the other groups. This observation is particularly relevant when placed in the context of exercise training.

Regularly performed exercise training increases the mitochondria content and respiratory capacity of skeletal muscle fibres (Holloszy 1967) which results in a smaller disturbance in the metabolic homeostasis of the trained muscle compared with untrained muscle during exercise of the same intensity (Constable *et al.* 1987). Specifically, with an increased mitochondria density, ATP resynthesis can be better maintained through oxidative phosphorylation. However, if ATP resynthesis can be adequately maintained during exercise training then the stimulus to increase mitochondrial density does not exist and thus, elevations in skeletal muscle oxidative capacity will not occur (Dudley *et al.* 1982). Consequently, the attenuation of the exercise-induced elevations in skeletal muscle oxidative capacity and GLUT4 protein concentration in the TR + CL animals may have been a result of the ATP levels being effectively maintained during training.

Further supporting the concept that skeletal muscle ATP levels play a major role in the contraction signal was that not only did citrate synthase activity increase in the TR + CL + β animals, but skeletal muscle GLUT4 protein concentration was elevated above that which occurred with exercise training alone. This appeared to be a result of the combined effects of exercise training and 2% β-GPA diet substantially reducing skeletal muscle ATP levels despite the presence of clenbuterol. Moreover, the exercise-induced adaptations could not be accounted for by differences in ATP/ADP or ADP_f among groups which implies that the ATP concentration, as opposed to the high energy phosphate state, regulates skeletal muscle adaptation. This agrees with the recent finding that rates of mitochondrial DNA transcription are regulated in direct response solely to changes in cytosolic ATP concentration (Enriquez *et al.* 1996). While it is unclear why the GLUT4 protein concentration was increased to a greater extent than citrate synthase in the TR + CL + β animals, it does appear that the stimulus which initiated the increased expression of these proteins is shared. These findings therefore indicate that if the muscle ATP concentration is reduced to a 'threshold level' by exercise and sustained for a critical period of time then increases in oxidative capacity and GLUT4 protein concentration will be initiated.

As chronic clenbuterol administration decreases β-adrenergic receptor density (Torgan *et al.* 1993a, b), then it is conceivable that clenbuterol treatment might also reduce adenylate cyclase activity. Adenylate cyclase activity is highly correlated with increases in muscle oxidative capacity and GLUT4 protein concentration (Kraus *et al.* 1992, Yaspelkis *et al.* 1997), and it has recently been suggested that activation of the adenylate cyclase system during exercise training may potentiate the exercise training effect (Yaspelkis *et al.* 1998). Consistent with this hypothesis, we found that GTP-, NaF- and isoproterenol-stimulated adenylate cyclase activity in the TR + CL animals was reduced below that of the CON animals and that forskolin-, NaF- and isoproterenol- stimulated adenylate cyclase activity was reduced below that of the TR animals. Of interest, however, was that although adenylate cyclase activity was not different between the CON and TR animals, skeletal muscle GLUT4 protein concentration and citrate synthase activities of the TR animals were elevated above the CON animals. Additionally, skeletal muscle GLUT4 protein concentration was greater in the TR + CL + β animals compared with the TR animals despite adenylate cyclase activity being similar between these two groups. These results therefore suggest that adenylate cyclase activation is not a primary component of the exercise signal. However, in order for a training effect to occur it does appear that this system may need to be fully functional as indicated by the lack of training adaptations that occurred in the TR + CL animals.

Our finding that skeletal muscle GLUT4 protein concentration can increase independent of an increase in adenylate cyclase activity is consistent with previous research (Yaspelkis *et al.* 1998). Furthermore, our observation that exercise training does not increase adenylate cyclase activity is not without previous corroboration (Plourde *et al.* 1993). Thus, the probability that activation of the β-receptor signalling pathway solely accounts for differences in exercise-induced elevations of skeletal muscle GLUT4 protein concentration and citrate synthase activity is quite small. This contention is supported by our finding that 40 min of low frequency electrical stimulation did not result in a significantly different cAMP concentration among the experimental groups. Moreover, it has been observed that a single 10 min exercise bout will double the concentration of cAMP in rat skeletal muscle (Goldfarb *et al.* 1989) but, if rats are exercise trained 10 min d^{-1}

for 13 weeks skeletal muscle oxidative capacity does not increase (Fitts *et al.* 1975). The results therefore suggest that exercise-induced skeletal muscle adaptations are not initiated through the β-adrenergic signalling pathway.

In summary, clenbuterol treatment was found to attenuate the exercise-induced elevations in skeletal muscle GLUT4 protein concentration and citrate synthase activity. However, when β-GPA was provided in combination with clenbuterol, exercise training-induced elevations in skeletal muscle GLUT4 protein and citrate synthase activity occurred. Although clenbuterol reduced adenylate cyclase activity, it does not appear that differences in adenylate cyclase activity fully accounted for the inhibition of training adaptations particularly because skeletal muscle GLUT4 protein concentration and oxidative capacity increased in the TR and TR + CL + β animals despite adenylate cyclase activity not being elevated above control values. Rather, it appears that the attenuated exercise training adaptations were caused by clenbuterol treatment maintaining more normal skeletal muscle ATP levels. Thus, exercise training-induced elevations in skeletal muscle citrate synthase activity and GLUT4 protein appear to occur in direct response to a reduced ATP concentration in the activated skeletal muscle.

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