

Rapamycin inhibits the growth and muscle-sparing effects of clenbuterol

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Kline WO, Panaro FJ, Yang H, Bodine SC. Rapamycin inhibits the growth and muscle-sparing effects of clenbuterol. *J Appl Physiol* 102: 740–747, 2007. First published October 26, 2006; doi:10.1152/jappphysiol.00873.2006.—Clenbuterol and other β_2 -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 and mammalian target of rapamycin (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 mTOR was involved in mediating the effects of clenbuterol. Clenbuterol administration increased the phosphorylation status of PKB/Akt, S6 kinase 1/p70^{S6k}, and eukaryotic initiation factor 4E binding protein 1/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 after 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.

β_2 -adrenergic agonists; mTOR; Akt/PKB; MuRF1; MAFbx

CHRONIC ADMINISTRATION OF clenbuterol and other synthetic compounds that activate β -adrenergic receptors has 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, hindlimb 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 β_2 -adrenergic receptor as demonstrated from experiments performed in mice lacking the β_1 -, β_2 -, or both β_1 - and β_2 -adrenergic receptors (20). The signaling pathways that are activated downstream of the recep-

tor to induce the growth and muscle-sparing responses, however, are unclear.

The β -adrenergic receptor subtypes (β_1 AR, β_2 AR, and β_3 AR) are members of the G-protein-coupled receptor (GPCR) superfamily. Ligand binding on the receptor promotes GDP-GTP exchange on the $G\alpha$ subunit and subsequent dissociation of $G\alpha$ from $G\beta\gamma$, leading to activation of $G\alpha$ and release of free $G\beta\gamma$ heterodimers (see Ref. 16 for review). Subsequently, $G\alpha$ and $G\beta\gamma$ function as signaling mediators to directly interact with a variety of effector proteins. The $G\alpha$ subunit is divided into four families based on primary sequence: G_s , G_i , G_q , and G_{12} . In general, specificity and selectivity in GPCR signaling are achieved by coupling of a given GPCR to a single class of G proteins. β_1 AR is coupled exclusively with $G_s\alpha$, which in turn activates adenylate cyclase (AC), catalyzing cAMP formation (30). Historically, β_2 ARs were also believed to couple exclusively with $G_s\alpha$. However, in cardiac tissue, β_2 ARs can couple to both $G_s\alpha$ and $G_i\alpha$ signaling pathways (34, 47).

The phosphatidylinositol 3-kinase (PI3-kinase)-PKB/Akt signaling pathway has been implicated in the regulation of cell growth and more specifically in muscle fiber growth in mammals (8, 13, 36, 41). The prevailing theory is that the growth and muscle-sparing effects of clenbuterol and other β_2 AR agonists in skeletal muscle are mediated through the classic β_2 AR- G_s -AC-cAMP signaling pathway. The possibility exists, however, that in skeletal muscle additional signaling pathways are activated by β_2 ARs. One likely pathway is the PI3-kinase/Akt pathway given that 1) β_2 AR stimulation in the heart couples to $G_i\alpha$ - $G\beta\gamma$ to activate PI3-kinase/Akt signaling pathways (34, 47) and 2) clenbuterol treatment acutely activates p70^{S6k}/S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) in skeletal muscle (44).

The objective of the present study was to determine whether activation of Akt and mammalian target of rapamycin (mTOR) signaling pathways mediate the growth and muscle-sparing effects of clenbuterol. Examinations of 1) the phosphorylation and activation status of Akt, S6K1/p70s6k, and 4E-BP1 after clenbuterol treatments and 2) the effects of coadministration of rapamycin on the growth and muscle-sparing effects of clenbuterol were 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

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initial body weights of 240–280 g. Animals were assigned to one of three experimental groups (normal, denervated, or hindlimb-suspended groups) and were 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 with the use of aseptic surgical techniques, the sciatic nerve was isolated in the midhigh region and cut with sharp scissors. Unloading of the lower limb muscles was accomplished with a noninvasive 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 hindlimbs unloaded. All animal procedures were approved by the Institutional Animal Care and Use Committee and conformed to the American Physiological Society's *Guiding Principles in the Care and Use of Animals*.

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

Western blots. Muscles were homogenized at 4°C in radioimmunoprecipitation assay lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in 50 mM NaCl, 20 mM Tris, pH 7.6) containing 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 5 mM benzamide, 1 mM 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 12,000 g for 20 min before determination of protein concentration by BCA assay (Pierce Chemical). SDS-PAGE was performed on 7.5% or 15% (4E-BP1) gels prepared with an acrylamide-to-bisacrylamide ratio of 100:1. Western blots were revealed with enhanced chemiluminescence (Renaissance-NEN). Antibodies against Akt [New England Biolabs (NEB)], p70^{s6k} (Santa Cruz), and 4E-BP1 (Zymed) were used to detect protein expression levels. Phosphorylation-specific antibodies against Ser⁴⁷³ (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/ml of packed beads) were incubated at 23°C for 60 min with nonimmune serum or antisera to p70^{s6k}. The beads were then washed five times with PBS (145 nM NaCl, 4 mM KCl, and 10 mM sodium P_i, pH 7.4) 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 p70^{s6k} 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 DTT, 9 µM cAMP-dependent protein kinase inhibitory peptide, 20 µM calmidazolium, 200 µM [γ-³²P]ATP (300–500 cpm/pmol), and 40S 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 by a modification of the lithium chloride method of RNA extraction described by Auffray and Rougeon (4). Muscles were homogenized at 4°C in 3 M LiCl-6 M urea, and the homogenate was precipitated overnight at 4°C. The recovered lith-

ium-RNA was acid phenol-chloroform extracted to remove contaminating proteins. RNA concentration and purity were determined by spectrophotometry at 260 nm. Samples (10 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 ± SE. 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 body wt, mean ± SD). Daily administration of clenbuterol (3 mg/kg) for 14 days induced significant growth in all muscles examined: tibialis anterior (TA), medial gastrocnemius (MG), plantaris, and soleus (Fig. 1). The increase in mean wet weight relative to control ranged from 27 to 41%. In

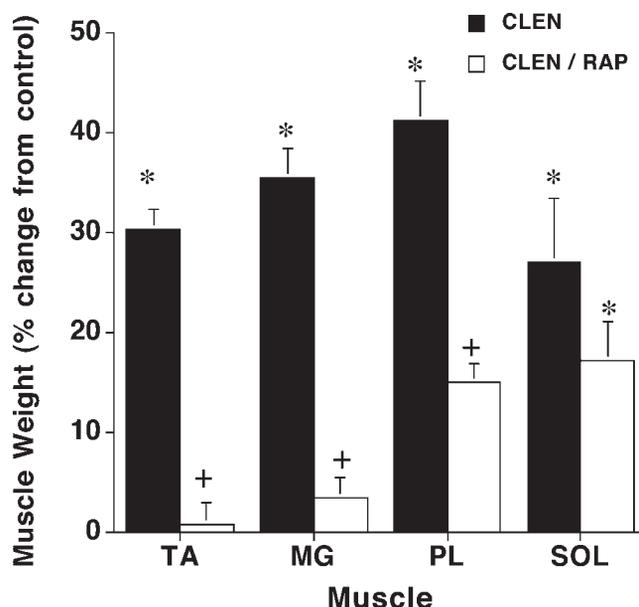


Fig. 1. Effects of 14 days of clenbuterol alone (Clen) or coadministration of clenbuterol and rapamycin (Clen/Rap) treatment on the weight of various hindlimb muscles of normal rats. Values are expressed as a percent change from control (means ± SE; *n* = 8–10). TA, tibialis anterior; MG, medial gastrocnemius; PL, plantaris; SOL, soleus. Significance was set at *P* < 0.05: *significant difference from control group; +significant difference from clenbuterol alone group.

general, muscles composed predominantly of fast-twitch fiber types showed the greatest response to clenbuterol treatment. Clenbuterol-induced growth in the TA, MG, and plantaris was significantly reduced by coadministration of rapamycin (Fig. 1). Muscle weights of the TA, MG, and plantaris were not significantly different from control after the combination treatment. In contrast, rapamycin coadministration resulted in only a 10% decrease in the wet weight of the soleus relative to clenbuterol alone.

Next, rapamycin was coadministered 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 after both denervation-induced (Fig. 2) and hindlimb suspension-induced (Fig. 3) atrophy. Ten days of clenbuterol treatment after sciatic nerve transection in young female rats (258 ± 11 g body wt) resulted in significantly less loss of muscle mass in all muscles studied than in untreated denervated muscles (Fig. 2). Administration of rapamycin alone following denervation had no effect on the progression of muscle atrophy. Moreover, coadministration 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 plantaris. In fact, coadministration of rapamycin further reduced (4–8%) the amount of muscle loss following denervation, with this effect being significant in the plantaris. Although rapamycin had no effect on clenbuterol in fast-twitch muscles, it reduced the effectiveness of clenbuterol in the soleus (Fig. 2). In these experiments, the sciatic nerve was transected only on the right side. Of note is that, although clenbuterol attenuated atrophy in the muscles of the right (denervated) leg, it induced hypertrophy in the muscles of the left (neurally intact) leg. Furthermore, although rapamycin had no effect on the muscles of the right leg, it was

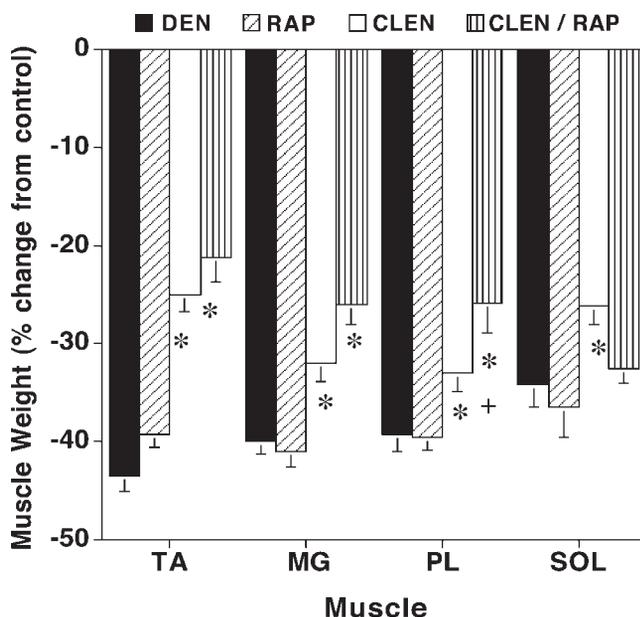


Fig. 2. Effects of 10 days of clenbuterol alone, rapamycin alone, or coadministration of clenbuterol and rapamycin on muscle loss after denervation. Effect of 10 days of denervation alone (Den) was also studied. Values are expressed as a percent change from control (means \pm SE; $n = 9-10$). Significance was set at $P < 0.05$: *significant difference from denervation group; + significant difference from clenbuterol alone group.

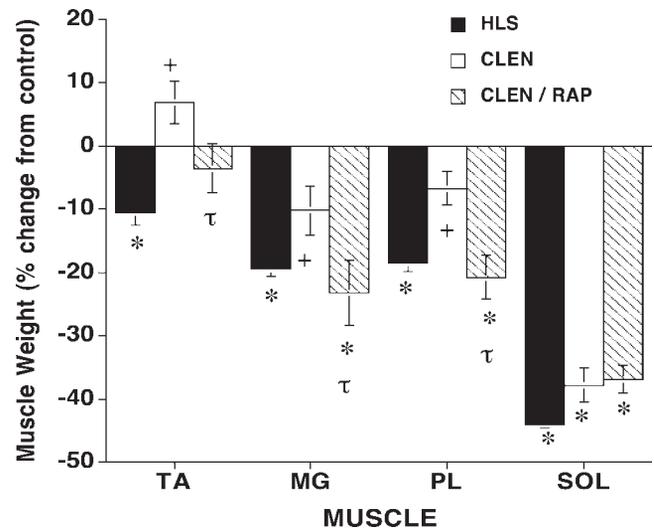


Fig. 3. Effects of 14 days of clenbuterol alone or coadministration of clenbuterol and rapamycin on muscle loss after hindlimb suspension. Effect of 14 days of hindlimb suspension alone (HLS) was also studied. Values are expressed as a percent change from control (means \pm SE; $n = 8-10$). Significance was set at $P < 0.05$: *significant difference from control group; + significant difference from hindlimb suspension group; τ significant difference from clenbuterol alone group.

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 body wt) led to significant atrophy (10–44%) in the TA, MG, plantaris, and soleus muscles. Daily administration of clenbuterol during the unloading period resulted in significant sparing of muscle mass in those muscles composed predominantly of fast-twitch fiber types (i.e., TA, MG, plantaris) but not in the soleus, a predominantly slow-twitch muscle (Fig. 3). The wet weights of the TA, MG, and plantaris were 17, 11, and 12% larger, respectively, in the hindlimb-suspended plus clenbuterol vs. hindlimb-suspended but untreated group. Coadministration 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 7 days had no significant effect on muscle loss.

Clenbuterol activates Akt-mediated signaling pathways. The results obtained with the combination of 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 S6K1/p70^{S6K} and the translational repressor 4E-BP1/PHAS-1. Chronic clenbuterol administration for 3, 7, and 14 days resulted in an elevation of the phosphorylation status of S6K1 and 4E-BP1 in the MG (Fig. 4A). Furthermore, the specific activity of S6K1, as measured by in vitro kinase assay, significantly increased in the MG after 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 PKB/Akt (13, 17). Akt activation was assessed after both acute and chronic administration of clenbuterol. After a single subcutaneous injection of clenbuterol, Akt phosphorylation in skeletal muscle increased within 1 h. Figure 5A shows the increase in Akt phosphorylation in the MG and TA 2 and 4 h after a single injection of clenbuterol.

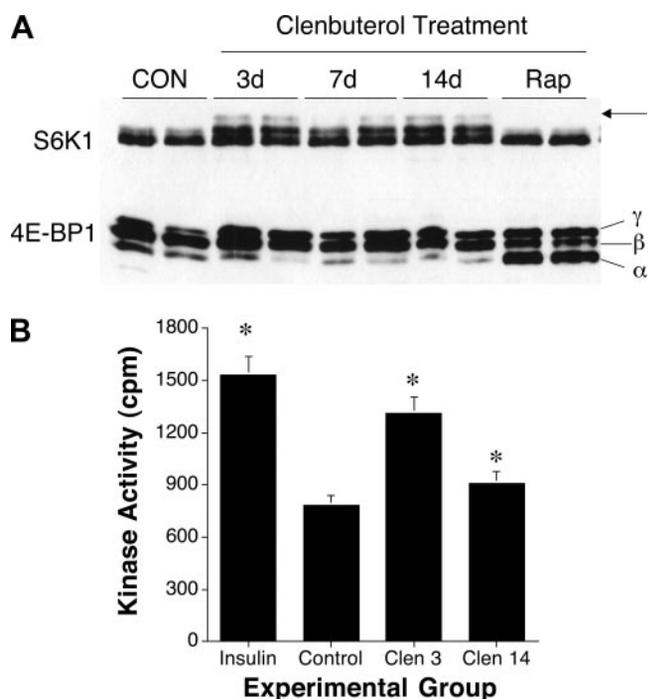


Fig. 4. A: Western blots of S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) in the MG of control rats (CON) or after clenbuterol treatment for 3, 7, or 14 days (d). The gel shift observed for S6K1 and 4E-BP1 after 14 days of clenbuterol treatment was inhibited by coadministration 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: specific activity of S6K1 was determined by ³²P incorporation into 40S ribosomes in the immune complex. S6K1 activity was measured in the MG muscle of rats after no treatment (control), insulin injection (insulin), 3 days clenbuterol treatment (Clen 3), or 14 days of clenbuterol treatment (Clen 14). Values are means ± SE; n = 4–5. *Significant difference from control group (P < 0.05).

At 2 h, Akt phosphorylation was elevated 24-fold in the MG and 52-fold in the TA. Elevated Akt phosphorylation levels were also detected after chronic administration of clenbuterol. Akt phosphorylation levels, measured 24 h after the last injection, were increased three- to sixfold in the MG after 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 after denervation (7) and numerous atrophy-inducing conditions (7, 32). The expression of *MuRF1* and *MAFbx* transcripts was examined after 1) denervation alone, 2) denervation + clenbuterol, and 3) denervation + clenbuterol + rapamycin. The upregulation of *MuRF1* and *MAFbx* transcript expression after 10 days of denervation was suppressed in the MG with daily clenbuterol treatment (Fig. 6). Coadministration 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 after 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 after 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* after unloading (Fig. 7B).

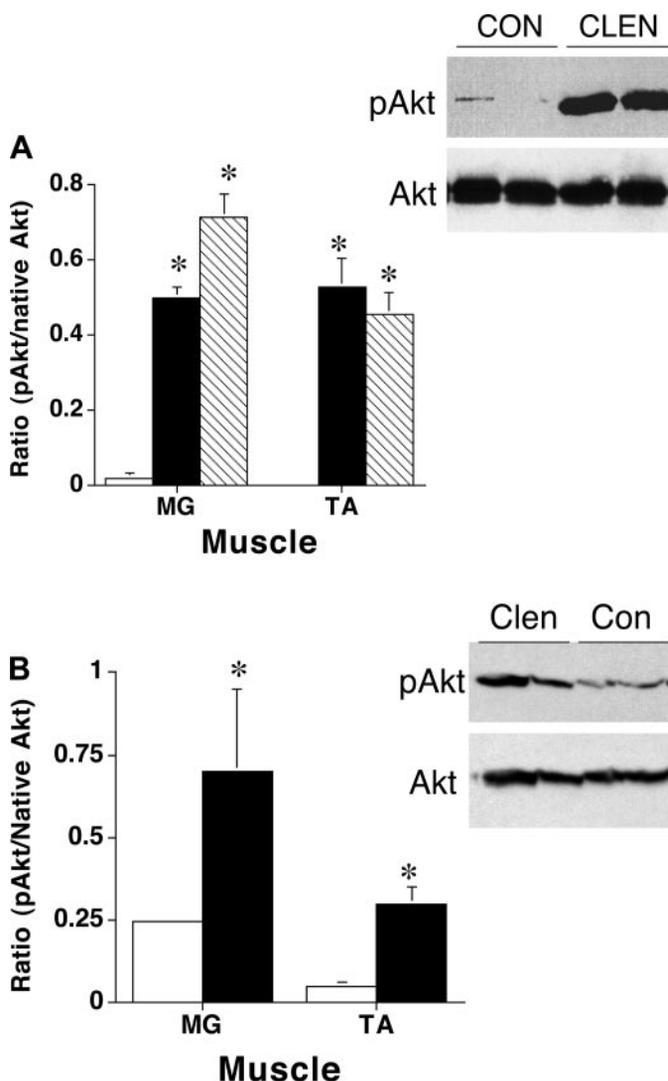


Fig. 5. Akt phosphorylation status in the MG after acute (A) and chronic (B) clenbuterol treatment (3 mg/kg). A: Western blots of native and phosphorylated Akt in MG after no treatment (CON) or 4 h after a subcutaneous injection of clenbuterol. Each lane represents 200 μg of total protein extracted from individual MG muscles. The ratio of phosphorylated to native Akt was calculated for control (open bars), 2 h clenbuterol treatment (solid bars), and 4 h clenbuterol treatment (hatched bars) in the MG and TA muscles. Values are means ± SE; n = 4. *Significant difference from control group (P < 0.05). B: Western blots of native and phosphorylated Akt in MG after no treatment or after 10 days of clenbuterol. Each lane represents 200 μg of total protein extracted from a pool of 3 MG muscles. Ratio of phosphorylated to native Akt was calculated for control (open bars) or clenbuterol treatment (solid bars) in the MG and TA muscles. Values are means ± SE; n = 4. *Significant difference from control group (P < 0.05).

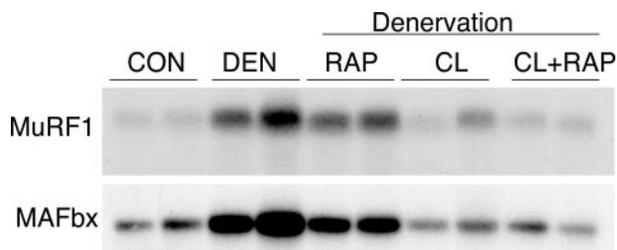


Fig. 6. Northern blots showing the effects of denervation and clenbuterol treatment on *MuRF1* and *MAFbx* transcripts. MG was obtained from rats after no treatment (CON), 10 days of denervation (DEN), 10 days of denervation + rapamycin (RAP), 10 days of denervation + clenbuterol (CL), or 10 days of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

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 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 affects protein synthesis or degradation processes. The reported effects of clenbuterol and other β_2 -adrenergic agonists on muscle protein turnover have been contradictory (23). A number of studies utilizing radioisotopically labeled amino acids have reported increases in the fractional synthesis rates in skeletal muscles after treatment with β_2 -adrenergic agonists (18, 26, 29). However, other studies have found no change in protein synthesis rates (28, 35). The conflicting results could be because of differences in the muscles studied and the timing of the measurements. In general, the growth effects induced by β_2 -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 after 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, which 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-twitch 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 area 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 β_2 -adrenergic agonists increase cAMP accumulation in skeletal muscle (30). A twofold increase in the cAMP concentration in

the rat gastrocnemius muscle was measured between 0.5 and 5 h after a single subcutaneous injection of clenbuterol (26). Consequently, it is generally presumed that the actions of clenbuterol are mediated through cAMP and downstream targets such as PKA and cAMP response element-binding protein (30). In cardiac tissue, however, β_2 -adrenergic agonists have also been shown to activate PI3-kinase and its downstream pathways through coupling to $G_i\alpha$ - $G\beta\gamma$ (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, which was the least responsive muscle to clenbuterol treatment and unresponsive to rapamycin. The reason for the conflicting data between fast- and slow-twitch 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 after 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 after 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 after treatment

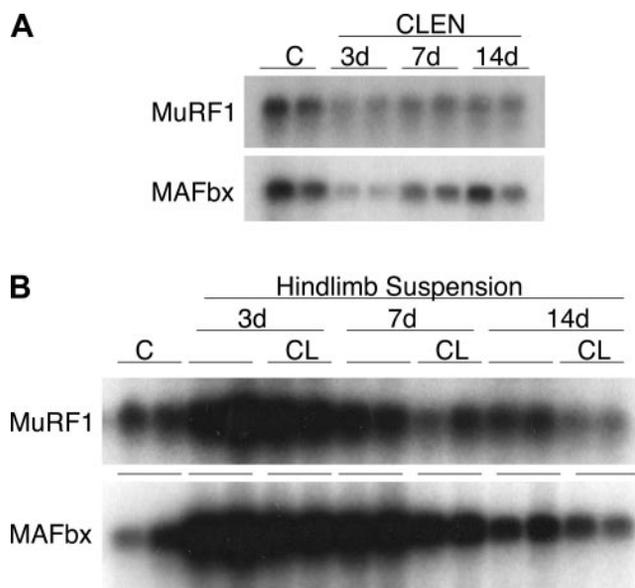


Fig. 7. Northern blots showing the effects of clenbuterol treatment on *MuRF1* and *MAFbx* transcripts in the MG muscle of normal (A) and hindlimb-suspended (B) rats. A: MG was obtained from rats after no treatment (C) or after clenbuterol (CLEN) treatment for 3, 7, or 14 days (d). B: MG was obtained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

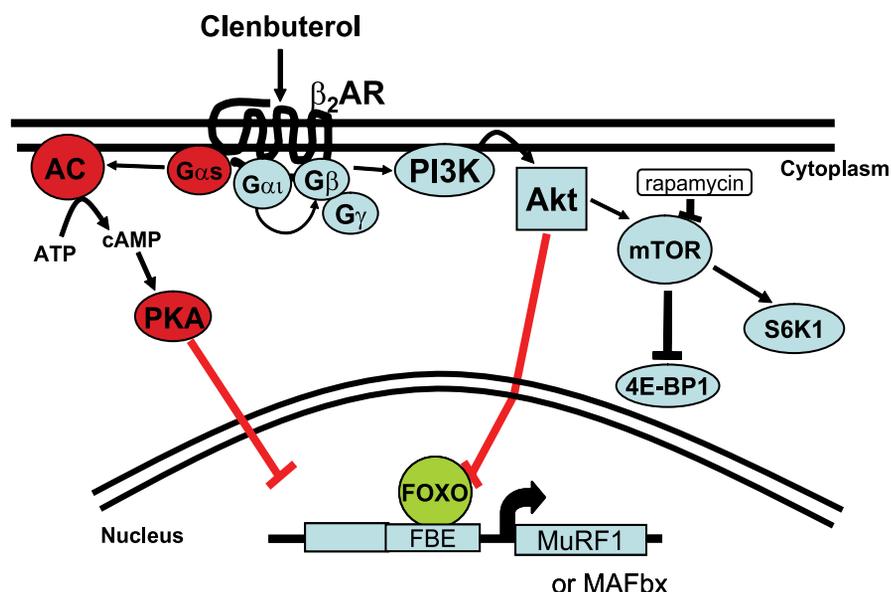


Fig. 8. Hypothetical model of β_2 -adrenergic signaling in skeletal muscle. It is proposed that activation of β_2 -adrenergic receptor (β_2 AR) by clenbuterol couples to both $G_s\alpha$ -adenylyl cyclase (AC)-cAMP and $G_i\alpha$ - $G\beta\gamma$ -phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathways. Activation of PKB/Akt leads to the activation of mammalian target of rapamycin (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.

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. Although 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 β_2 AR to G_i - $G\beta\gamma$ -PI3-kinase as occurs in cardiac tissue (Fig. 8). Although activation of PI3-kinase can occur through other growth factor receptors such as IGF-I, no increases in IGF-I protein levels have been observed in muscle (Ref. 48 and unpublished observations) or plasma (44) after clenbuterol treatment. Although we have no direct evidence that PI3-kinase is activated after clenbuterol administration, it is a likely candidate for activating Akt/PKB. Additional experiments are required to determine whether PI3-kinase is activated by clenbuterol administration and whether the activation of PI3-kinase is linked to G_i - $G\beta\gamma$ 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, by the Akt/mTOR pathway; however, other pathways are likely involved. Clenbuterol treatment after both denervation and hindlimb suspension reduced muscle loss as previously reported (3, 12, 43, 46, 49). Interestingly, clenbuterol was effective in sparing soleus mass during denervation but not for hindlimb suspension. The responses to coadministration of rapamycin and clenbuterol were in complete contrast between the two atrophy models, suggesting that different pathways mediate the effects. Whereas rapamycin inhibited the effect of clenbuterol in unloaded fast-twitch muscles, it had no effect on denervated fast-twitch muscles. If anything, rapamycin treatment improved the muscle-sparing effect of clenbuterol in the denervated fast-twitch 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 after denervation and hindlimb suspension occurs because of the combination of increased protein degradation and decreased protein synthesis. A suppression of protein synthesis, rather than increased 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 after 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. Furthermore, 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). Furthermore, Yimlamai et al. (48) recently demonstrated that clenbuterol was able to suppress the upregulation of various components of the ubiquitin-proteasome 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 forkhead transcription factors, which prevent translocation of 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 after 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 that the phosphodiesterase 4-specific inhibitor rolipram partially spares muscle mass in denervation (19).

In summary, the present findings demonstrate that the β_2 -adrenergic agonist clenbuterol does activate Akt and its downstream effector mTOR. Activation of mTOR and its downstream targets 4E-BP1 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 proteasome 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 $G_i\alpha$ - $G\beta\gamma$ -PI3-kinase signaling pathways (see Fig. 8), similar to what occurs in cardiac tissue. Additional experiments are required to determine whether PI3-kinase 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 $G_s\alpha$ -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 β_2 -adrenergic receptor agonists in skeletal muscle could lead to the development of compounds to treat muscle atrophy.

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