J Appl Physiol 102: 740–747, 2007.
First published October 26, 2006; doi:10.1152/japplphysiol.00873.2006.

Rapamycin inhibits the growth and muscle-sparing effects of clenbuterol

William O. Kline,[2] Frank J. Panaro,[2] Hayung Yang,[1] and Sue C. Bodine[1,2]

[1]*Section of Neurobiology, Physiology & Behavior, University of California, Davis, California; and* [2]*Regeneron Pharmaceuticals, Tarrytown, New York*

Submitted 7 August 2006; accepted in final form 15 October 2006

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/japplphysiol.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 receptor to induce the growth and muscle-sparing responses, however, are unclear.

The β-adrenergic receptor subtypes (β_1AR, β_2AR, and β_3AR) are members of the G-protein-coupled receptor (GPCR) superfamily. Ligand binding on the receptor promotes 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 Ref. 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: 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_1AR is coupled exclusively with $G_s\alpha$, which in turn activates adenylate cyclase (AC), catalyzing cAMP formation (30). Historically, β_2ARs were also believed to couple exclusively with $G_s\alpha$. However, in cardiac tissue, β_2ARs 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_2AR agonists in skeletal muscle are mediated through the classic β_2AR-G_s-AC-cAMP signaling pathway. The possibility exists, however, that in skeletal muscle additional signaling pathways are activated by β_2ARs. One likely pathway is the PI3-kinase/Akt pathway given that *1*) β_2AR stimulation in the heart couples to $G_i\alpha$-Gβγ 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

Address for reprint requests and other correspondence: S. C. Bodine, Univ. of California, Davis, Section of Neurobiology, Physiology, and Behavior, 196 Briggs Hall, One Shields Ave., Davis, California 95616 (e-mail: scbodine@ucdavis.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

 8750-7587/07 $8.00 Copyright © 2007 the American Physiological Society

Downloaded from http://jap.physiology.org/ by 10.220.33.3 on May 22, 2017

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 midthigh 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 benzamidine, 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 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/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 $MgCl_2$, 1 mM DTT, 9 μM cAMP-dependent protein kinase inhibitory peptide, 20 μM calmidazolium, 200 μM [γ-^{32}P]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 lithium-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 ^{32}P-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

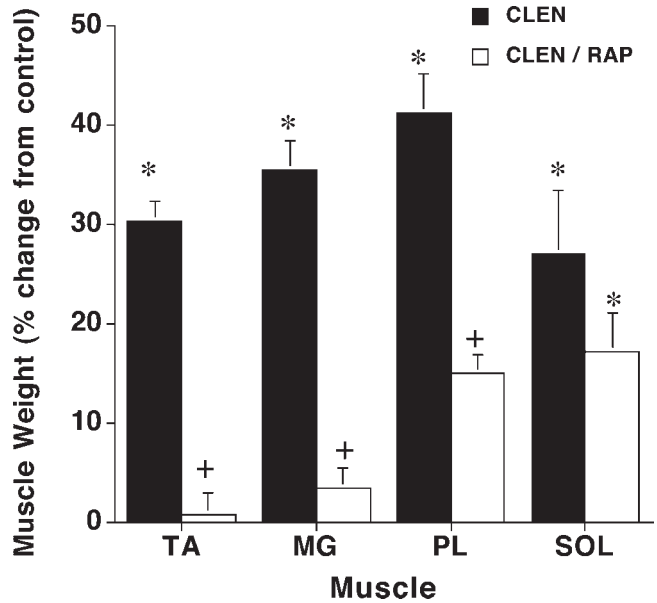

Fig. 1. Effects of 14 days of clenbuterol alone (Clen) or coadministration of clenbuterol and rapamycin (Clen/Rap) treatment on the weight of various hindlimb muscles of normal rats. Values are expressed as a percent change from control (means ± SE; $n = 8$–10). TA, tibialis anterior; MG, medial gastrocnemius;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

Downloaded from http://jap.physiology.org/ by 10.220.33.3 on May 22, 2017

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

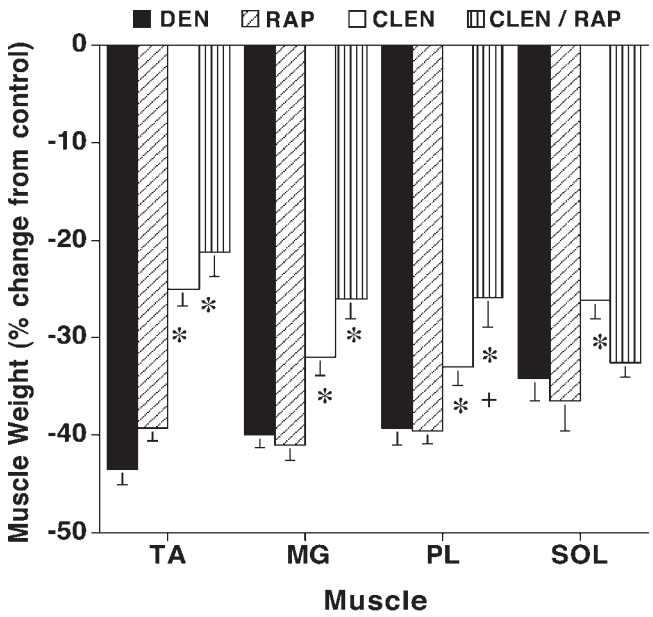

Fig. 2. Effects of 10 days of clenbuterol alone, rapamycin alone, or coadministration of clenbuterol and rapamycin on muscle loss after denervation. Effect of 10 days of denervation alone (Den) was also studied. Values are expressed as a percent change from control (means ± SE; n = 9–10). Significance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

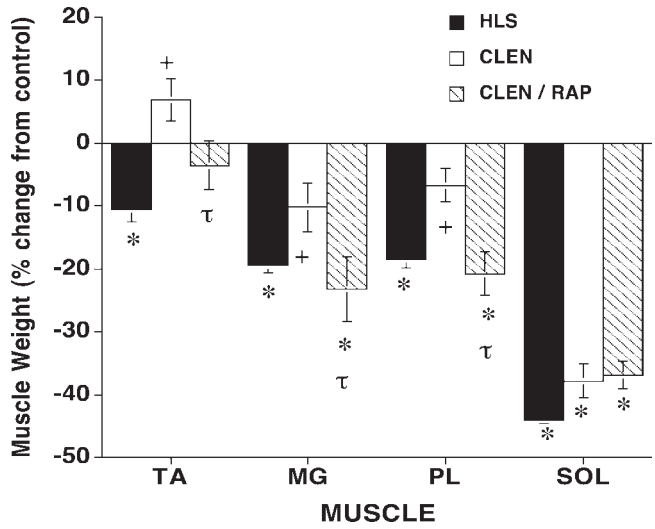

Fig. 3. Effects of 14 days of clenbuterol alone or coadministration of clenbuterol and rapamycin on muscle loss after hindlimb suspension. Effect of 14 days of hindlimb suspension alone (HLS) was also studied. Values are expressed as a percent change from control (means ± SE; n = 8–10). Significance was set at $P < 0.05$: *significant difference from control group; +significant difference from hindlimb suspension group; τsignificant 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. 4*A*). 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. 4*B*). The activation of S6K1 and 4E-BP1 by clenbuterol was suppressed by rapamycin (Fig. 4*A*).

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 5*A* shows the increase in Akt phosphorylation in the MG and TA 2 and 4 h after a single injection of clenbuterol.

Downloaded from http://jap.physiology.org/ by 10.220.33.3 on May 22, 2017

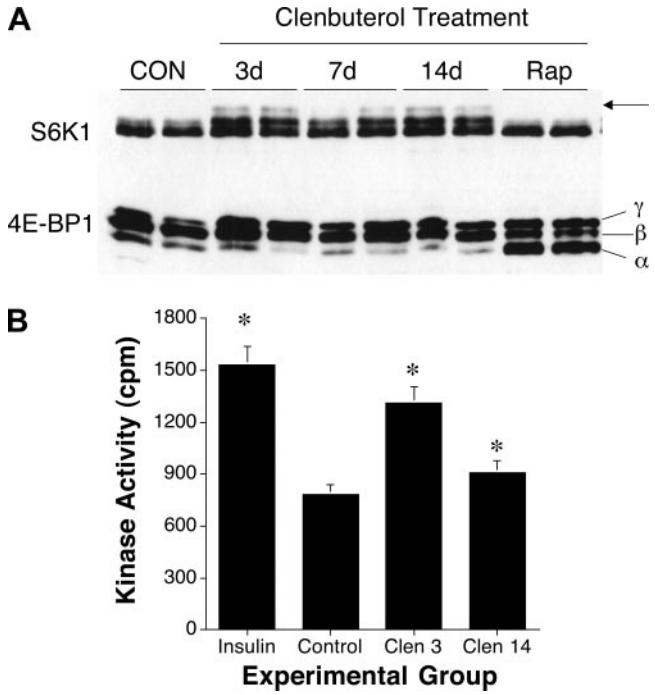

Fig. 4. *A*: Western blots of S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) in the MG of control rats (CON) or after clenbuterol treatment for 3, 7, or 14 days (d). The gel shift observed for S6K1 and 4E-BP1 after 14 days of clenbuterol treatment was inhibited by coadministration 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 ^{32}P 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. 5*B*).

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. 7*A*) and hindlimb-suspended rats (Fig. 7*B*). Clenbuterol was able to suppress baseline expression of *MuRF1* and *MAFbx* in normal rats (Fig. 7*A*) and reduce the upregulated expression of *MuRF1* and *MAFbx* after unloading (Fig. 7*B*).

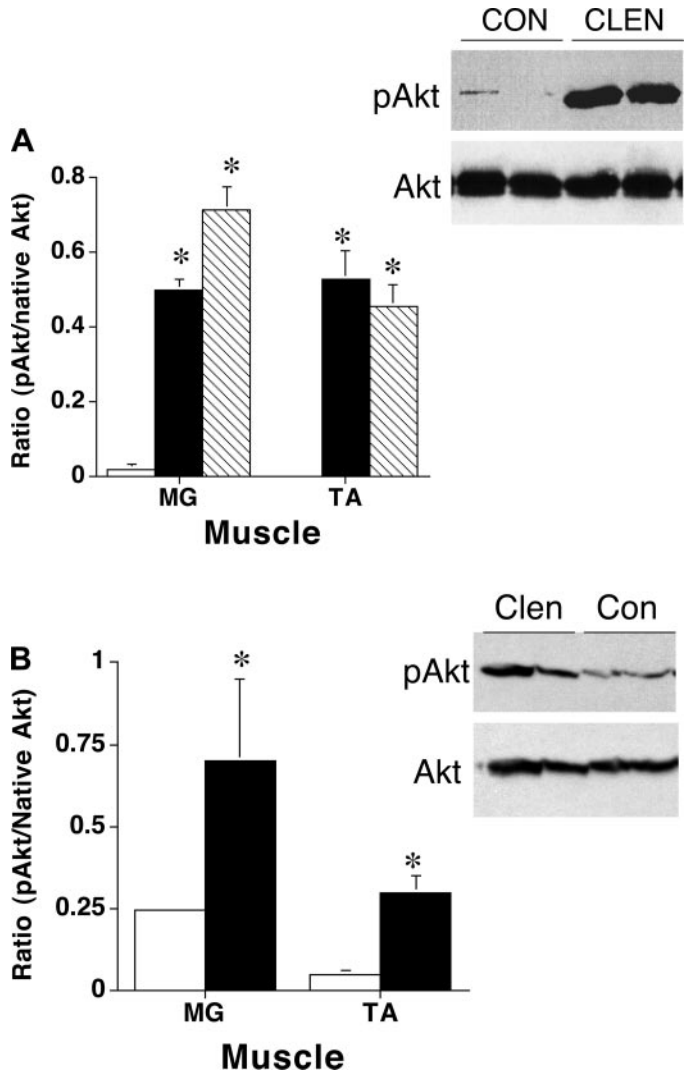

Fig. 5. Akt phosphorylation status in the MG after acute (*A*) and chronic (*B*) clenbuterol treatment (3 mg/kg). *A*: Western blots of native and phosphorylated Akt in MG after no treatment (CON) or 4 h after a subcutaneous injection of clenbuterol. Each lane represents 200 μg of total protein extracted 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 ($P < 0.05$).

Downloaded from http://jap.physiology.org/ by 10.220.33.3 on May 22, 2017

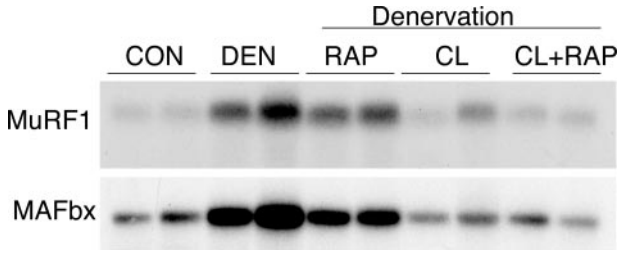

Fig. 6. Northern blots showing the effects of denervation and clenbuterol treatment on *MuRF1* and *MAFbx* transcripts. MG was obtained from rats after no treatment (CON), 10 days of denervation (DEN), 10 days of denervation + rapamycin (RAP), 10 days of denervation + clenbuterol (CL), or 10 days of denervation + clenbuterol + rapamycin (CL+RAP). Each lane represents 10 μg 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

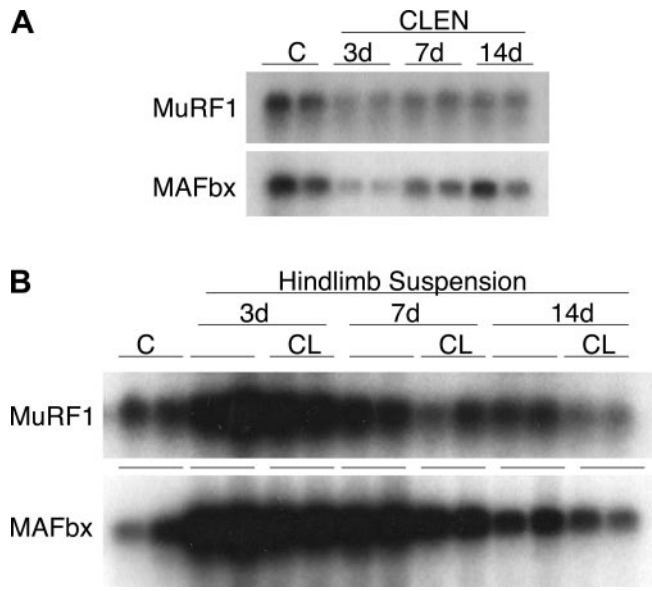

Fig. 7. Northern blots showing the effects of clenbuterol treatment on *MuRF1* and *MAFbx* transcripts in the MG muscle of normal (*A*) and hindlimb-suspended (*B*) rats. *A*: MG was obtained from rats after no treatment (C) or after clenbuterol (CLEN) treatment for 3, 7, or 14 days (d). *B*: MG was 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 μg of total RNA extracted from a pool of 3 MG muscles. For each group, duplicate lanes represent different pools of MG muscle.

Downloaded from http://jap.physiology.org/ by 10.220.33.3 on May 22, 2017

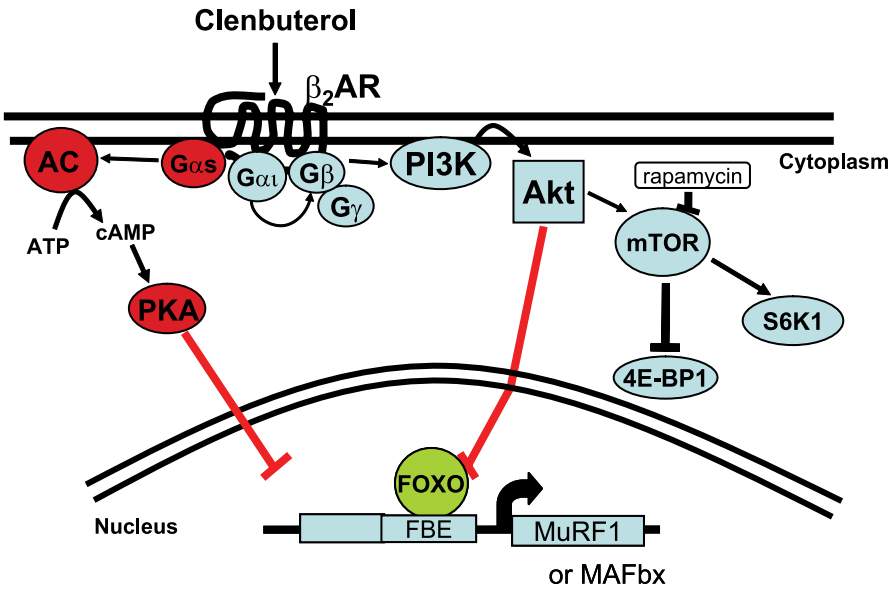

Fig. 8. Hypothetical model of β_2-adrenergic signaling in skeletal muscle. It is proposed that activation of β_2-adrenergic receptor (β_2AR) by clenbuterol couples to both $G_s\alpha$-adenylate cyclase (AC)-cAMP and $G_i\alpha$-G$\beta\gamma$-phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathways. 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_2AR 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 determined 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-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

Downloaded from http://jap.physiology.org/ by 10.220.33.3 on May 22, 2017

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 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 $G_i\alpha$-$G\beta\gamma$-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.

ACKNOWLEDGMENTS

We thank our colleagues at Regeneron Pharmaceuticals and the University of California, Davis, for support and technical expertise.

REFERENCES

1. **Agbenyega ET, Wareham AC.** Effect of clenbuterol on skeletal muscle atrophy in mice induced by the glucocorticoid dexamethasone. *Comp Biochem Physiol Comp Physiol* 102: 141–145, 1992.
2. **Agrawal S, Thakur P, Katoch SS.** Beta adrenoceptor agonists, clenbuterol, and isoproterenol retard denervation atrophy in rat gastrocnemius muscle: use of 3-methylhistidine as a marker of myofibrillar degeneration. *Jpn J Physiol* 53: 229–237, 2003.
3. **Apseloff G, Girten B, Walker M, Shepard DR, Krecic ME, Stern LS, Gerber N.** Aminohydroxybutane bisphosphonate and clenbuterol prevent bone changes and retard muscle atrophy respectively in tail-suspended rats. *J Pharmacol Exp Ther* 264: 1071–1078, 1993.
4. **Auffray C, Rougeon F.** Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur J Biochem* 107: 303–314, 1980.
5. **Azpiazu I, Saltiel AR, DePaoli-Roach AA, Lawrence JC.** Regulation of both glycogen synthase and PHAS-I by insulin in rat skeletal muscle involves mitogen-activated protein kinase-independent and rapamycin-sensitive pathways. *J Biol Chem* 271: 5033–5039, 1996.
6. **Babij P, Booth FW.** Clenbuterol prevents or inhibits loss of specific mRNAs in atrophying rat skeletal muscle. *Am J Physiol Cell Physiol* 254: C657–C660, 1988.
7. **Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, Glass DJ.** Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 294: 1704–1708, 2001.
8. **Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD.** Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol* 3: 1014–1019, 2001.
9. **Choo JJ, Horan MA, Little RA, Rothwell NJ.** Anabolic effects of clenbuterol on skeletal muscle are mediated by β2-adrenoceptor activation. *Am J Physiol Endocrinol Metab* 263: E50–E56, 1992.
10. **Cockman MD, Jones MB, Prenger MC, Sheldon RJ.** Magnetic resonance imaging of denervation-induced muscle atrophy: effects of clenbuterol in the rat. *Muscle Nerve* 24: 1647–1658, 2001.
11. **Costelli P, Garcia-Martinez C, Llovera M, Carbo N, Lopez-Soriano FJ, Agell N, Tessitore L, Baccino FM, Argiles JM.** Muscle protein waste in tumor-bearing rats is effectively antagonized by a beta 2-adrenergic agonist (clenbuterol). Role of the ATP-ubiquitin-dependent proteolytic pathway. *J Clin Invest* 95: 2367–2372, 1995.
12. **Dodd SL, Koesterer TJ.** Clenbuterol attenuates muscle atrophy and dysfunction in hindlimb-suspended rats. *Aviat Space Environ Med* 73: 635–639, 2002.
13. **Fingar DC, Blenis J.** Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene* 23: 3151–3171, 2004.
14. **Grindeland RE, Roy RR, Edgerton VR, Grossman EJ, Mukku VR, Jiang B, Pierotti DJ, Rudolph I.** Interactive effects of growth hormone and exercise on muscle mass in suspended rats. *Am J Physiol Regul Integr Comp Physiol* 267: R316–R322, 1994.
15. **Haddad F, Adams GR, Bodell PW, Baldwin KM.** Isometric resistance exercise fails to counteract skeletal muscle atrophy processes during the initial stages of unloading. *J Appl Physiol* 100: 433–441, 2006.
16. **Hall RA.** β-Adrenergic receptors and their interacting proteins. *Semin Cell Dev Biol* 15: 281–288, 2004.
17. **Harris TE, Lawrence JC Jr.** TOR signaling. *Sci STKE* 2003: re15, 2003.
18. **Hesketh JE, Campbell GP, Lobley GE, Maltin CA, Acamovic F, Palmer RM.** Stimulation of actin and myosin synthesis in rat gastrocnemius muscle by clenbuterol; evidence for translational control. *Comp Biochem Physiol C* 102: 23–27, 1992.
19. **Hinkle RT, Dolan E, Cody DB, Bauer MB, Isfort RJ.** Phosphodiesterase 4 inhibition reduces skeletal muscle atrophy. *Muscle Nerve* 32: 775–781, 2005.
20. **Hinkle RT, Hodge KM, Cody DB, Sheldon RJ, Kobilka BK, Isfort RJ.** Skeletal muscle hypertrophy and anti-atrophy effects of clenbuterol are mediated by the β2-adrenergic receptor. *Muscle Nerve* 25: 729–734, 2002.
21. **Horne Z, Hesketh J.** Increased association of ribosomes with myofibrils during the skeletal-muscle hypertrophy induced either by the beta-adrenoceptor agonist clenbuterol or by tenotomy. *Biochem J* 272: 831–833, 1990.

Downloaded from http://jap.physiology.org/ by 10.220.33.3 on May 22, 2017

22. **Kim YS, Lee YB, Ashmore CR.** Cimaterol-induced growth in rats: growth pattern and biochemical characteristics. *Growth Dev Aging* 52: 41–45, 1988.
23. **Kim YS, Sainz RD.** Beta-adrenergic agonists and hypertrophy of skeletal muscles. *Life Sci* 50: 397–407, 1992.
24. **Kimball SR, Farrell PA, Jefferson LS.** Invited Review: Role of insulin in translational control of protein synthesis in skeletal muscle by amino acids or exercise. *J Appl Physiol* 93: 1168–1180, 2002.
25. **Kissel JT, McDermott MP, Mendell JR, King WM, Pandya S, Griggs RC, Tawil R.** Randomized, double-blind, placebo-controlled trial of albuterol in facioscapulohumeral dystrophy. *Neurology* 57: 1434–1440, 2001.
26. **MacLennan PA, Edwards RH.** Effects of clenbuterol and propranolol on muscle mass. Evidence that clenbuterol stimulates muscle β-adrenoceptors to induce hypertrophy. *Biochem J* 264: 573–579, 1989.
27. **Maltin CA, Delday MI.** Satellite cells in innervated and denervated muscles treated with clenbuterol. *Muscle Nerve* 15: 919–925, 1992.
28. **Maltin CA, Hay SM, Delday MI, Smith FG, Lobley GE, Reeds PJ.** Clenbuterol, a beta agonist, induces growth in innervated and denervated rat soleus muscle via apparently different mechanisms. *Biosci Rep* 7: 525–532, 1987.
29. **Maltin CA, Hay SM, McMillan DN, Delday MI.** Tissue specific responses to clenbuterol: temporal changes in protein metabolism of striated muscle and visceral tissues from rats. *Growth Regul* 2: 161–166, 1992.
30. **Mersmann HJ.** Overview of the effects of beta-adrenergic receptor agonists on animal growth including mechanisms of action. *J Anim Sci* 76: 160–172, 1998.
31. **Murphy RJ, Beliveau L, Gardiner PF, Calderone A.** Nifedipine does not impede clenbuterol-stimulated muscle hypertrophy. *Proc Soc Exp Biol Med* 221: 184–187, 1999.
32. **Nader GA.** Molecular determinants of skeletal muscle mass: getting the "AKT" together. *Int J Biochem Cell Biol* 37: 1985–1996, 2005.
33. **Navegantes LC, Resano NM, Migliorini RH, Kettelhut IC.** Role of adrenoceptors and cAMP on the catecholamine-induced inhibition of proteolysis in rat skeletal muscle. *Am J Physiol Endocrinol Metab* 279: E663–E668, 2000.
34. **Pavoine C, Defer N.** The cardiac β2-adrenergic signalling a new role for the cPLA2. *Cell Signal* 17: 141–152, 2005.
35. **Reeds PJ, Hay SM, Dorwood PM, Palmer RM.** Stimulation of muscle growth by clenbuterol: lack of effect on muscle protein biosynthesis. *Br J Nutr* 56: 249–258, 1986.
36. **Rennie MJ, Wackerhage H, Spangenburg EE, Booth FW.** Control of the size of the human muscle mass. *Annu Rev Physiol* 66: 799–828, 2004.
37. **Roberts P, McGeachie JK.** The effects of clenbuterol on satellite cell activation and the regeneration of skeletal muscle: an autoradiographic and morphometric study of whole muscle transplants in mice. *J Anat* 180: 57–65, 1992.
38. **Ryall JG, Gregorevic P, Plant DR, Sillence MN, Lynch GS.** β_2-Agonist fenoterol has greater effects on contractile function of rat skeletal muscles than clenbuterol. *Am J Physiol Regul Integr Comp Physiol* 283: R1386–R1394, 2002.
39. **Ryall JG, Plant DR, Gregorevic P, Sillence MN, Lynch GS.** Beta 2-agonist administration reverses muscle wasting and improves muscle function in aged rats. *J Physiol* 555: 175–188, 2004.
40. **Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH, Goldberg AL.** Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* 117: 399–412, 2004.
41. **Sartorelli V, Fulco M.** Molecular and cellular determinants of skeletal muscle atrophy and hypertrophy. *Sci STKE* 2004: re11, 2004.
42. **Signorile JF, Banovac K, Gomez M, Flipse D, Caruso JF, Lowensteyn I.** Increased muscle strength in paralyzed patients after spinal cord injury: effect of β_2 adrenergic agonist. *Arch Phys Med Rehabil* 76: 55–58, 1995.
43. **Sneddon AA, Delday MI, Maltin CA.** Amelioration of denervation-induced atrophy by clenbuterol is associated with increased PKC-α activity. *Am J Physiol Endocrinol Metab* 279: E188–E195, 2000.
44. **Sneddon AA, Delday MI, Steven J, Maltin CA.** Elevated IGF-II mRNA and phosphorylation of 4E-BP1 and p70(S6k) in muscle showing clenbuterol-induced anabolism. *Am J Physiol Endocrinol Metab* 281: E676–E682, 2001.
45. **Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, Gonzalez M, Yancopoulos GD, Glass DJ.** The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol Cell* 14: 395–403, 2004.
46. **Wineski LE, von Deutsch DA, Abukhalaf IK, Pitts SA, Potter DE, Paulsen DF.** Muscle-specific effects of hindlimb suspension and clenbuterol in mature male rats. *Cells Tissues Organs* 171: 188–198, 2002.
47. **Xiao RP.** β-Adrenergic signaling in the heart: dual coupling of the β_2-adrenergic receptor to G(s) and G(i) proteins. *Sci STKE* 2001: RE15, 2001.
48. **Yimlamai T, Dodd SL, Borst SE, Park S.** Clenbuterol induces muscle-specific attenuation of atrophy through effects on the ubiquitin-proteasome pathway. *J Appl Physiol* 99: 71–80, 2005.
49. **Zeman RJ, Ludemann R, Etlinger JD.** Clenbuterol, a β_2-agonist, retards atrophy in denervated muscles. *Am J Physiol Endocrinol Metab* 252: E152–E155, 1987.

Downloaded from http://jap.physiology.org/ by 10.220.33.3 on May 22, 2017