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Attenuation of insulin resistance by chronic β_2-adrenergic agonist treatment Possible muscle specific contributions

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Abstract

A possible mechanism by which chronic clenbuterol treatment causes multiple physiological changes in skeletal muscle that leads to reduced insulin resistance in the obese Zucker rat (*fa*/*fa*) was investigated. Animals were gavaged with clenbuterol (CB) (0.8 $mg \cdot kg^{-1} \cdot day^{-1}$), terbutaline (TB) (1.0 $mg \cdot kg^{-1} \cdot day^{-1}$), or control (CT) vehicle for six weeks. Oral glucose tolerance and insulin responses were markedly improved in CB rats and impaired in TB rats. CB treatment caused a 24–34% gain in muscle mass in all muscle fiber types, and increases in 3-O-methyglucose transport (2-fold) and GLUT4 concentration (57%) in fast twitch glycolytic (FG) muscle. Oxidative capacity was reduced in both FG (47%) and fast twitch oxidative (FO) muscle (30%), but not in slow twitch oxidative (SO) muscle. Null model analysis for receptor occlusion demonstrated that most functional β-adrenoceptors were lost in FO (82%) and FG (89%) fibers, but not in SO fibers. We propose that hypertrophy is the result of continuous direct activation of β-adrenoceptors while loss in oxidative capacity may be the result of receptor down regulation. Improvements in insulin resistance may have been due, in part, to both increases in lean body mass and specific adaptations in FG muscle.

Keywords: Diabetes; Glucose transport; GLUT4; Insulin resistance; Muscle hypertropy

Introduction

Clenbuterol, a long acting β_2-adrenoceptor agonist, has been intensely studied as a repartitioning agent [1,2,3,4,5,6,7]. Specifically, clenbuterol increases muscle mass and decreases

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fat mass when given to animals daily for several weeks. In addition to these well-established effects, we observed improvements in oral glucose tolerance and decreases in oxidative capacity of fast but not slow twitch muscle in the obese Zucker rat [7,8]. The obese Zucker rat is a well established model for the etiology of obesity related type 2 diabetes [9,10]. They are homozygous recessive (*fa*/*fa*) for a missense mutation in the extracellular domain of the leptin receptor and exhibit hyperphagia [11,12]. Furthermore, the Zucker rat has elevated circulating leptin levels [13]. Although born at normal weight, they become obese and insulin resistant as they reach maturity.

Despite intensive investigation the mechanism by which clenbuterol acts is unknown. At high concentrations the β-adrenoceptor agonist propranolol prevents repartitioning suggesting direct activation of β-adrenoceptors as the cause of changes in body composition [1]. However, chronic treatment with a shorter duration β_2-agonist does not cause repartitioning unless chronic slow releasing implants containing the agonist are used [1]. This indicates a continual presence of the agonist is necessary for repartitioning to occur. We have previously demonstrated that β-adrenoceptor number decreases in fast twitch muscle after chronic clenbuterol treatment suggesting that repartitioning, the decrease in the oxidative capacity of fast twitch muscle fibers, and the improvement in oral glucose tolerance may be related to receptor down regulation in muscle tissue [6,8].

The present study addressed the association of β-adrenoceptor function with the effects of clenbuterol. We investigated whether direct activation or down regulation of β-adrenoceptor signal transduction is responsible for the multiple effects of clenbuterol on muscle and if these changes can explain the whole body improvement in insulin resistance seen after treatment. We examined the receptor mediated and post receptor activation of adenylyl cyclase after prolonged (six weeks) clenbuterol and terbutaline treatment. Terbutaline is a β_2-adrenoceptor agonist with a four-hour half elimination time in the rat, whereas the half elimination time of clenbuterol is 20 hours [14,15]. Furthermore, we examined the oral glucose tolerance of the obese Zucker rat, and insulin-stimulated glucose transport, GLUT4 transporter concentration, oxidative capacity and hypertrophy of muscle in response to treatment with these drugs.

We demonstrated that several of these physiological parameters differ in their relationship to adrenergic signal transduction alterations after clenbuterol treatment. Clenbuterol was highly effective in reversing whole body insulin resistance and terbutaline was contraindicated for treatment of pre-diabetic insulin resistance.

Methods

Study design

Obese Zucker rats (Harlen Sprague-Dawley, Indianapolis, IN) were pair weighted to one of three groups: control (CT), clenbuterol (CB), and terbutaline (TB). Rats in the CB and TB groups were gavaged with 0.8 and 1.0 $mg \cdot kg^{-1}$, respectively, once per day , five days per week for six-weeks. CT rats were gavaged with vehicle only. The dosages selected for the β_2-agonists were maximally effective but constituted a minimal potential for crossreactivity with other beta receptors (14,15). After five weeks, all animals were given an oral glucose tolerance test following a 12-hour fast and a 48-hour withdrawal from treatment. After six weeks, drug

treatment was withdrawn for 48 hours and the hind limbs were perfused after a 12-hour fast. The animal care and use committee of the University of Texas at Austin approved all procedures.

Oral glucose tolerance test and insulin response

Resting blood glucose and insulin were obtained via the tail vein prior to intubating each rat with 1 g glucose per kg body weight (50% glucose solution). Tail blood samples (0.5 ml) were collected at 15, 30 and 60 min after glucose intubation. Plasma was extracted and assayed for glucose using a glucose analyzer (Yellow Springs Instruments 23A, Yellow Springs, OH) and insulin was measured by RIA (Linco, St Charles, MO).

Hind limb perfusion

The hind limbs of rats were surgically isolated and perfused in a non-recirculating system as previously described [8]. Animals were anesthetized with 65 $mg \cdot kg^{-1}$ pentobarbital. The soleus (slow twitch, oxidative fibers), plantaris (mixed fast twitch fibers), red (fast twitch, oxidative fibers) and white (fast twitch, glycolytic fibers) sections of the gastrocnemius and quadriceps were removed from the right leg, freeze clamped and stored at $-80°C$ for later measurements. The left leg was isolated, washed with 25 ml Krebs- Henseleit buffer following an intracardiac injection of sodium pentobarbital. Following washout, the left leg was perfused at 5 $ml \cdot min^{-1}$ for 10 min with an equilibration medium consisting of 4% BSA, 30% human red blood cells, 8 mM glucose, and 1,000 $\mu U \cdot ml^{-1}$ insulin (Lilly R-100, Indianapolis, IN) that was gassed with 95% O_2 and 5% CO_2. After this equilibration period, the leg was perfused with a media that contained radioactive tracers for an additional 10 min. This tracing media was identical to the equilibration media except it did not contain glucose but contained 2 mM pyruvate, 8 mM 3-O-methylglucose (0.3 $\mu Ci \cdot ml^{-1}$ ^3H), 2 mM mannitol (0.1 $\mu Ci \cdot ml^{-1}$ ^{14}C). The tracing period (10 min) selected allowed for the determination of intracellular 3-O-methylglucose accumulation during the linear phase of transport [16,17]. At the end of this period the same muscles were trimmed of connective tissue and weighed. The soleus and plantaris muscles were excised and weighed intact to determine muscle hypertrophy. Glucose transport capacity was based on the accumulation of 3-O-methylglucose, which is transported but not metabolized by muscle fibers. Adjustment was made for the accumulation of mannitol, which is not transported into the muscle fibers but retained within the extracellular space. Values were expressed as $\mu mol \cdot g^{-1} \cdot h^{-1}$.

Adenylyl cyclase assay

Preparation of muscle samples for adenyly cyclase assay was carried out as previously described [18] and cyclic AMP was measured via a competitive binding assay with ^3H labeled cyclic AMP (Diagnostic Products, Los Angeles, CA). Production of cyclic AMP was measured with the following present in the reaction medium; isoproterenol (RBI-102, Nutick, MA) at a concentration between 10^{-9} and 10^{-4} M or 200 μM forskolin (Sigma F6886, St. Louis, MO) as a positive control. Results were reported as $pmol \cdot mg^{-1}protein \cdot min^{-1}$.

Adenylyl cyclase calculations

Once dose-response curves were determined to differ among experimental groups by repeated measure ANOVA analysis, non-linear regression was used to describe the three curves.

Curves were fitted to the following equation using a Levenberg-Marquardt algorithm provided with SPSS®:

$$\text{Response} = (\text{Min}-\text{Max})/(1+\text{EXP}(\text{slope} \times (\ln(\text{drug concentration})-\ln(\text{ED}_{50})))) + \text{Max}.$$

Where response is the rate of cAMP production, Min and Max are the predicted basal and maximal rates of cAMP production, slope is the exponential slope of the fitted curves, and ED_{50} is the concentration of isoproterenol that is predicted to give a half maximal response. Both ANOVA analysis and F test on the residuals of successive models were used to test these curves for statistical significance. The clenbuterol curves were different from the control and the terbutaline curves in fast oxidative muscle. Hence, further models were used to describe the nature of these relationships.

A following null model for receptor occlusion was used to analyze the shift in dose response curve and determine how many receptors were functionally lost, where response (R) equals:

$$R = (\text{Min} - \text{Max})/(1+\text{EXP}(\text{slope} \times (\ln(\text{drug concentration})-\ln(\text{ED}_{50})-\ln(\text{shift})))) + \text{Max}.$$

$$\text{Shift} = 1/(1-\text{Yi}) + (\text{Yi}/(1-\text{Yi})) \times (1/\text{Ka}) \times \text{drug concentration}.$$

Yi is the predicted fraction of functional receptors lost after clenbuterol induced down regulation, and Ka is the concentration at which half of the receptors are predicted to be activated.

Western blots and citrate synthase

The GLUT4 concentration within muscle samples was determined by Western blot analysis and expressed as a percentage of a heart standard. Samples were homogenized in 20 volumes N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HES) buffer and approximately 75 μg protein, determined by the Bradford method (BioRad 500, Hercules, CA), were separated by a 12% SDS-PAGE resolving gel and semi-dry transferred to PVDF membranes. The membranes were blotted for GLUT4 using an ECL detection system (Amersham RPN 2109, Piscataway, NJ). Affinity purified primary antibody (Michael Gibbs, Pfizer Inc., Groton, CT) was used at 1/5,000 dilution. The exposed film was digitized with a mirror scan 600+ flatbed scanner with a transparency adapter (Mirror Technologies Inc, Taiwan) and then analyzed using NIH Image software. An aliquot of the HES homogenate was used to determine citrate synthase activity by the method of Sere (19).

Statistics

ANOVA analysis with a rejection criteria of $p<0.05$ was used for all analyses except the null models. These models were analyzed by ANOVA, when appropriate, and by F test on the ratio of residuals between models described above using rejection criteria of $p<0.05$.

Results

Oral glucose tolerance and insulin response

Oral glucose tolerance tests (OGTT) were affected by both clenbuterol (CB) and terbutaline (TB) treatments. Resting and peak glucose concentrations and the area under the curve were reduced in CB animals and increased in TB animals (Fig 1).

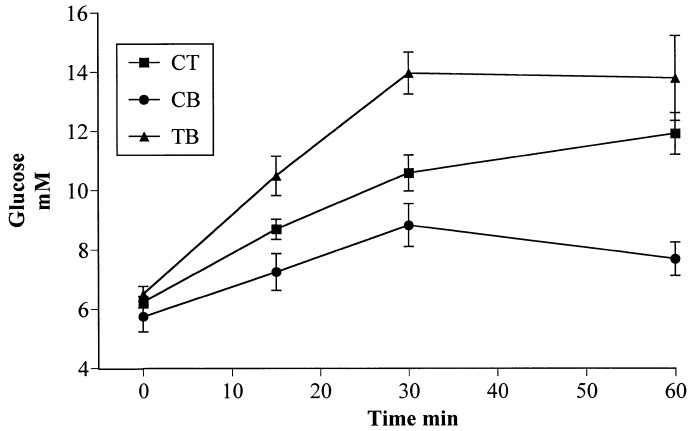

Fig. 1. Oral glucose tolerance test after a five-week treatment with CT, control; CB, clenbuterol; or TB, terbutaline. Error bars represent standard error of the mean for 6–8 animals.

CB treatment resulted in a substantial reduction in both basal insulin level and insulin response to an OGTT (Fig 2). TB treatment, however, did not alter the basal insulin level nor was there a significant effect on the insulin response to the OGTT.

3-O-methylglucose transport and GLUT4

Despite the large improvement in the OGTT (Fig 1) only fast twitch glycolytic (FG) muscle fibers exhibited differences in 3-O-methylglucose transport among experimental groups (Fig 3). Transport was increased (200%) by CB treatment in muscle composed of FG fibers compared to the control. Western blots revealed a small but significant increase (57%) in total GLUT4 concentration in FG muscle of the CB group (Fig 4). Slow twitch oxidative (SO) or fast twitch oxidative (FO) fibers did not differ among experimental groups.

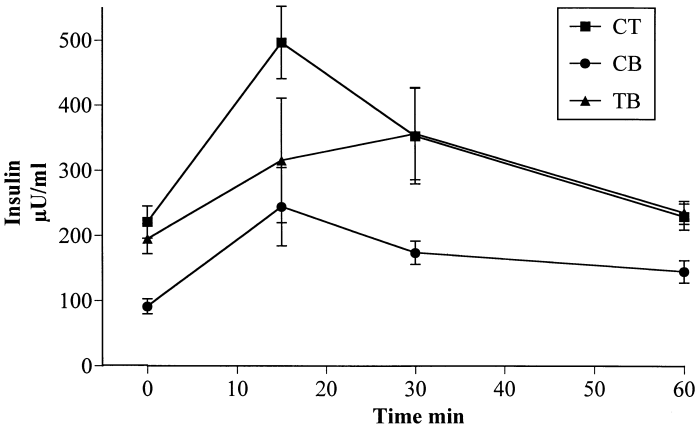

Fig. 2. Insulin response to an oral glucose tolerance test after a five-week treatment with CT, control; CB, clenbuterol; or TB, terbutaline. Error bars represent standard error of the mean for 6–8 animals.

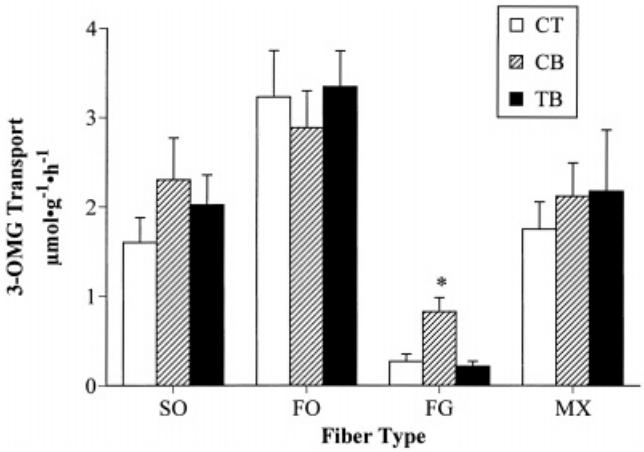

Fig. 3. 3-O-methylglucose transport in different muscle fiber types during hindlimb perfusion. CT, control; CB, clenbuterol; and TB, terbutaline. SO, slow twitch oxidative; FO, fast twitch oxidative; FG, fast twitch glycolytic; and MX, mixed FO and FG fibers of the plantaris muscle.

Muscle hypertrophy

As expected CB increased muscle mass in both slow twitch fibers of the soleus (35%) and the fast twitch fibers of the plantaris (24%) (Fig 5). TB did not affect muscle size. No differences in total body weight were detected among experimental and control groups.

Oxidative capacity

Citrate synthase activity did not change after CT and TB treatment in SO muscle, while both FO and FG muscle had a lower citrate synthase activity compared to control after CB

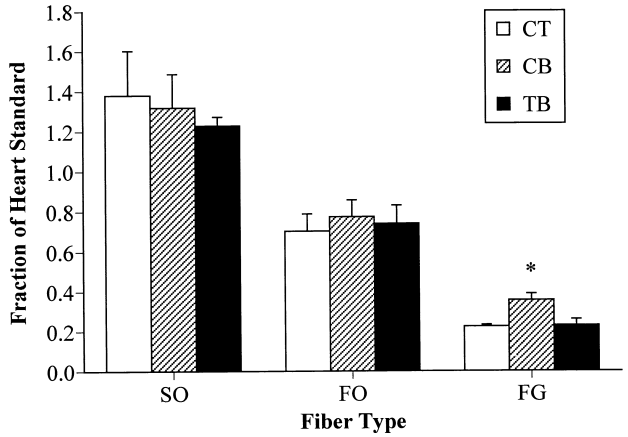

Fig. 4. GLUT4 concentration in different fiber types as determined by Western blotting and normalization to a heart standard. CT, control; CB, clenbuterol; and TB, terbutaline. SO, slow twitch oxidative; FO, fast twitch oxidative; FG, fast twitch glycolytic fibers.

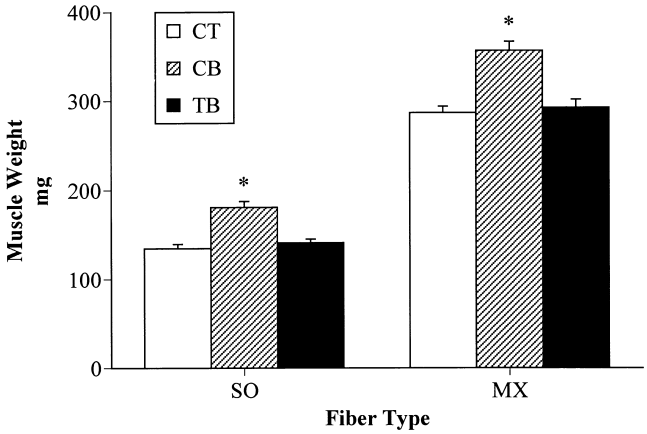

Fig. 5. Muscle hypertrophy determined by weighing intact muscles. CT, control; CB, clenbuterol; and TB, terbutaline. Slow twitch oxidative (SO) soleus muscle and MX, mixed fast twitch oxidative (FO) and fast twitch glycolytic (FG) fibers of he plantaris muscle.

treatment (Fig 6). In CB animals, citrate synthase activity decreased by 30% in FO and by 47% in FG fibers.

Signal transduction

Individual dose-response curves were fitted by nonlinear regression analysis for each experimental treatment on the three muscle fiber types. The dose-response curves in the CB group differed from their respective controls based on repeated measure ANOVA. Null model analysis for receptor occlusion was used to calculate the expected loss in receptors that would account for the rightward shift in the EC_{50} and downward shift in response of the CB group compared to the pooled CT and TB curves (Fig. 7 & 8).

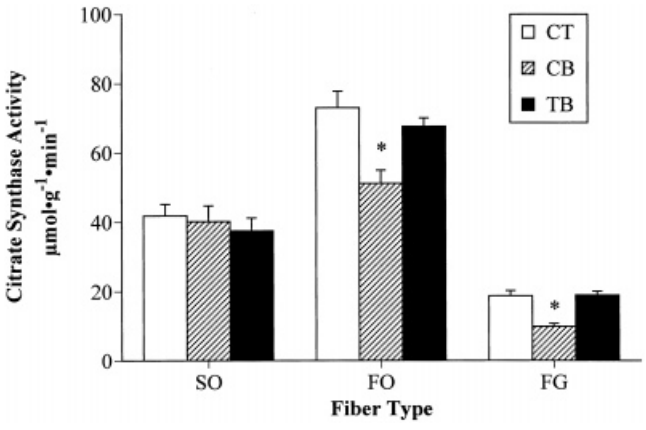

Fig. 6. Oxidative capacity of different fiber types as determined by citrate synthase activity. CT, control; CB, clenbuterol; and TB, terbutaline. SO, slow twitch oxidative; FO, fast twitch oxidative; FG, fast twitch glycolytic fibers.

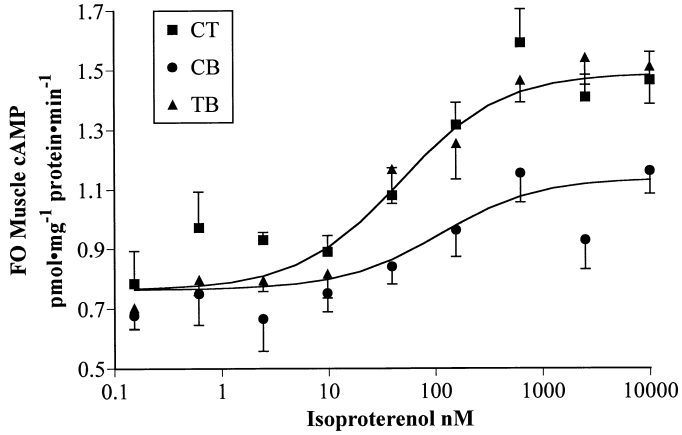

Fig. 7. Nonlinear regression and null model for cAMP response to isoproterenol in fast twitch oxidative (FO) muscle. CT, control; CB, clenbuterol; and TB, terbutaline.

Based on this analysis FO and FG muscle lost approximately 82% and 89% of their functional receptors which resulted in a 50% and 59% loss in responsiveness (cAMP production), respectively. Therefore receptor reserve was lost and approximately half the functional receptors needed for a full response were left. The concentration of isoproterenol at which one-half of the receptors are expected to be activated (Ka) could also be estimated at 220 nM and 257 nM for FO and FG, respectively. The SO muscle showed no significant change in the dose-response curves among treatments, therefore no down regulation or receptor activation values could be calculated (Fig. 9). Forskolin stimulated adenylyl cyclase activity did not differ among experimental treatments (Table 1) and changes in responsiveness were assumed to be receptor related.

Discussion

In response to insulin, blood glucose is primarily moved into muscle cells [20] by glucose transporters (GLUT4) that are translocated from the interior of the muscle cell to the cell surface membrane [21]. In the insulin resistant fatty Zucker rat, the primary defect is in the translocation of GLUT4 in response to insulin [21,22]. However, it has been shown that insulin-stimulated muscle glucose transport is improved if GLUT4 concentration of the fatty rat is increased [3,22,23,24]. The increase in 3-O-methylglucose transport in FG muscle in response to insulin after CB treatment would suggest that a greater fraction of glucose transporters can be moved to the surface of this muscle fiber type, or there are more total glucose transporters available to move to the surface (Fig 3). Total cellular GLUT4 as assessed by Western blotting increased after CB treatment suggesting transport was improved by increasing the total glucose transporter pool (Fig 4). Therefore, a change in the total number of GLUT4 transporters translocated to the surface of muscle fibers in response to insulin could explain in part the improvement in OGTT for CB treated rats.

The relatively low transport rate of the FG fiber type would suggest, however, that mechanisms other than enhancement in insulin-stimulated muscle glucose transport were involved

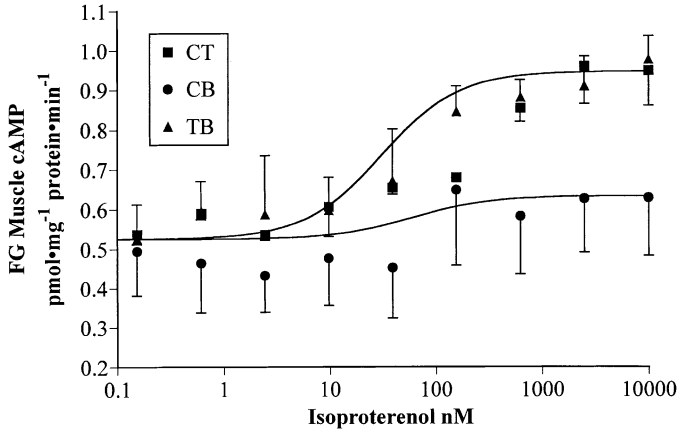

Fig. 8. Nonlinear regression and null model for cAMP response to isopoterenol in fast twitch glycolytic (FG) muscle. CT, control; CB, clenbuterol; and TB, terbutaline.

in the improvement in the OGTT observed. Most likely the relative increase in muscle mass to total body weight also contributed to the reduction in insulin resistance since any increase in muscle mass would effectively augment total skeletal muscle GLUT4 and blood glucose clearance. Changes in blood glucose depend on the difference between rate of appearance of glucose from the liver and gut, and the rate of disappearance of glucose by muscle in the postprandial state [20]. A small difference between these rates can cause blood glucose to rise and therefore a small variance in muscle mass could contribute disproportionately to the clearance of blood glucose. Likewise, the effect of a small difference in total FG GLUT4 could be amplified by the same mechanism.

In addition, a mechanism not directly involving skeletal muscle may have lead to some of the improvements in oral glucose tolerance found in our study. A recent study demonstrated improvements in glucose intolerance of the Zucker rat after chronic treatment with a β_3-agonist [15]. This would suggest that activation of fat tissue alone could explain at least some of the antidiabetic effects of chronic β-agonist treatment since β_3-adrenoceptors are found primarily in brown and white adipose tissue. β_3-agonists are more likely specific for β_3-adrenoceptors on adipocytes and induce lipolysis, whereas CB is more specific for β_2-adrenoceptors found in muscle. However, the possibility of some cross-over effect cannot be excluded [25]. It is also possible that clenbuterol improved glucose tolerance by increasing the livers response to insulin. This could result in a reduced hepatic glucose output, as well as an increase in hepatic glucose uptake. However, we have found no information on the response of the liver following chronic clenbuterol treatment.

Although CB treatment reveals possible relationships between changes in muscle function and improvements in OGTT, the TB-induced impairment of OGTT cannot be easily explained by the measures made in this study. β-agonists can act as insulin secretagogues [26,27]. Therefore, a possible pancreatic beta cell burnout mechanism could lead to the decrease in insulin response from TB animals (Fig 2) and subsequent increase in blood glucose for the OGTT (Fig 1). However, since CB did not cause this effect, it would have to either de-

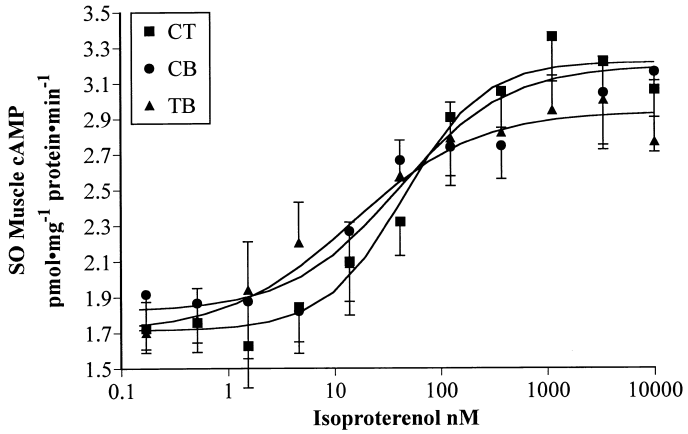

Fig. 9. Nonlinear regression and null model for cAMP response to isoproterenol in slow twitch oxidative (SO) muscle. CT, control; CB, clenbuterol; and TB, terbutaline.

sensitize the beta cells to adrenergic stimulation or the peripheral effects on muscle and adipose tissue would have to override any negative effects on insulin secretion. Future studies on pancreatic function following short and long duration chronic agonists treatments need to be done to address this question before the usefulness of CB for treatment of insulin resistance can be fully accepted.

Functional adrenoceptor number, as determined by isoproterenol stimulation, was reduced in FO and FG muscle fibers by CB treatment (Fig. 7, 8). Other studies have reported a loss in adrenoceptor binding by Scatchard analysis in both normal and insulin resistant rats following CB treatment [7,28], but this is the first study to report functional changes in receptor activity. The loss in response to isoproterenol in FO and FG fibers was likely due to reduced receptor number since no changes in forskolin-stimulated adenylyl cyclase activity occurred (data not shown). TB had no effect on either FO or FG fibers suggesting sustained activation of adrenoceptors is necessary for chronic down regulation of adrenoceptor number and activity.

β-adrenoceptor down regulation has been previously suggested as a possible mechanism for muscle hypertrophy because long-acting agonists that cause hypertrophy in fast twitch muscle also cause a decrease in β-adrenoceptor number [1, 28]. In the present study, the hypertrophy of the FO and FG fibers that occurred was accompanied by the down regulation of

Table 1
Muscle adenylyl cyclase response to 200 μM forskolin stimulation as determined from cAMP production

	Muscle fiber type		
Treatments	Slow-twitch oxidative	Fast-twitch oxidative	Fast-twitch glycolytic
Control	5.12±0.79	3.78±0.37	3.53±1.29
Clenbuterol	6.73±1.19	3.52±0.35	3.35±1.16
Terbutaline	5.24±0.81	3.94±0.27	2.29±0.13

Mean values (±SEM) for cAMP are given in pmol·mg^{-1} protein·min^{-1}.

the β-adrenoceptors supporting this hypothesis. However, hypertrophy of SO muscle occurred despite no noticeable down regulation of β-adrenoceptors. Thus our results suggest that sustained direct activation, and not down regulation of the β-adrenoceptors as the cause of CB-induced hypertrophy. Although indirect endocrine effects have been proposed as a cause of muscle hypertrophy, castration and adrenalectomization does not prevent CB-induced muscle hypertrophy [29,30]. The lack of a TB-induced effect also indicates that a long acting β-adrenergic agonist is necessary for hypertrophy to occur. Some recent evidence suggests that chronic activation of the adrenergic pathway could in theory activate growth promoting signal transduction pathways, although support for β_2-adrenergic activation of growth pathways is limited [31].

The finding that β-adrenoceptors in the SO fibers of the soleus muscle did not functionally down regulate is important and suggests that β-adrenoceptors in the SO fibers are different than those in FO and FG fibers. Although there is evidence to support the presence of the non-desensitizing β_3-adrenoceptors in soleus muscle, they do not appear to be the majority of beta receptors in this tissue [32,33,34]. Consequently, another mechanism must prevent the down regulation in the SO muscle. Also, any effect of CB on SO fibers cannot be directly attributed to down regulation of β-adrenoceptors.

While down regulation of the β-adrenoceptor does not appear to be required for muscle hypertropy, evidence from this and previous studies from our laboratory suggest it is a factor regulating muscle oxidative capacity [7]. This is consistent with the observation that oxidative capacity and β-adrenoceptor density tend to cooperatively upregulate in response to exercise training or chronic electrical stimulation [3,35,36,37,38]. Further support for this hypothesis is our finding that SO fibers did not exhibit a decrease in oxidative capacity or a decrease in β-adrenoceptor density. Whereas exercise training and chronic electrical stimulation increase oxidative capacity, β-adrenoceptor activity and post receptor adenylyl cyclase activity [35,39], post receptor activity did not change in any fiber type with CB treatment. Therefore, it would appear that post receptor changes in adrenergic function do not have to accompany changes in β-adrenoceptor density to modify the muscle oxidative capacity.

Finally, FO and FG muscle had similar receptor pharmacological changes, but only the FG muscle demonstrated an increase in GLUT4 concentration and glucose transport. Thus, it is difficult to explain the increase in GLUT4 in the FG muscle. Although adenylyl cyclase activity positively correlates with GLUT4 concentration and glucose transport capacity in skeletal muscle in vivo [18], cAMP clearly down regulates the transcription of GLUT4 in adipose cell lines [40]. Hence, both cis and trans factors may be mediated by cAMP or associated factors that can change GLUT4 concentration. One possible explanation therefore is that the low adenylyl cyclase activity of FG muscle may favor GLUT4 induction due to lack of cAMP suppression of GLUT4 transcription.

Conclusion

The results of the present study demonstrate the effectiveness of CB to attenuate and TB to exacerbate insulin resistance in the *fa*/*fa* rat despite both compounds being β agonists. The CB-induced improvement in insulin resistance may be partially due to both general muscle hypertrophy and specific improvements in the response of FG muscle fibers to insulin. Mus-

cle hypertrophy following CB treatment was likely due to sustained β-adrenergic activation rather than down regulation of β-adrenoceptors in skeletal muscle, while the loss of oxidative capacity appeared to be related to functional down regulation of β-adrenoceptors.

Acknowledgments

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