Rapamycin Inhibits the Growth and Muscle Sparing Effects of Clenbuterol.

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ABSTRACT

Clenbuterol and other β2-adrenergic agonists are effective at inducing muscle growth and attenuating muscle atrophy through unknown mechanisms. This study tested the hypothesis that clenbuterol-induced growth and muscle sparing is mediated through the activation of Akt/mTOR signaling pathways. Clenbuterol was administered to normal weight-bearing adult rats to examine the growth-inducing effects, and to adult rats undergoing muscle atrophy as the result of hindlimb suspension or denervation to examine the muscle sparing effects. The pharmacological inhibitor, rapamycin, was administered in combination with clenbuterol in vivo to determine whether activation of the mammalian target of rapamycin (mTOR) was involved in mediating the effects of clenbuterol. Clenbuterol administration increased the phosphorylation status of PKB/Akt, S6K1/p70^s6k, and 4E-BP1/PHAS-1. Clenbuterol treatment induced growth by 27-41% in normal rats and attenuated muscle loss during hindlimb suspension by 10-20%. Rapamycin treatment resulted in a 37-97% suppression of clenbuterol-induced growth and a 100% reduction of the muscle sparing effect. In contrast, rapamycin was unable to block the muscle sparing effects of clenbuterol following denervation. Clenbuterol was also shown to suppress the expression of the MuRF1 and MAFbx transcripts in muscles from normal, denervated and hindlimb suspended rats. These results demonstrate that the effects of clenbuterol are mediated, in part, through the activation of Akt and mTOR signaling pathways.
INTRODUCTION

Chronic administration of clenbuterol and other synthetic compounds that activate β-adrenergic receptors have been shown to induce skeletal muscle growth in multiple mammalian species including rats, cows, sheep, pigs and humans (23, 25, 30, 42). In addition to promoting growth in normal animals, β-adrenergic agonists can attenuate skeletal muscle atrophy induced by a variety of conditions including denervation, hind limb unloading, dexamethasone treatment, cachexia, and aging (1, 10-12, 39, 49). The mechanisms by which clenbuterol and other β-adrenergic agonists promote growth or inhibit atrophy in skeletal muscle are unclear, and could involve both an increase in the rate of protein synthesis and/or a decrease in protein degradation (23). The effects of clenbuterol are mediated through binding to the β2-adrenergic receptor as demonstrated from experiments performed in mice lacking the β1, β2 or both β1/β2 adrenergic receptors (20). The signaling pathways that are activated downstream of the receptor to induce the growth and muscle sparing responses, however, are unclear.

The β-adrenergic receptor subtypes (β1AR, β2AR and β3AR) are members of the G protein-coupled receptor (GPCR) superfamily. Ligand binding on the receptor promotes guanosine diphosphate-guanosine triphosphate (GDP-GTP) exchange on the Gα subunit and subsequent dissociation of Gα from Gβγ, leading to activation of Gα and release of free Gβγ heterodimers (see (16) for review). Subsequently, Gα and Gβγ function as signaling mediators to directly interact with a variety of effector proteins. The Gα subunit is divided into four families based on primary sequence: Gs, Gi, Gq and G12. In general, specificity and selectivity in GPCR signaling is achieved by coupling of a given GPCR to a single class of G proteins. The β1-adrenergic receptor is coupled
exclusively with Gαs, which in turn activates adenylate cyclase (AC), catalyzing 3’, 5’-adenosine monophosphate (cAMP) formation (30). Historically, the β2-adrenergic receptors were also believed to couple exclusively with Gαs. However, in cardiac tissue, β2-adrenergic receptors can couple to both Gαs and Gαi signaling pathways (34, 47).

The phosphatidylinositol 3-kinase (PI3k)-protein kinase B/Akt signaling pathway has been implicated in the regulation of cell growth, and more specifically muscle fiber growth, in mammals (8, 13, 36, 41). The prevailing theory is that the growth and muscle sparing effects of clenbuterol, and other β2AR agonists, in skeletal muscle are mediated through the classic β2AR-Gs-AC-cAMP signaling pathway. The possibility exists, however, that in skeletal muscle additional signaling pathways are activated by β2-adrenergic receptors. One likely pathway is the PI3k/Akt pathway given that (1) β2AR stimulation in the heart couples to Gαi-Gβγ to activate PI3k/Akt signaling pathways (34, 47), and (2) clenbuterol treatment acutely activates p70s6k/S6K1 and 4E-BP1 in skeletal muscle (44).

The objective of the present study was to determine whether activation of Akt and mTOR signaling pathways mediate the growth and muscle sparing effects of clenbuterol. Examination of (1) the phosphorylation/activation status of Akt, S6K1/p70s6k and 4E-BP1 following clenbuterol treatments, and (2) the effects of co-administration of rapamycin on the growth and muscle sparing effects of clenbuterol, was used to assess involvement of the Akt and mTOR pathway. The present findings provide support for the hypothesis that Akt and mTOR signaling pathways partially mediate the growth and muscle sparing effects of clenbuterol.
METHODS

Animals and Treatment Protocols. All experiments were performed in young adult female Sprague Dawley rats (Taconic Farms) with initial body weights of 240-280 grams. Animals were assigned to one of three experimental groups (normal adults, denervation or hindlimb suspension) and treated with vehicle, clenbuterol, rapamycin or a combination of clenbuterol and rapamycin. Rats were randomized to treatment or vehicle groups so that mean starting body weights of each group were equal. Drug treatment began on the day of surgery or the first day of hindlimb unloading. Clenbuterol (Sigma) was delivered once daily via a subcutaneous injection at a dose of 3 mg/ml, dissolved in water. Rapamycin (Calbiochem) was delivered once daily via intraperitoneal injection at a dose of 1.5 mg/kg, dissolved in 2% carboxymethylcellulose.

Muscle Atrophy Models. Denervation of the lower limb muscles in the right leg only was induced through transection of the sciatic nerve. Under isoflurane anesthesia and using aseptic surgical techniques, the sciatic nerve was isolated in the midthigh region and cut with a sharp scissor. Unloading of the lower limb muscles was accomplished using a non-invasive tail suspension model (14). The tail was attached via traction tape and a plastic bar to a swivel mounted at the top of the cage, allowing free 360° rotation. The rats were maintained in ~ 30° head-down tilt position with their hind limbs unloaded. All animal procedures were approved by the Institutional Animal Care and Use Committee and conformed to the Guiding Principles in the Care and Use of Animals of the American Physiological Society.

Tissue collection. Tissue samples were collected at various time points (up to 14 days) following drug treatment and experimental manipulation. Rats were anesthetized
with ketamine/xylazine (85 mg/kg, 5 mg/kg) and the hind limb muscles were dissected free of connective tissue, weighed, frozen in liquid nitrogen and stored at -80 °C for later analysis. Upon completion of the tissue removal, the rats were killed by exsanguination.

**Western Blots.** Muscles were homogenized at 4°C in RIPA lysis buffer (1% NP40, 0.5% Na deoxycholate, 0.1% SDS in 50 mM NaCl, 20 mM Tris, pH 7.6) containing 1 mM PMSF, 10 µg ml⁻¹ aprotinin, 10 µg ml⁻¹ leupeptin, 5 mM benzamidine, 1mM EDTA, 5 mM N-ethylmaleimide, 50 mM NaF, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 100 nM Okadaic acid, and 5 nM microcystin LR. Homogenates were clarified by centrifugation at 12,000g for 20 min prior to determination of protein concentration by BCA assay (Pierce Chemical Co.). SDS-PAGE was performed on 7.5% or 15% (4E-BP1) gels prepared with an acrylamide: bisacrylamide ratio of 100:1.

Western blots were revealed with enhanced chemiluminescence (Renaissance, NEN). Antibodies against Akt (NEB), p70⁶⁶k (Santa Cruz), and 4E-BP1 (Zymed) were used to detect protein expression levels. Phosphorylation specific antibodies against Ser 473 (NEB) were used to detect the catalytically activated form of Akt.

**Kinase Assay of S6K1:** Protein A-agarose beads (Bio-Rad, 0.1 ml of serum per ml of packed beads) were incubated at 23 °C for 60 min with nonimmune serum or antisera to p70⁶⁶k. The beads were then washed five times with phosphate-buffered saline (PBS) (145 nM NaCl, 4 mM KCl and 10 mM sodium Pi, pH7.4) and once with homogenization buffer. Samples of extract (100 µl) were incubated with beads (10 µl) for 60 min at 4 °C with constant mixing and then washed twice (0.5 ml of homogenization buffer/wash) and suspended in 100 µl of homogenization buffer. To measure p70⁶⁶k activity, immune complexes were incubated with 10 µl of solution containing 50 mM sodium β-
glycerophosphate (pH 7.4), 14 mM sodium fluoride, 10 mM MgCl₂, 1 mM dithiothreitol, 9 µM cAMP-dependent protein kinase inhibitory peptide, 20 µM calmidazolium, 200 µM [γ⁻³²P] ATP (300-500 cpm/pmol) and 40 S ribosomes (2 mg/ml final concentration) (provided by John C. Lawrence Jr.). (5).

RNA Extraction and Northern Blots. Total RNA was extracted from frozen muscle samples using a modification of the lithium chloride method of RNA extraction described by Auffray and Rougeon (4). Muscles were homogenized at 4°C in 3M LiCl-6M Urea and the homogenate was precipitated overnight at 4°C. The recovered lithium-RNA was acid phenol-choroform extracted to remove contaminating proteins. RNA concentration and purity will be determined by spectrophotometry at 260 nm. Ten µg were subjected to Northern blot analysis with ³²P-labeled DNA probes for MuRF1 and MAFbx transcripts. To control for the amount of total RNA loaded, the agarose gels were stained with ethidium bromide and photographed to assess ribosomal RNA bands. Probes were prepared with a random-priming kit (Prime-It II, Stratagene). Northern probes for rat MuRF1 were made by PCR, spanning bp 24 - 612 of coding sequence. For rat MAFbx, the probe was made by PCR, and spanned bp 21 - 563 of coding sequence.

Statistical Analysis. All data are expressed as means ± standard error. A one-way ANOVA using Fisher's post hoc correction for multiple paired comparisons was used for comparisons between groups (Statview). Statistical significance was set at p<0.05.
RESULTS

Effect of Rapamycin on Clenbuterol Induced Growth and Muscle Sparing.

The ability of clenbuterol to induce growth and attenuate atrophy is well known, however the pathways responsible for mediating the effects of clenbuterol are poorly understood. To determine whether the effects of clenbuterol are mediated by mTOR signaling pathways, the pharmacological inhibitor, rapamycin, was administered in combination with clenbuterol. Rapamycin is a relatively selective inhibitor of mTOR (13, 17), and does not induce atrophy when given to normal adult rats (8).

The growth-promoting effects of clenbuterol were assessed in the lower limb muscles of normal young adult female rats (254 ± 12 g BW, mean ± SD). Daily administration of clenbuterol (3 mg/kg) for fourteen days induced significant growth in all muscles examined: tibialis anterior (TA), medial gastrocnemius (MG), plantaris (PL) and soleus (SOL) (Fig. 1). The increase in mean wet weight relative to control ranged from 27 to 41%. In general, muscles composed predominantly of fast fiber types showed the greatest response to clenbuterol treatment. Clenbuterol induced growth in the TA, MG and PL was significantly reduced by co-administration of rapamycin (Fig. 1). Muscle weights of the TA, MG and PL were not significantly different from control following the combination treatment. In contrast, rapamycin co-administration resulted in only a 10% decrease in the wet weight of the SOL relative to clenbuterol alone.

Next, rapamycin was co-administered with clenbuterol during two atrophy-inducing events: 10 days of denervation and 14 days of hindlimb suspension. Daily administration of clenbuterol alone was able to attenuate muscle loss following both denervation (Fig. 2) and hindlimb suspension (Fig. 3) induced atrophy. Ten days of
clenbuterol treatment following sciatic nerve transection in young female rats (258 ±11g, BW) resulted in significantly less loss of muscle mass in all muscles studied compared to untreated denervated muscles (Fig 2). Administration of rapamycin alone following denervation had no effect on the progression of muscle atrophy. Moreover, co-administration of rapamycin (1.5 mg/kg) with clenbuterol (3 mg/kg) had no effect on the ability of clenbuterol to suppress muscle atrophy in the TA, MG or PL. In fact, co-administration of rapamycin further reduced (4-8%) the amount of muscle loss following denervation; this effect being significant in the PL. While rapamycin had no effect on clenbuterol in fast muscles, it reduced the effectiveness of clenbuterol in the SOL (Fig 2). In these experiments, the sciatic nerve was transected only on the right side. Of note is that while clenbuterol attenuated atrophy in the muscles of the right (denervated) leg, it induced hypertrophy in the muscles of the left (neurally intact) leg. Further, while rapamycin had no effect on the muscles of the right leg, it was able to suppress the growth-effects of clenbuterol in the muscles of the left leg (data not shown).

Fourteen days of hindlimb suspension in young female rats (264 ±22 g,BW) lead to significant atrophy (10-44%) in the TA, MG, PL and Sol muscles. Daily administration of clenbuterol during the unloading period resulted in significant sparing of muscle mass in those muscles composed predominantly of fast fiber types (i.e., TA, MG, PL), but not the SOL, a predominantly slow muscle (Fig 3). The wet weights of the TA, MG and PL were 17, 11 and 12% larger, respectively, in HLS plus clenbuterol versus HLS untreated. Co-administration of rapamycin inhibited the effects of clenbuterol on the fast-twitch muscles, but had no effect on the soleus (Fig 3).
Rapamycin given alone during hindlimb suspension for seven days had no significant effect on muscle loss.

_Clenbuterol Activates Akt-mediated Signaling Pathways._

The results obtained with the combination rapamycin and clenbuterol treatment suggested that, in some models, the effects of clenbuterol were mediated through mTOR and its downstream targets. Two well-documented targets of mTOR are the ribosomal protein S6 kinase (S6K1/p70^66k_) and the translational repressor eukaryotic initiation factor 4E binding protein 1 (4E-BP1/PHAS1). Chronic clenbuterol administration for 3, 7 and 14 days resulted in an elevation of the phosphorylation status of S6K1 and 4E-BP1 in the medial gastrocnemius (Fig. 4A). Further, the specific activity of S6K1, as measured in an in vitro kinase assay, significantly increased in the MG following 3 and 14 days of clenbuterol treatment (Fig. 4B). The activation of S6K1 and 4E-BP1 by clenbuterol was suppressed by rapamycin (Fig. 4A).

A potential upstream activator of mTOR is protein kinase B/Akt (13, 17). Akt activation was assessed following both acute and chronic administration of clenbuterol. Following a single subcutaneous injection of clenbuterol, Akt phosphorylation in skeletal muscle increased within 1 hour. Figure 5A shows the increase in Akt phosphorylation in the MG and TA two and four hours following a single injection of clenbuterol. At two-hours, Akt phosphorylation was elevated 24-fold in the MG and 52-fold in the TA. Elevated Akt phosphorylation levels were also detected following chronic administration of clenbuterol. Akt phosphorylation levels, measured 24 hours following the last injection, were increased 3-6 fold in the MG following 9 days of clenbuterol treatment (Fig 5B).
Clenbuterol Inhibits MuRF1 and MAFbx Expression.

The inability of rapamycin to inhibit the effects of clenbuterol in denervated muscles suggested that additional pathways are involved in suppressing denervation-induced atrophy. Potential targets for regulation are the E3 ubiquitin ligases, MuRF1 and MAFbx. MuRF1 and MAFbx expression is upregulated following denervation (7) and numerous atrophy-inducing conditions (7, 32). The expression of MuRF1 and MAFbx transcripts was examined following: (1) denervation alone, (2) denervation + clenbuterol, and (3) denervation + clenbuterol + rapamycin. The up-regulation of MuRF1 and MAFbx transcript expression following 10 days of denervation was suppressed in the MG with daily clenbuterol treatment (Fig. 6). Co-administration of rapamycin (1.5 mg/kg) with clenbuterol (3 mg/kg) did not block the ability of clenbuterol to suppress MuRF1 and MAFbx expression. Consequently, the suppression of MuRF1 and MAFbx appears to occur through activation of pathways upstream of mTOR. These data suggest that the ability of clenbuterol to reduce muscle loss following denervation are due, in part, to the suppression of MuRF1 and MAFbx expression, however, additional experiments are required to prove that the suppression of MuRF1 and MAFbx is necessary and sufficient to mediate the muscle sparing effects of clenbuterol in denervated muscle.

MuRF1 and MAFbx expression were also examined following clenbuterol treatment of normal (Fig 7A) and hindlimb suspended rats (Fig 7B). Clenbuterol was able to suppress baseline expression of MuRF1 and MAFbx in normal rats (Fig 7A) and reduce the upregulated expression of MuRF1 and MAFbx following unloading (Fig 7B).

DISCUSSION
The present findings provide new insights into the signaling pathways through which clenbuterol exerts its growth and muscle sparing effects. The data provide evidence that clenbuterol activates Akt and its downstream targets to induce muscle growth in normal rats and suppress muscle loss under selective atrophy-inducing conditions. The findings illustrate that the actions of clenbuterol are likely mediated through multiple pathways, and underscore the necessity to examine multiple muscles under a variety of conditions in order to establish a complete understanding of clenbuterol and its mode of action.

The growth and muscle sparing effects of clenbuterol could be mediated through a single or multiple pathway(s) that affect protein synthesis or degradation processes. The reported effects of clenbuterol and other β2-adrenergic agonists on muscle protein turnover have been contradictory (23). A number of studies utilizing radioisotopically-labelled amino acids have reported increases in the fractional synthesis rates in skeletal muscles following treatment with β2-adrenergic agonists (18, 26, 29). However, other studies have found no change in protein synthesis rates (28, 35). The conflicting results could be accountable to differences in the muscles studied and the timing of the measurements. In general, the growth effects induced by β2-adrenergic agonists are more pronounced in predominantly fast-twitch muscle than slow-twitch muscle (18, 23, 31, 38). Increases in fractional synthesis rates have been observed at 1-3 days following the start of clenbuterol treatment, returning to normal within 7-10 days (18, 29). For example, Hesketh et al (18) observed a 37% increase in the fractional rate of protein synthesis in the rat gastrocnemius after 1 day of clenbuterol treatment, that returned to control levels by 4 days.
Alterations in protein synthesis can occur as the result of changes in the capacity for protein translation and/or the efficiency of protein translation (24). Clenbuterol treatment of normal adult rats results in increases in both RNA content (6, 9, 29) and translational efficiency (protein synthesized per RNA) (21, 29) in both slow and fast muscles. In contrast, no changes in DNA content have been reported in response to clenbuterol (22, 27, 37). Consequently, the increase in fiber cross-sectional does not appear to be due to an increase in the number of nuclei per fiber.

It has clearly been shown that clenbuterol and other β2-adrenergic agonists increase cyclic AMP accumulation in skeletal muscle (30). A 2-fold increase in the cAMP concentration in the rat gastrocnemius muscle was measured between 0.5 and 5 hours after a single subcutaneous injection of clenbuterol (26). Consequently, it is generally presumed that the actions of clenbuterol are mediated through cyclic AMP and downstream targets such as protein kinase A (PKA) and cyclic AMP response element-binding protein (CREB) (30). In cardiac tissue, however, β2-adrenergic agonists, have also been shown to activate PI3k and its downstream pathways through coupling to Gαi-Gβγ (see Fig. 8). The knowledge that (1) clenbuterol can stimulate protein synthesis in skeletal muscles, (2) protein translation can be increased through activation of mTOR and its downstream targets (13, 17, 24), and (3) activation of mTOR can lead to muscle growth (8) provided the rationale for investigating whether rapamycin could block the effects of clenbuterol.

The data demonstrate that clenbuterol-induced muscle growth in normal weight-bearing animals was inhibited by rapamycin, suggesting that mTOR activation by clenbuterol is critical for inducing muscle growth. The one exception was the soleus
muscle that was the least responsive muscle to clenbuterol treatment and unresponsive to rapamycin. The reason for the conflicting data between fast and slow muscle is unclear. The soleus has often given contradictory results in response to clenbuterol. For example, one report found an increase in protein synthesis in the denervated soleus following clenbuterol treatment, but no change in protein synthesis in the innervated soleus (28). The findings that phosphorylation/activation of Akt, S6K1, and 4E-BP1 are increased following clenbuterol treatment provide further support for a role of Akt/mTOR pathways in mediating the growth effects of clenbuterol. An increase in the activation of mTOR and its downstream targets, S6K1 and 4E-BP1, could explain the findings of increases in RNA and translational efficiency following treatment with clenbuterol (23). The present findings are consistent with those of Sneddon et al (44) who reported an increase in both 4E-BP1 and S6K1 phosphorylation in the plantaris of young male rats during clenbuterol treatment. While Sneddon et al (44) found increases in 4E-BP1 and S6K1 phosphorylation for only 2 days with repeated clenbuterol treatment, we observed increases for up to 14 days. One proposed mechanism through which clenbuterol could activate Akt/mTOR is through the coupling of the β2-adrenergic receptor to Gαi-Gβγ–PI3K, as occurs in cardiac tissue (Fig 8). While activation of PI3K can occur through other growth factor receptors such as IGF-1, no increases in IGF1 protein levels have been observed in muscle ((48) and unpublished observations) or plasma (44) following clenbuterol treatment. While we have no direct evidence that PI3K is activated following clenbuterol administration, it is a likely candidate for activating Akt/PKB. Additional experiments are required to determined whether PI3K is activated by
clenbuterol administration and whether the activation of PI3K is linked to Gi-Gβγ or some other upstream mechanism.

The ability of clenbuterol to suppress muscle loss under a variety of atrophy-inducing conditions is well established. However, the mechanism of action of the muscle sparing effect of clenbuterol is unknown. Our findings suggest that the muscle sparing effects are mediated, in part, the Akt/mTOR pathway; but other pathways are likely involved. Clenbuterol treatment following both denervation and hindlimb suspension was able to reduce muscle loss as previously reported (3, 12, 43, 46, 49). Interestingly, clenbuterol was effective in sparing soleus mass during denervation, but not hindlimb suspension. The responses to co-administration of rapamycin and clenbuterol were in complete contrast between the two atrophy models, suggesting that different pathways mediate the effects. While rapamycin inhibited the effect of clenbuterol in unloaded fast muscles, it had no effect on denervated fast muscles. If anything, rapamycin treatment improved the muscle sparing effect of clenbuterol in the denervated fast muscles. The difference in the responses could be related to differences in the primary mechanisms responsible for muscle loss in denervation vs hindlimb suspension.

Muscle loss following denervation and hindlimb suspension occurs due to a combination of increases in protein degradation and decreases in protein synthesis. A suppression of protein synthesis, rather than an increase in protein degradation, could be of greater significance to the induction of muscle loss following hindlimb unloading than denervation. Previous reports have shown inactivation of the Akt/mTOR signaling pathway following unloading (8, 15). Our findings suggest that activation of Akt/mTOR pathways by clenbuterol is sufficient to suppress muscle loss during unloading, most
likely through an increase in protein translation. Further, the ability of rapamycin to inhibit the effects of clenbuterol suggests that activation of signaling pathways downstream of mTOR is necessary for mediating the muscle sparing effects, especially during unloading.

In addition to activating protein translation pathways via mTOR activation, clenbuterol also suppressed the transcriptional upregulation of the E3 ubiquitin ligases, MuRF1 and MAFbx, which are thought to be involved in protein degradation. Suppression of MuRF1 and MAFbx mRNA expression was evident under both normal and atrophy conditions. Clenbuterol administration has been reported to decrease protein degradation rates during cancer-induced cachexia (11) and denervation (2). Further, Yimlamai et al (48) recently demonstrated that clenbuterol was able to suppress the upregulation of various components of the ubiquitin-proteosome pathway during hindlimb suspension. The present study is the first, however, to demonstrate specific suppression of MuRF1 and MAFbx by clenbuterol. During hindlimb suspension, suppression of MuRF1 and MAFbx alone was not sufficient to prevent muscle loss. Haddad et al (15) reported similar findings in a recent study in which isometric resistance exercise was utilized in an attempt to counter unloading induced muscle atrophy. The resistance exercise protocol used during hindlimb suspension was unable to prevent muscle atrophy even though it was able to suppress MuRF1 and MAFbx expression (15). The lack of a muscle sparing effect was thought to be due to insufficient activation of Akt/mTOR pathways (15).

In contrast to hindlimb suspension, suppression of denervation-induced atrophy by clenbuterol was independent of mTOR, but might be related to the repression of
MuRF1 and MAFbx expression. The clenbuterol-induced repression of MuRF1 and MAFbx mRNA could occur through an Akt-independent pathway such as cAMP, or through an Akt-dependent, rapamycin insensitive pathway. Treatment of denervated muscle with both clenbuterol and rapamycin revealed that clenbuterol-induced repression of the MuRF1 and MAFbx transcripts occurred upstream of mTOR activation. One hypothesis is that clenbuterol-induced repression of MuRF1 and MAFbx occurs through the activation of Akt, which leads to the phosphorylation of the forkhead transcription factors, which prevents translocation of the FOXOs from the cytoplasm to the nucleus (Fig 8). The FOXO family of transcription factors (FOXO1, FOXO3a, FOXO4) has been implicated in the regulation of MuRF1 and MAFbx transcription (40, 45). In the present study, we did not measure the expression or cellular localization of FOXO transcription factors. Additional experiments are needed to determine whether translocation of FOXO transcription factors from the cytoplasm to the nucleus increases following denervation, and whether clenbuterol can prevent the translocation of the FOXOs, leading to repression of MuRF1 and MAFbx expression. Alternatively, it is possible that the muscle sparing effects of clenbuterol during denervation are mediated through the cAMP-PKA pathway since elevated cAMP levels can inhibit proteolysis in isolated myofibers (33) and the phosphodiesterase 4 specific inhibitor, rolipram, partially spares muscle mass in denervation (19).

In summary, the present findings demonstrate that the β2 adrenergic agonist, clenbuterol, does activate Akt and its downstream effector, mTOR. Activation of mTOR and its downstream targets, 4EBP1 and S6K1, can lead to increases in protein translation and could explain previous findings of increased protein synthesis following clenbuterol
treatment. The ability of clenbuterol to repress MuRF1 and MAFbx transcripts and components of the ubiquitin proteosome pathways (48), suggests that clenbuterol can also affect protein degradation. The findings suggest that activation of mTOR is critical for clenbuterol-induced muscle growth in normal animals and muscle sparing during unloading. The mechanism through which clenbuterol activates mTOR is likely through the activation of Akt, although other mechanisms cannot be ruled out. We postulate that Akt is activated through a Gαi-Gβγ-PI3k signaling pathways (see Figure 8), similar to what occurs in cardiac tissue. Additional experiments are required to determine whether PI3K is activated by clenbuterol and responsible for the activation of Akt. Further analysis is also needed to parse out the roles of the Akt/mTOR and Gαs-AC-cAMP pathways in mediating the actions of clenbuterol in skeletal muscle. It is clear from the data that multiple pathways mediate the effects of clenbuterol in skeletal muscle. A better understanding of the actions of β2 adrenergic receptor agonists in skeletal muscle could lead to the development of compounds to treat muscle atrophy.

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**FIGURE LEGENDS**

**Figure 1:** Effects of 14 days of clenbuterol alone (solid) or co-administration of clenbuterol and rapamycin (empty) treatment on the weight of various hind limb muscles of normal rats. Values are expressed as a percent change from control (means ± sem; n=8-10). Significance was set at p < 0.05; * denotes significant difference from control group; + denotes significant difference from clenbuterol alone group.

**Figure 2:** Effects of 10 days of clenbuterol alone (empty), rapamycin alone (hatched) or co-administration of clenbuterol and rapamycin (vertical lines) on muscle loss following denervation. The solid bars represent the effect of 10 days of denervation alone. Values are expressed as a percent change from control (means ± sem; n=9-10). Significance was set at p < 0.05; * denotes significant difference from denervation group; + denotes significant difference from clenbuterol alone group.

**Figure 3:** Effects of 14 days of clenbuterol alone (empty) or co-administration of clenbuterol and rapamycin (hatched) on muscle loss following hindlimb suspension. The solid bars represent the effect of 14 days of hindlimb suspension alone. Values are expressed as a percent change from control (means ± sem; n=8-10). Significance was set at p < 0.05; * denotes significant difference from control group; + denotes significant difference from treatment group.
difference from hindlimb suspension group; * denotes significant difference from clenbuterol alone group.

**Figure 4**: (A) Western blots of S6K1 and 4E-BP1 in the medial gastrocnemius of control rats (CON) or after clenbuterol treatment for 3, 7 or 14 days. The gel shift observed for S6K1 and 4E-BP1 after 14 days of clenbuterol treatment was inhibited by co-administration of rapamycin (Rap). Each lane represents 25 µg (S6K1) or 100 µg (4E-BP1) of total protein extracted from a pool of 3 MG muscles. For each group, duplicate lanes represent different pools of MG muscle. (B) The specific activity of S6K1 was determined by $^{32}$P incorporation into 40S ribosomes in the immune complex. S6K1 activity was measured in the medial gastrocnemius muscle of rats following no treatment (control), insulin injection (insulin), 3 days clenbuterol treatment (clen3), or 14 days of clenbuterol treatment (clen14). Values are means ±sem; n=4-5. Significance was set at $p < 0.05$; * denotes significant difference from control group.

**Figure 5**: Akt phosphorylation status in the medial gastrocnemius following acute (A) and chronic (B) clenbuterol treatment (3 mg/kg). (A) Western blots of native and phosphorylated Akt in MG following no treatment (CON) or four hours following a subcutaneous injection of clenbuterol (CLEN). Each lane represents 200 µg of total protein extracted from individual MG muscles. The ratio of phosphorylated to native Akt was calculated for control (empty), 2 hr clenbuterol treatment (solid), and 4 hr clenbuterol treatment (hatched) in the MG and TA muscles. Values are means ±sem, n=4. Significance was set at $p < 0.05$; * denotes significant difference from control.
group. (B) Western blots of native and phosphorylated Akt in MG following no
treatment (CON) or 10 days of clenbuterol (CLEN). Each lane represents 200 µg of total
protein extracted from a pool of 3 MG muscles. The ratio of phosphorylated to native
Akt was calculated for control (empty), or clenbuterol treatment (solid) in the MG and
TA muscles. Values are means ±sem, n=4. Significance was set at p < 0.05; * denotes
significant difference from control group.

Figure 6: Northern blots showing the effects of denervation and clenbuterol treatment on
MuRF1 and MAFbx transcripts. Medial gastrocnemius was obtained from rats following
no treatment (CON), 10 days of denervation (DEN), 10 days of denervation + rapamycin,
(RAP), 10 days of denervation + clenbuterol (CL) or 10days of denervation + clenbuterol
+ rapamycin (CL+RAP). Each lane represents 10 µg of total RNA extracted from a pool
of 3 MG muscles. For each group, duplicate lanes represent different pools of MG
muscle.

Figure 7: Northern blots showing the effects of clenbuterol treatment on MuRF1 and
MAFbx transcripts in the medial gastrocnemius muscle of normal (A) and hindlimb
suspended (B) rats. (A) Medial gastrocnemius was obtained from rats following no
treatment (C) or clenbuterol (CLEN) treatment for 3 (3d), 7 (7d) and 14 (14d) days. (B)
Medial gastrocnemius was obtained from rats following no treatment (C) or hindlimb
suspension for 3 (3d), 7 (7d) or 14 (14d) days. In addition, rats under suspension were
also treated with clenbuterol (CL) for 3, 7 or 14 day. Each lane represents 10 µg of total
RNA extracted from a pool of 3 MG muscles. For each group, duplicate lanes represent different pools of MG muscle.

**Figure 8**: Hypothetical model of β2-adrenergic signaling in skeletal muscle. It is proposed that activation of the β2-AR by clenbuterol couples to both Gαs-AC-cAMP and Gαi-Gβγ-PI3K-Akt signaling pathways. Activation of Protein Kinase B/Akt leads to the activation of mTOR which stimulates protein translation through S6K1 and 4E-BP1, and phosphorylation of the FOXO transcription factors which leads to their sequestration in the cytoplasm and the repression of MuRF1 and MAFbx expression.
A

Clenbuterol Treatment

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<td>4E-BP1</td>
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</tbody>
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B

Kinase Activity (cpm)

- Insulin
- Control
- Clen 3
- Clen 14

Experimental Group