Clenbuterol suppresses proteasomal and lysosomal proteolysis and atrophy-related genes in denervated rat soleus muscles independently of Akt


1Departments of Physiology and 2Biochemistry/Immunology, School of Medicine of Ribeirão Preto University of São Paulo, São Paulo, Brazil

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Gonçalves DA, Silveira WA, Lira EC, Graça FA, Paula-Gomes S, Zanon NM, Kettelhut IC, Navegantes LC. Clenbuterol suppresses proteasomal and lysosomal proteolysis and atrophy-related genes in denervated rat soleus muscles independently of Akt. Am J Physiol Endocrinol Metab 302: E123–E133, 2012. First published September 27, 2011; doi:10.1152/ajpendo.00188.2011.—Although it is well known that administration of the selective β2-adrenergic agonist clenbuterol (CB) protects muscle following denervation (DEN), the underlying molecular mechanism remains unclear. We report that in vivo treatment with CB (3 mg/kg sc) for 3 days induces antiproteolytic effects in normal and denervated rat soleus muscle via distinct mechanisms. In normal soleus muscle, CB treatment stimulates protein synthesis, inhibits Ca2+-dependent proteolysis, and increases the levels of calpastatin protein. On the other hand, the administration of CB to DEN rats ameliorates the loss of muscle mass, enhances the rate of protein synthesis, attenuates hyperactivation of proteasomal and lysosomal proteolysis, and suppresses the transcription of the lysosomal protease cathepsin L and of atrogin-1/MAFbx and MuRF1, two ubiquitin (Ub) ligases involved in muscle atrophy. These effects were not associated with alterations in either IGF-I content or Akt phosphorylation levels. In isolated muscles, CB (10−6 M) treatment significantly attenuated DEN-induced overall proteolysis and upregulation in the mRNA levels of the Ub ligases. Similar responses were observed in denervated muscles exposed to β-2-BNZ-cAMP (500 μM), a PKA activator. The in vitro addition of triciribine (10 μM), a selective Akt inhibitor, did not block the inhibitory effects of CB on proteolysis and Ub ligase mRNA levels. These data indicate that short-term treatment with CB mitigates DEN-induced atrophy of the soleus muscle through the stimulation of protein synthesis, downregulation of cathepsin L and Ub ligases, and consequent inhibition of lysosomal and proteasomal activities and that these effects are independent of Akt and possibly mediated by the cAMP/PKA signaling pathway.

β2-agonist; muscle atrophy; protein degradation; adenosine 3′,5′-cyclic monophosphate

The role of β-adrenergic signaling in the regulation of skeletal muscle protein metabolism is far from being completely established. Numerous studies (reviewed in Refs. 27 and 31) show that the dietary administration of β2-agonists to many species markedly stimulates skeletal muscle growth. Indeed, β2-agonist treatment enhances the functional repair of regenerating rat skeletal muscle after injury (5) and prevents muscle wasting in different atrophic states, including denervation (DEN) (18), muscular dystrophies (14), cancer cachexia (7), and sarcopenia (40). The mechanisms by which β2-agonists induce growth and muscle-sparing responses are uncertain and may involve an increase in the rate of protein synthesis and/or a decrease in protein degradation (10, 29, 34, 36). A large body of evidence indicates that the effects of β2-agonists are in part due to inhibition of muscle proteolysis. Indeed, it has been shown that the administration of β2-agonists is accompanied by a reduction in calpain 1 activity and an increase in the activity of calpastatin, an endogenous inhibitor of calpains, suggesting suppression of the Ca2+-dependent proteolysis (2). More recently, a few studies have demonstrated that β2-agonist treatment might attenuate muscle atrophy through inhibitory effects on the ubiquitin (Ub)-proteasome system (UPS), the main intracellular pathway for protein degradation in skeletal muscle (10, 53). In a variety of atrophy models, the processes of ubiquitination and degradation are regulated by two muscle-specific Ub ligases: atrogin-1/muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1) (6, 16). The expression of these genes, known as “atrogenes”, is inhibited by the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway through the phosphorylation and nuclear exclusion of forkhead box class O (Foxo) transcription factors (42, 47). Costelli et al. (10) were the first to demonstrate that clenbuterol (CB) treatment suppresses the expression of Ub mRNA in rats inoculated with AH-130 Yoshida ascites hepatoma cells. It has also been shown that CB treatment attenuated the hindlimb unweighting-induced muscle atrophy in rats by reducing the hyperactivation of UPS in fast-twitch muscles but not slow-twitch muscles, such as the soleus muscle (53). The reasons for the differences between fast- and slow-twitch muscles are unclear. The autophagic/lysosomal system is also implicated in the turnover of soluble constituents of the cytoplasm and of cellular organelles during basal and catabolic conditions, and the activation of this system may be the consequence of an ordered transcriptional program involving a set of genes, e.g., microtubule-associated protein 1 light-chain 3β (LC3b), γ-aminobutyric acid (GABA) A receptor-associated protein-like 1 (GABARAPL1), and cathepsin L (4, 23). Indeed, the expression and activity of the autophagic/lysosomal genes are upregulated in many models of muscle atrophy (4, 23), but their regulation by β2-agonists is controversial and not fully understood.

β2-adrenoceptors account for the majority of the effects of β2-agonists on skeletal muscle. Stimulation of the GTP-binding protein stimulatory (Gs)-coupled β2-adrenergic receptor activates adenylyl cyclase, leading to an increase in cAMP and activation of PKA and the cAMP response element-binding protein (CREB). The link between the activation of this pathway and the inhibition of the Ca2+-dependent proteolytic system is well supported in the literature (37). In contrast, the role of the cAMP cascade in the regulation of the UPS and autophagy/lysosomal systems remains elusive. It was proposed recently that CB might activate Akt through the direct coupling

Address for reprint requests and other correspondence: L. C. C. Navegantes, Dept. of Physiology, School of Medicine, USP, 14049-900 Ribeirão Preto, SP, Brazil (e-mail address: navegantes@fmrp.usp.br).
of the β2-adrenergic receptor to the GTP-binding inhibitory protein G\(\alpha\)-Qβγy-PISK signaling pathway (21). However, the discovery of the exchange protein directly activated by cAMP (EPAC), a guanine nucleotide exchange factor for the Ras-like small GTPases Rap1 and Rap2 (11, 20), has opened up an entirely novel signaling pathway activated by cAMP because this factor seems to be involved in the stimulation of Akt upon an increase in the intracellular levels of cAMP. Another possibility is that PKA itself could directly mediate the antiproteolytic effect of β2-agonists, given that this kinase reduces protein breakdown in vitro (3) and has been shown recently to phosphorylate Foxo1 in vascular endothelial cells (24).

The current study was undertaken to clarify the molecular mechanisms and signaling events involved in the reversal of DEN-induced atrophy by CB in rat soleus muscles, which are composed mainly of slow-twitch fibers. We demonstrate that the short-term treatment with CB in vivo and in vitro suppresses the DEN-induced hyperactivation of the UPS and lysosomal system and upregulation of genes involved in muscle atrophy (atrogin-1, MuRF1, and cathepsin L) and show that these effects are independent of Akt but may be mediated by PKA.

MATERIALS AND METHODS

Animals, Treatment, and DEN Surgery

Because the incubation procedure required intact muscles of a sufficient thinness to allow for the adequate diffusion of metabolites and oxygen, fed 4-wk-old male Wistar rats (∼70 g) were used in all experiments. For most experiments, the following four experimental groups were used: sham-operated rats treated with saline (CON), sham-operated rats treated with CB (CB), 3-day-denervated rats treated with saline (DEN), and 3-day-denervated rats treated with CB (DEN + CB). DEN was chosen as an atrophy model because it induces a significant loss of contractile proteins that is due primarily to the enhancement of protein breakdown (13). The duration of DEN was chosen because both proteolysis (13) and the mRNA levels of Ub ligases (41) peak at 3 days after surgery. The muscles of two hindlimbs were denervated by cutting out an ∼2-mm section of the sciatic nerve ∼1 cm above the popliteal fossa while the animals were under anesthesia (10 mg/kg ketamine and 85 mg/kg xylazine). DEN was performed bilaterally to study the proteolytic process activities (see Measurements of Protein Degradation). In vivo treatment with CB or saline began on the day of surgery. CB (3 mg/kg in saline) was subcutaneously injected once daily for 3 days, and animals were euthanized 24 h after the last injection or as described in Experimental Protocols (below). This dose has been shown previously to mitigate atrophy in denervated muscles (46). With the exception of experiment 1, the rats were killed by cervical dislocation. All animals were housed in a room with a 12:12-h light-dark cycle and were given free access to water and a normal laboratory chow diet. All experiments and protocols were performed in accordance with the ethical principles for animal research adopted by Brazilian College of Animal Experimentation and were approved by the School of Medicine of Ribeirão Preto of the University of São Paulo-Ethical Commission of Ethics in Animal Research (no. 116/2006).

Isolated Skeletal Muscles

The soleus muscle was rapidly dissected, weighed, and maintained at approximately its resting length by securing the tendons in aluminum wire supports. Tissues were incubated at 37°C in Krebs-Ringer bicarbonate buffer (pH 7.4) equilibrated with 95% oxygen and 5% carbon dioxide; the buffer contained 5 mM glucose and 10 mM ascorbic acid to prevent CB oxidation.

Rates of Protein Synthesis

Muscles were incubated as described above in a buffer that contained all amino acids at concentrations similar to those of rat plasma (43). After a 1-h equilibration period, -[U-14C]lysine (0.05 µCi/ml) was added to the replacement medium, in which the muscles were incubated for the next 2 h. At the end of this period, the specific activity of acid-soluble lysine (intracellular lysine pool) in each muscle was measured by estimating the radioactivity and the concentration of lysine in this pool, which was determined by the method of Waalkes and Udenfriend (51). After measurement of the radioactivity was incorporated into total protein of the same muscle, the rate of synthesis was calculated using the specific activity of the intracellular pool of lysine of each muscle, assuming that there was no recycling of the label during the incubation period (12, 49).

Measurements of Protein Degradation

Briefly, overall proteolysis and the activity of the proteolytic systems (UPS, lysosomal, and Ca\(^{2+}\)-dependent) were measured by following the tyrosine released into the medium in the presence of cycloheximide (0.5 nM), which prevented protein synthesis and reincorporation of tyrosine back into proteins. Tissues were preincubated for 1 h and then incubated for 2 h in fresh medium with the identical composition. Because muscle cannot synthesize or degrade tyrosine, its release reflects protein breakdown. The overall proteolysis was evaluated by measuring the tyrosine released into the medium. For measurement of UPS activity, muscles from one limb were incubated under conditions that prevent activation of the lysosomal [10 nM methylamine, 1 µM insulin, and branched-chain amino acid (170 µM leucine, 100 µM isoleucine, and 200 µM valine) and Ca\(^{2+}\)-dependent (Ca\(^{2+}\)-free medium with cysteine-protease inhibitors, including 25 µM E64 and 50 µM leupeptin)] proteolytic systems. In addition, muscles from the contralateral limb were incubated with the proteasome inhibitor MG132 (20 µM). For measurement of lysosomal proteolysis, muscles from one limb were incubated in the absence of methylene, insulin, and branched-chain amino acids, a condition in which the lysosomal system is activated. Contralateral muscles were incubated in the presence of insulin (1 µU/ml, leucine (170 µM), isoleucine (100 µM), valine (200 µM), and methylene (10 mM), a weak base that increases intralysosomal pH and inhibits lysosomal proteolysis. For measurement of Ca\(^{2+}\)-dependent proteolytic activity, muscles from one limb were incubated in the presence of Ca\(^{2+}\) and inhibitors of the lysosomal system (methylene, insulin, and branched-chain amino acids). Contralateral muscles were incubated in a Ca\(^{2+}\)-free medium that contained lysosomal inhibitors and cysteine-protease inhibitors (50 µM E64 and 25 µM leupeptin). UPS, lysosomal, and Ca\(^{2+}\)-dependent proteolytic activities were calculated from the difference in tyrosine release between the left and right muscles. Tyrosine release was assayed using the fluorometric method (51).

Quantitative PCR

Skeletal muscles were harvested and immediately frozen in liquid nitrogen. RNA was subsequently isolated from individual skeletal muscles using TRIzol (Invitrogen, Carlsbad, CA). Reverse transcription into cDNA was performed using 2 µg of total cellular RNA, 20 pmol of oligo(dT) primer (Invitrogen), and Advantage ImProm-II reverse transcriptase (Promega, Madison, WI). Real-time PCR was carried out using an ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA), a SuperScript III Platinum SYBR Green One-Step RT-qPCR kit with ROX (Invitrogen), and primers for rat atrogin-1 (forward 5′-GCA GAG AGT CGG CAA GTC-3′ and reverse 5′-CAG GTC GTG CAT CGT GAG-3′), MuRF1 (forward 5′-TGG ACA TCT ACA AGC AGA AA-3′ and reverse 5′-CTG CCT TTG GAA GAT GCT TT-3′), GABARAP1 (forward 5′-CCC AGT TGT GCC AGT AGA CA-3′ and reverse 5′-GAC TGA TCC TGA
GCG TCC TG-3'), LC3b (forward 5'-TTT GTA AGG GCG GTT CTG AC-3' and reverse 5'-CAG GTA GCA GGA AGC AGA GG-3'), cathepsin L (forward 5'-GTG GAC TGT TCT CAC GCT CAAG-3' and reverse 5'-TCC GTC CTT CGC TTT ATA GG-3'), and cyclophilin B (forward 5'-GCA TAC AGG TGC TGG CAT CT-3' and reverse 5'-CTT CCC AAA GAC CAC ATG CT-3'). The relative quantitation of mRNA levels was plotted as the fold increase compared with the CON group values. Transcripts of interest were normalized to cyclophilin B levels. The level of the target transcripts was calculated using the standard curve method (8).

**Western Blotting Analysis**

Skeletal muscles were homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 5 μg/ml aprotonin, 1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride at 4°C. The homogenate was centrifuged at 21,000 g at 4°C for 20 min, with the supernatant retained, and protein content was determined using BSA as a standard (26). An equal volume of sample buffer (20% glycerol, 125 mM Tris·HCl, 4% SDS, 100 mM dithiothreitol, and 0.02% bromophenol blue, pH 6.8) was added to the supernatant, and the mixture was boiled. Fifty to one hundred micrograms of total proteins was separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-Akt (1:750), anti-phospho (p)-Ser473-Akt (1:750), anti-p-(Thr308)-Akt (1:500), anti-Foxo1 (1:1,000), anti-p-(Ser253)-Foxo3a (1:750), anti-p-(Ser256)-Foxo1, anti-CREB 1,000), anti-p-(Ser253)-Foxo3a, anti-Foxo3a (1:500), anti-p-(Ser256)-Foxo1, anti-Foxo1, anti-p-(Ser473)-Akt (1:750), anti-p-(Thr 308)-Akt (1:500), anti-Foxo1 (1:1,000), anti-p-(Ser239)-Foxo1 (1:750), anti-Foxo3a (1:500), anti-p-(Ser239)-Foxo3a (1:750), anti-p-(Ser313)-CREB (1:750), anti-CREB (1:750), anti-GABARAP (1:750), anti-LC3 (1:1,000), anti-atrogin-1 (1:1,000), anti-proteasome 20S C2 subunit (1:750), anti-IGF-1 (1:3,000), anti-caspase-3 (1:1,000), anti-β-actin (1:2,000), and anti-α-tubulin (1:750). Primary antibodies (Ab) were detected using peroxidase-conjugated secondary Ab (1:10,000 for IGF-1, 1:13,000 for caspase-3, 1:1,000 for GABARAP, Foxo3a, p-Ser253 Foxo3a, Foxo1, p-Ser256 Foxo1, Akt, p-Thr308 Akt, p-Ser239 Akt, and 1:1,500 for CREB, and p-Ser133 CREB and 1:5,000 for the other primary Ab) and visualized using ECL reagents on film or in an ImageQuant 350 detection system (GE Healthcare, Piscataway, NJ). Band intensities were quantified using ImageJ (version 1.43u; National Institutes of Health).

**Metabolites and Hormone Measurements**

Glucose concentrations were determined using the YSI 2700 select biochemical analyzer (Yellow Springs Instruments, Yellow Springs, OH). Free fatty acids (FFAs) were determined by spectrophotometry using a Random NEFA kit (Random Laboratories, Crumlin, County Antrim, UK). Insulin was measured by radioimmunoassay, and muscular cAMP levels were measured using a method based on a competitive enzyme immunoassay system (GE Healthcare).

**Antibodies, Drugs, and Reagents**

Rabbit polyclonal anti-p-(Ser473)-Akt, anti-p-(Thr308)-Akt, anti-Akt, anti-p-(Ser239)-Foxo1, anti-Foxo1, anti-p-(Ser239)-Foxo3a, anti-Foxo3a, anti-p-(Ser313)-CREB, and CREB were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-atrogin-1, anti-cathepsin B, anti-cathepsin L, anti-GABARAP, anti-caspase-3, and anti-β-actin and mouse anti-α-tubulin Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human anti-IGF-1 antibody was purchased from Upstate (Lake Placid, NY). Rabbit anti-LC3 was purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-proteasome 20S C2 subunit was purchased from Boston Biochem (Cambridge, MA). Rabbit anti-goat IgG and goat anti-rabbit IgG antibodies (secondary antibodies) were purchased from Santa Cruz Biotechnology and Cell Signaling Technology, respectively. All drugs and reagents were purchased from Sigma-Aldrich (St. Louis, MO), Thermo Scientific HyClone (Pittsburgh, PA), Invitrogen (Carlsbad, CA), Calbiochem EMD Biosciences (La Jolla, CA), or Amersham Biosciences (Piscataway, NJ).

**Experimental Protocols**

**Experiment 1:** metabolic effects of CB treatment. Rats were divided into CON, CB, DEN, and DEN + CB groups. Three days after surgery and treatment, animals were decapitated, and their trunk blood, soleus muscle, heart, and adipose tissue were collected. Tissues were rapidly weighed, and soleus muscles were frozen in liquid nitrogen and stored at −80°C for determination of cAMP levels. FFAs, glucose, and insulin were measured in the plasma samples.

**Experiment 2:** the in vivo effect of CB on protein synthesis and protein degradation in normal and denervated soleus. Muscles from CON, CB, DEN, and DEN + CB rats were rapidly dissected and incubated for 2 h for measurement of protein synthesis or protein degradation.

**Experiment 3:** the in vivo effect of CB on Akt phosphorylation and markers of atrophy and autophagy in normal and denervated soleus muscles. Muscles from CON, CB, DEN, and DEN + CB rats were harvested, immediately frozen in liquid nitrogen, and stored at −80°C for measurement of the mRNA expression of atrogin-1, MuRF1, LC3b, GABARAP, and cathepsin L, the protein expression of atrogin-1, the C2 subunit of the 20S proteasome, LC3, GABARAP, calpastatin, calpain 1 and 2, caspase-3, and IGF-I, and the phosphorylation of Akt.

**Experiment 4:** the in vivo effect of a single injection of CB on Akt phosphorylation in normal muscles from rats and mice. To evaluate the acute temporal effects of CB on Akt phosphorylation, the rats received an injection of CB or saline. After 1 or 2 h, soleus and extensor digitorum longus (EDL) muscles were harvested. To compare the effects of CB on Akt phosphorylation in soleus muscles from different rodent species, groups of mice (12 wk-old C57Bl6) were treated with CB or saline, and the soleus muscle was harvested 1 h after treatment. Muscles were stored as described above until further processing.

**Experiment 5:** the in vitro effects of CB on protein degradation, expression of Ub ligases, and Akt/Foxo and PKA/CREB signaling. To identify the concentration of CB that was able to suppress DEN-induced overall proteolysis and Ub induced fusion in liquid nitrogen, and in vitro, denervated soleus muscles were incubated for 2 h with CB dissolved in ascorbic acid at various concentrations (10⁻⁸, 10⁻⁷, and 10⁻⁶ M). Once the minimum concentration required for inhibition was determined, normal and denervated soleus muscles were incubated with CB (10⁻⁶ M) for 2 h and used to analyze the phosphorylation status of Akt, Foxo1, Foxo3a, and CREB.

**Experiment 6:** the in vitro effects of triciribine, an Akt inhibitor, on protein degradation and Ub ligase expression in denervated soleus muscle incubated with CB. For measurement of overall proteolysis and the mRNA expression of Ub ligases, denervated soleus muscle from one limb was incubated for 2 h with CB (10⁻⁶ M, dissolved in 10 mM ascorbic acid) and/or triciribine (10 μM, dissolved in 0.01% DMSO). Contralateral denervated and normal muscles were incubated with ascorbic acid and DMSO. Muscles were exposed to triciribine for 30 min before the addition of CB.

**Experiment 7:** the in vitro effects of H89, a PKA inhibitor, on protein degradation and Akt phosphorylation in denervated soleus muscles incubated with CB. For measurement of overall proteolysis and the mRNA expression of Ub ligases, denervated soleus muscle from one limb was incubated for 2 h with CB (10⁻⁶ M, dissolved in 10 mM ascorbic acid) and/or H89 (50 μM dissolved in 0.01% DMSO). Contralateral denervated and normal muscles were incubated with ascorbic acid and DMSO. Muscles were exposed to H89 for 30 min before the addition of CB.

**Experiment 8:** involvement of PKA and EPAC in the antiproteolytic effect of CB in vitro. For the measurement of overall proteolysis and the mRNA levels of Ub ligases, normal and denervated soleus
muscles were incubated for 2 h in the presence or absence of 6-BNZ-cAMP (500 μM), a cAMP analog that activates PKA or 8-pCPT-2′-O-Me-cAMP (8-pCPT; 300 μM), an EPAC-specific activator.

**Statistical Analysis**

The data are presented as means ± SE. The means from different groups were analyzed using paired or unpaired Student’s t-tests. Multiple comparisons were made using two-way ANOVA followed by a Holm-Sidak post hoc test or one-way ANOVA followed by a Student-Newman-Keuls post hoc test. P ≤ 0.05 was taken as the criterion for significance.

**RESULTS**

**Metabolic Effects of CB Treatment**

CB treatment of normal rats did not affect the wet mass of the soleus muscle (Table 1) but led to an increase (11%) in the wet mass of the heart. CB-treated rats had a marked reduction in the wet mass of epididymal white adipose tissue and an increase in plasma FFA levels. The plasma levels of glucose and insulin were unchanged 3 days after CB treatment. In heart muscle mass and in plasma FFA levels increased in the heart mass and in plasma FFA levels served as controls for the efficiency of the treatment because CB, like other β2-agonists, causes cardiac hypertrophy and lipolytic effects. DEN reduced the wet muscle mass of the soleus muscle by 30% (Table 1). Prior studies (13, 15) have demonstrated that short-term treatment with CB was effective at promoting the well-known antiatrophy effect of β2-agonists. As shown in Table 1, the cAMP content did not change significantly in muscles from normal rats treated with CB. DEN induced a 74% increase in cAMP levels in soleus muscles, and this effect was abolished by 3 days of CB treatment.

**In Vivo Effects of CB on Protein Synthesis and Protein Degradation and Markers of Atrophy and Autophagy in Normal and Denervated Soleus Muscles**

Normal and denervated soleus muscles from rats treated with CB were isolated to evaluate the in vitro protein synthesis rate, protein degradation rate, and activity of various proteolytic systems. CB promoted anabolic actions in soleus muscles from normal rats by increasing (CON: 0.325 ± 0.010; CB: 0.410 ± 0.027 nmol Tyr·mg⁻¹·2 h⁻¹; n = 6–7, P < 0.05) protein synthesis and by decreasing overall proteolysis (13%; Fig. 1A). The antiproteolytic effect was paralleled by inhibition (41%; Fig. 1B) of Ca²⁺-dependent proteolysis and an increase (2-fold; Fig. 2B) in calpastatin protein levels. As described previously, the removal of the NH₂-terminal region of the 80-kDa catalytic subunit of the calpains by autolysis can be used as a marker of calpain activation (32). Thus we assessed calpain 1 autolysis in the soluble fraction of our extracts. As shown in Fig. 2B, neither the full-length (80-kDa) or autolyzed (75-kDa) forms of calpain 1 nor the total content of calpain 2 was altered by CB. In addition, no changes were observed in the activity (Fig. 1B) or expression (Fig. 2) of components of the UPS and lysosomal systems. DEN did not alter the rate of protein synthesis (DEN: 0.362 ± 0.026 nmol Tyr·mg⁻¹·2 h⁻¹; n = 8, P = 0.262) but led to an increase (60%; Fig. 1A) in overall proteolysis, which was accompanied by stimulation of the UPS (140%) and lysosomal (110%) proteolytic activities (Fig. 1B). Although the Ca²⁺-dependent activity estimated in isolated muscles was not altered, DEN increased calpain 1 autolysis, whereas calpain 2 protein levels remained un-

**Table 1. Effects of treatment for 3 days with CB and/or DEN on tissue mass and metabolic parameters**

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>CB</th>
<th>DEN</th>
<th>DEN + CB</th>
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<tbody>
<tr>
<td>Soleus mass/BM, mg/100 g</td>
<td>44.3 ± 1.2 (8)</td>
<td>45.9 ± 0.1 (8)</td>
<td>31.6 ± 1.5 (8)*</td>
<td>41.2 ± 0.9 (10)#</td>
</tr>
<tr>
<td>cAMP levels, fmol/mg muscle</td>
<td>1.003 ± 91 (7)</td>
<td>1.203 ± 79 (7)</td>
<td>1.743 ± 175 (7)*</td>
<td>804 ± 73 (6)#</td>
</tr>
<tr>
<td>Heart mass/BM, mg/100 g</td>
<td>516 ± 13 (8)</td>
<td>553 ± 9 (8)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epididymal adipose tissue, g/100 g</td>
<td>71 ± 6 (8)</td>
<td>33 ± 5 (10)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFAs, μmol/ml</td>
<td>0.244 ± 0.025 (6)</td>
<td>0.317 ± 0.019 (7)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>116 ± 3 (8)</td>
<td>114 ± 3 (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin, μIU/ml</td>
<td>8 ± 0.8 (7)</td>
<td>11 ± 1.2 (6)</td>
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</table>

Values are means ± SE (n). CB, clenbuterol; DEN, denervation; CON, control; BM, body mass; FFA, free fatty acids. *P < 0.05 vs. CON; #P < 0.05 vs. DEN.
changed (Fig. 2B). This effect was associated with a decrease in calpastatin protein levels (Fig. 2B). As shown in Fig. 2A, DEN upregulated the mRNA levels of the Ub ligases atrogin-1 (−10-fold) and MuRF1 (3.5-fold) as well as the lysosomal protease cathepsin L (54%). Atrogin-1 protein content was also increased (3-fold) by DEN (Fig. 2A). Additionally, DEN increased the mRNA expression of the autophagy-related genes LC3b (2-fold) and GABARAP1 (3-fold) but did not alter the levels of the LC3-I and LC3-II forms of the protein (Fig. 2B). GABARAP protein was not detected by immunoblotting in soleus muscles from any group. Otherwise, the muscle-sparing effects of CB on denervated muscles were associated with stimulation of protein synthesis (DEN/CB: 0.516 ± 0.038 nmol Tyr·mg⁻¹·h⁻¹; n = 8, P < 0.05) and inhibition of total protein degradation (20%; Fig. 1A) and the proteolytic activities (Fig. 1B) of the UPS (−30%) and lysosomal system (−40%). CB completely prevented the DEN-induced upregulation of the mRNA levels of atrogin-1, MuRF1, and cathepsin L (Fig. 2A) and the protein content of atrogin-1 (Fig. 2B) but had no effect on the mRNA levels of LC3b and GABARAP1 (Fig. 2A). Either CB nor DEN changed the levels of the C2 subunit of the 20S proteasome, pro-caspase-3, or its active form caspase-3 p17 (Fig. 2B). Taken together, our results support the hypothesis that the suppression of atrogenes is one mechanism by which β2-agonists induce inhibition of the UPS and lysosomal systems in denervated rat soleus muscles in vivo.

In Vivo Effect of CB on IGF-I/Akt Signaling in Muscles from Rats and Mice

Because IGF-I/Akt signaling has recently been proposed to mediate the actions of β2-agonists on protein metabolism, we analyzed the protein levels of IGF-I and the phosphorylation status of Akt by immunoblotting. Neither DEN nor CB changed the IGF-I levels, Akt levels, or Ser473 phosphorylation of Akt in the soleus muscle (Fig. 3). To test whether β2-agonists activate Akt at times <3 days or in other muscle types or species, Akt phosphorylation was analyzed in the soleus muscle and EDL, a typical fast-twitch muscle, from rats and mice 1 and 2 h after a single CB injection. Again, CB was ineffective at inducing Ser phosphorylation of Akt in normal rat soleus muscle at 1 and 2 h (Fig. 4A). A similar response was observed in denervated soleus muscles (data not shown).
lack of effect on Akt phosphorylation in response to β2-adrenergic agonists seems to be specific for the soleus muscle because there were two- and 3.5-fold increases in Akt phosphorylation after 1 and 2 h of treatment, respectively, in EDL (Fig. 4B). In contrast to rats, the treatment of mice with CB induced three- and sixfold increases in Ser473 and Thr308 phosphorylation of Akt, respectively, in soleus muscles (Fig. 4D). Together, these findings indicate that the effect of CB on Akt is muscle type and species specific. Moreover, these data suggest that the IGF-I/Akt signaling might not be involved in the antitrophic effects of CB treatment on soleus muscles from denervated rats.

In Vitro Effects of CB on Protein Degradation, Ub Ligase Expression, and Akt/Foxo Signaling

CB, at various concentrations, was added to the incubation medium for soleus muscle isolated from denervated rats. The addition of 10⁻⁷ and 10⁻⁶ M CB to the incubation medium reduced the total protein degradation by 10% (Fig. 5A). As shown in Fig. 5B, DEN increased the mRNA levels of both atrogin-1 (~18-fold) and MuRF1 (~3-fold), and these catabolic effects were inhibited by the highest concentration of CB used (10⁻⁶ M). The MuRF1 mRNA levels returned to basal values, and atrogin-1 mRNA was decreased by ~60% within 2 h of CB incubation (Fig. 5B). Figure 6 shows that these effects were not associated with Akt activation. In both normal and denervated soleus muscles, the phosphorylation status of Akt (at Ser and Thr) was unchanged by CB (Fig. 6). DEN decreased (76%) the protein levels of Foxo1 but did not alter the Ser phosphorylation Foxo1 when the phosphoprotein value was normalized to β-actin (Fig. 6). The phosphorylation status and protein levels of Foxo3a were not altered in any group. To further investigate the role of Akt in these antitrophy effects of CB, the overall proteolysis and Ub ligase expression were assessed in denervated soleus muscles incubated with the highest concentration of CB (10⁻⁶ M) in the absence or presence of triciribine, a selective Akt inhibitor. Triciribine alone increased overall proteolysis in denervated soleus muscles but had no effect on either atrogin-1 or MuRF1 mRNA levels (Fig. 7). Furthermore, triciribine did not alter the decreases in protein breakdown and Ub ligase mRNA levels induced by CB in denervated soleus muscles (Fig. 7). These in vitro results are consistent with the in vivo data and show that the suppressive effects of CB on proteolysis and Ub ligase expression in denervated soleus muscles are mediated directly by the activation of β2-adrenoceptors in denervated soleus muscles independent of Akt signaling.

Involvement of PKA and EPAC in the Antiproteolytic Effects of CB In Vitro

Because the majority of the intracellular effects of β2-adrenergic agonists in skeletal muscle are mediated by cAMP, we investigated the effects of PKA and EPAC, the main effectors activated by this nucleotide, by incubating soleus muscles in the presence of 6-BNZ-cAMP (a PKA activator) or 8-pCPT (an EPAC activator). The addition of 6-BNZ-cAMP to the incubation medium for soleus muscle isolated from normal and denervated rats reduced overall proteolysis by ~15% (Fig. 8A). In normal soleus muscle, 6-BNZ-cAMP significantly suppressed the expression of MuRF1 mRNA by ~47% but had no effect on atrogin-1 mRNA expression (Fig. 8B). Interestingly, the DEN-induced upregulation of MuRF1 mRNA expression was completely abolished by incubation with the PKA agonist, whereas atrogin-1 expression showed a trend toward lower expression (~35%) that was not statistically significant (Fig. 8B). In turn, 8-pCPT reduced the overall proteolysis in normal soleus muscle by 11% but did not affect denervated soleus muscle (Fig. 8A). Neither atrogin-1 nor MuRF1 mRNA expression was altered by 8-pCPT under any condition. Collectively, these findings show that 6-BNZ-cAMP mimics most effects of CB in vitro and implicate the PKA signaling pathway in the inhibitory effect of CB on the expression of atrogenes in soleus muscles from denervated rats.

To further investigate the involvement of the PKA signaling pathway in the antiproteolytic effects of CB, the phosphorylation of CREB, a downstream target of PKA, was assessed in...
isolated soleus muscles from normal and denervated rats. As shown in Fig. 6, CB increased the Ser phosphorylation of CREB by 57% in normal soleus muscles in vitro. Interestingly, DEN increased the phosphorylation (45%) and protein levels (26%) of CREB in soleus muscles, but CB did not cause additional effects on Ser-CREB phosphorylation in DEN muscles (Fig. 6). Overall proteolysis was also analyzed in denervated soleus muscles in the presence of CB and/or the PKA inhibitor H89. The addition of 10^{-6} M CB to the incubation medium significantly decreased the overall proteolysis (nmol Tyr·mg^{-1}·2 h^{-1}) in denervated muscles (CON: 0.474 ± 0.021; DEN: 0.806 ± 0.026; DEN + CB: 0.667 ± 0.027; n = 6–7, P < 0.05). Surprisingly, H89 alone also promoted a decrease (0.663 ± 0.023; n = 7, P < 0.05) in the increased proteolysis induced by DEN. No further inhibition of proteolysis was observed when denervated muscles were coincubated with CB and H89 (0.656 ± 0.030; n = 7, P = 0.556). The antiproteolytic effect induced by H89 in vitro was associated with a marked increase in the phosphorylation of Akt at Thr308 (15-fold) and Ser473 (6-fold) (data not shown).

**DISCUSSION**

The current work shows that CB, a selective β_{2}-agonist, acutely inhibits proteolysis in innervated and denervated rat soleus muscles via distinct mechanisms. In normal muscle, in addition to increasing the rate of protein synthesis, CB inhibits the Ca^{2+}-dependent proteolytic system, consistent with data from other in vitro studies in which CB was shown to reduce Ca^{2+}-dependent protein degradation (33, 36, 37). On the other hand, it has been demonstrated (35) that guanethidine-induced acute adrenergic neural blockade increases Ca^{2+}-dependent proteolysis in rat soleus muscle, suggesting the existence of an inhibitory tonus that restrains Ca^{2+}-dependent proteolysis. There is a growing body of evidence that the activity and gene expression of calpastatin, an endogenous inhibitor of calpains, are increased after β_{2}-agonist treatment (2, 38), suggesting that this molecular mechanism underlies the inhibitory effect of β_{2}-agonists on Ca^{2+}-dependent proteolysis. This hypothesis is supported by the current study’s findings that CB treatment increases calpastatin protein levels in normal rat soleus muscle. Because β-agonists activate PKA and may act via stimulation of cAMP-responsive elements in the calpastatin promoter regions (9), it has been proposed that calpastatin is a target for this kinase. Indeed, it has been shown that the calpastatin gene promoter is activated in L6G8 cells treated with dibutyryl-cAMP or forskolin (45). From these observa-
tions, it can be concluded that β2-agonists induce antiproteolytic effects in normal muscles, at least in part, by reducing Ca2+-dependent protein degradation via PKA-mediated effects on calpastatin expression.

The main goal of the present work was to investigate how β2-agonists counteract the loss of muscle mass in the face of catabolic muscle disuse. In agreement with previous studies (13, 29, 50), our data show that the rate of protein synthesis did not change in denervated soleus muscles and that most of the increase in protein degradation is due to the hyperactivation of the UPS and lysosomal systems. Although we observed an increase in calpain 1 autolysis and a reduction in calpastatin content following DEN, neither the maximal activity of Ca2+-dependent proteolysis nor the levels of calpain 1 or 2 were altered. Similarly, Tischler et al. (50) have observed that this degradative system is not involved in DEN-induced proteolysis. Caspase-3 is another cysteine protease that may mediate muscle atrophy induced by chronic DEN (39); however, the present data show that the levels of the caspase-3 precursor pro-caspase-3 and its mature/active form caspase-3 p17 were not changed in denervated muscles. On the other hand, DEN upregulates the expression of several atrogens (atrogin-1, MuRF1, and cathepsin L), as first reported by Sacheck et al. (41), and the mRNA expression of the autophagy-related genes LC3b and GABARAP1 but did not affect the levels of LC3-II, an autophagosomal marker in mammals (48). Although the present data show that DEN did not alter the content or phosphorylation status of Akt and Foxo3a, it seems highly likely that any decrease in phosphorylated Akt or Foxo in response to DEN would have occurred acutely, i.e., long before...
we sampled the muscle at 3 days after DEN. This hypothesis is supported by the finding that only a few hours of DEN (1 and 6 h) are needed to reduce the phosphorylation of Akt in rat diaphragm muscles (1).

This study confirmed other reports (29, 46) that concluded that acute treatment with CB exerts muscle-sparing effects following atrophic conditions. Although the rate of protein synthesis was stimulated by CB treatment, as described previously by Maltin et al. (29), treatment with this β2-agonist significantly inhibited the hyperactivation of both UPS and lysosomal proteolysis in denervated soleus muscles but did not affect either caspase-3 or the Ca\(^{2+}\)-dependent proteolytic system. The reason for these differences in response between the normal and denervated soleus muscles is unclear, but it may be due to a change in sensitivity to adrenergic stimulation, which seems to be increased after DEN. In fact, it has been shown that DEN results in increased cAMP levels, PKA activity, and cAMP binding to PKA regulatory subunits in rat soleus muscle (19). Accordingly, the current data show that DEN increased the intramuscular levels of cAMP and the phosphorylation of CREB, a downstream target of PKA. The inhibition of lysosomal proteolysis induced by CB in denervated muscles was associated with the downregulation of cathepsin L mRNA but not with the expression of the autophagy-related genes LC3b and GABARAP1. Koopman et al. (22) have recently reported similar effects on LC3b expression in skeletal muscle from normal mice treated with formoterol. In addition to its inhibition of the lysosomal system, CB is able, as shown here for the first time, to inhibit UPS activity not only in fast-twitch muscles, as proposed by Yimlamai et al. (53), but also in slow-twitch soleus muscles. We also found that CB treatment in vivo and in vitro prevented the transcriptional upregulation of atrophy-related Ub ligases (i.e., atrogin-1 and MuRF1) in denervated soleus muscles. To our knowledge, this study is the first to directly demonstrate an in vitro inhibitory effect of a β2-agonist on Ub ligase expression, a finding that strongly supports similar conclusions from in vivo experiments (17, 21). Taken together, these data suggest that the CB-induced protection of muscle following DEN occurs through the inhibition of lysosomal and UPS proteolysis, which is accompanied by the suppression of genes controlling atrophy but not autophagy.

It is well established that most of the actions of CB are mediated through cAMP. Although we did not detect any increase in muscle cAMP levels 24 h after the third injection of CB, such increases have been reported at 0.5 and 4 h after a single injection of CB in muscles from rats (28) and mice (17), respectively. Thus, it has been postulated that the increase in intracellular cAMP levels promoted by β2-adrenoceptors could be one of the regulatory mechanisms preventing excessive protein degradation in skeletal muscle. This view is consistent with the finding that the increase in muscle cAMP levels induced by IBMX, a nonselective phosphodiesterase inhibitor, reduces the activity of UPS, levels of Ub protein conjugates, and expression of atrogin-1 mRNA in isolated rat soleus muscles (17). Furthermore, IBMX blocked the dexamethasone-induced atrogin-1 expression in cultured muscle cells (17), whereas rolipram, a selective phosphodiesterase 4 inhibitor, attenuated the fasting-induced Ub ligase expression in rats (25). Therefore, the increase in the intracellular cAMP levels promoted by β2-agonists may play a direct role in suppressing Ub ligase expression and preventing the muscle protein breakdown induced by UPS during atrophy conditions.

Because it has been shown previously that, at least in fast-twitch skeletal muscles such as EDL, the production of cAMP upon epinephrine stimulation may activate Akt and inhibit proteolysis via a novel protein termed EPAC (3), we expected that CB administration would affect this kinase. The present data show that, in contrast to EDL, CB treatment in vivo (for 1 h, 2 h, or 3 days) or in vitro (for 2 h) does not stimulate Akt phosphorylation in the soleus muscle. Our data contrast those of Kline et al. (21), who found increased phosphorylation of Akt after acute and chronic CB treatment. A major difference between the two studies is that these authors studied muscles composed predominantly of fast-twitch fiber types (i.e., tibialis anterior and medial gastrocnemius) and not muscles like the soleus, which are rich in slow-twitch fibers. Investigation of the β2-agonist-induced activation of Akt in soleus muscles from rats and mice revealed that this kinase is differentially regulated in these two species. Unlike the rat soleus muscle, the mouse soleus muscle showed an increase in the phosphorylation levels of Akt 1 h after a single injection of CB. The available data do not allow for a conclusion to be reached regarding the reason for these differences, but the higher amount of fast-twitch fiber types in the mouse soleus muscle (52) compared with the rat soleus muscle could account for the difference in the Akt response between mice and rats. It is well established that different muscles show different responses to β-adrenergic stimulation. For example, it has been described that fast-twitch muscles such as the EDL are more responsive to the hypertrophic effect of β-adrenoceptor agonists than slow-twitch muscles (e.g., soleus) (31). This conclusion appears consistent with the present finding that Akt is activated by CB in the EDL but not in the soleus and with the previous verification that the reduction in proteolysis induced by isoproterenol in vitro is greater in the EDL than in the soleus (37). Further experiments are needed to confirm this hypothesis.

The above data suggest that the antiproteolytic effect of CB on denervated soleus muscles from rats occurs through Akt-independent signaling. To further support this hypothesis, in vitro studies with denervated soleus muscles were performed with triciribine, a selective Akt inhibitor. As expected, the CB-induced repression of the atrogin-1 and MuRF1 transcripts was not blocked by Akt inactivation. Next, we investigated the involvement of PKA and EPAC pathways. The data show that 6-BNZ-cAMP, a selective PKA agonist, mimicked the inhibitory effects of CB on Ub ligases and proteolysis, whereas 8-pCPT, a selective EPAC agonist, had no effect in denervated soleus muscles. Furthermore, 6-BNZ-cAMP inhibited the dexamethasone-induced Ub ligase expression in cultured C2C12 myotubes (Silveira W, Kettelhut IC, and Navegantes LC, unpublished observations). In line with the previous discussion of the mechanisms of the antiatrophic effect of CB, these data suggest that the repressive effect of β2-adrenoceptor stimulation on atrogene expression in denervated soleus muscle is mediated, at least in part, by cAMP/PKA signaling. To further support this hypothesis, studies were performed with denervated muscles incubated in the presence of CB and H89, a PKA inhibitor. However, these experiments were inconclusive because H89 alone decreased the higher overall proteolysis induced by DEN. Furthermore, H89 increased Akt phos-
phorylation even in the absence of β-adrenergic stimulation, supporting the results from a previous study showing that PKA inhibits Akt activation independent of receptor stimulation (30). The mechanism by which CB/cAMP/PKA signaling influences the transcription of atrogenes cannot be explained by the present data. Recently, Senf et al. (44) demonstrated that Foxo is regulated by the histone acetyltransferase (HAT) activity of p300/CBP repressed Foxo activation in cell-implanted soleus muscle and in C2C12 cells in response to dexamethasone and acute starvation. Thus, it seems reasonable to speculate that the inhibitory effect of CB on atrogenes observed here may be mediated through cAMP/PKA activation, leading to the activation of CBP and inhibition of the forkhead transcriptional program by acetylation.

In summary, the data in the present work show clearly that short-term treatment with CB, a selective β2-adrenergic agonist, enhances the rates of protein synthesis and reduces the baseline level of Ca2+-dependent proteolysis, probably by increasing calpastatin, in normal soleus muscle. In addition, CB mitigates DEN-induced atrophy by stimulating protein synthesis and inhibiting proteasomal and lysosomal proteolysis and the expression of several atrogenes (atrogin-1, MuRF1, and cathepsin L) in rat soleus muscle. Last, the present work provides evidence that the canonical cAMP/PKA/CREB signaling pathway is involved in the anticytotoxic effects of CB following DEN, which are independent of Akt. Further studies are needed to define the mechanisms by which PKA signaling reduces protein breakdown.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are reported by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

CLENBUTEROL EFFECTS ON PROTEOLYSIS


