Activating cAMP/PKA signaling in skeletal muscle suppresses the ubiquitin-proteasome-dependent proteolysis: implications for sympathetic regulation

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Silveira WA, Gonçalves DA, Graça FA, Andrade-Lopes AL, Bergantin LB, Zanon NM, Godinho RO, Kettelhut IC, Navegantes LC. Activating cAMP/PKA signaling in skeletal muscle suppresses the ubiquitin-proteasome-dependent proteolysis: implications for sympathetic regulation. J Appl Physiol 117: 11–19, 2014. First published May 15, 2014; doi:10.1152/japplphysiol.01055.2013.—Although we have recently demonstrated that plasma catecholamines induce antiproteolytic effects on skeletal muscle (Graça FA, Gonçalves DA, Silveira WA, Lira EC, Chaves VE, Zanon NM, Garófalo MAR, Kettelhut IC, Navegantes LC, Am J Physiol Endocrinol Metab. 305: E1483-E1494, 2013), the role of the muscle sympathetic innervation and, more specifically, norepinephrine (NE) in regulating the ubiquitin (Ub)-proteasome system (UPS) remains unknown. Based on previous findings that chemical sympathectomy acutely reduces UPS activity, we hypothesized that muscle NE depletion induces adrenergic supersensitivity in rat skeletal muscles. We report that surgical sympathetic denervation (SDEN), a condition in which only muscle NE from both hindlimbs is depleted, transiently reduced the overall proteolysis and the UPS activity (~25%) in both soleus and extensor digitorum longus muscles. This antiproteolytic response was accompanied by increased activity of adenyl cyclase (112%), levels of cyclic adenosine monophosphate (cAMP; 191%), and the serine phosphorylation of cAMP response element-binding protein (CREB). The consequent activation of cAMP-dependent protein kinase (PKA) in extensor digitorum longus from normal rats, NE (10⁻⁷ M) in vitro increased the levels of cAMP (115%) and the serine phosphorylation of both cAMP response element-binding protein (2.7-fold) and forkhead box class O1 transcription factor. Similar effects were observed in C2C12 cells incubated with forskolin (10 µM). In parallel, NE significantly reduced the basal UPS (21%) activity and the mRNA levels of atrophy-related Ub-ligases. Similar responses were observed in isolated muscles exposed to 6-BNZ-cAMP (500 µM), a specific PKA activator. The phosphorylation levels of Akt were not altered by SDEN, NE, forskolin or 6-BNZ-cAMP. Our results demonstrate that SDEN induces muscle adrenergic supersensitivity for cAMP leading to the suppression of UPS, and that the suppressive effects of NE on UPS activity and expression of Ub-ligases can be mediated by the activation of cAMP/PKA signaling, with the inhibition of forkhead box class O1 transcription factor.

Although it is well known that skeletal muscles receive a cholinergic motor innervation, evidence indicates that noradrenergic nerve terminals make close contact with striated muscle fibers in mammals (5, 6). However, the physiological role of this autonomic innervation in regulating protein metabolism remains unclear.

Most of the metabolic actions of catecholamines in peripheral tissues are exerted through a β-adrenoceptor-mediated increase in intracellular cyclic adenosine monophosphate (cAMP) and subsequent activation of cAMP-dependent protein kinase (PKA) and cAMP response element-binding protein (CREB). The intracellular concentration of cAMP in cells is determined via the balance between cAMP production by adenyl cyclase (AC), and cAMP degradation by phosphodiesterases (PDEs). Catecholamines have been traditionally viewed as catabolic, based on their lipolytic and glycogenolitic functions (27). However, numerous pharmacological studies, reviewed in Refs. 25 and 33, have shown that β₂-adrenergic agonists induce hypertrophy of skeletal muscle and blunt muscle atrophy in different conditions, including motor denervation, tumors, sepsis, and sarcopenia (13, 26, 40). The precise mechanism through which β₂-adrenergic agonists produce these effects is not completely known, but they seem to be due, at least in part, to a reduction in the rate of muscle protein breakdown (8, 16, 39). In fact, the in vitro addition of epinephrine (EPI) or NE induces a reduction in the rate of overall proteolysis (37), similar to that observed in human beings in vivo (17, 42). More recently, our laboratory (21) and others (47) have demonstrated that the short-term (3 days) or long-term (10 or 14 days) clenbuterol treatment, a selective β₂-adrenergic agonist, might attenuate muscle atrophy through inhibitory effects on the ubiquitin (Ub)-proteasome system (UPS), the main intracellular pathway for protein degradation in skeletal muscle (21, 26, 47). The Ub-conjugation to a protein substrate initiates its degradation by the 26S proteasome complex. In a variety of atrophy conditions, the processes of ubiquitination and degradation are regulated by two muscle-specific Ub-protein ligases (E3): atrogin-1/muscle atrophy F-box and MuRF1 (muscle RING finger 1) (10, 19). These genes, coined “atrogenes,” are regulated by Akt, a critical insulin signaling molecule that regulates muscle mass. One mechanism by which Akt reduces the expression of atrogenes involves the phosphorylation and subsequent nuclear exclusion of the forkhead box transcription factor Foxo (41, 44). Recent evidence indicates that atrogenes may also be regulated by molecules that act through the cAMP signaling pathway (9, 22). Indeed, the acute and chronic

NOREPINEPHRINE (NE) IS A NEUROTRANSMITTER produced by sympathetic nervous system (SNS), and it is released from sympathetic fibers that are distributed widely to the whole body.

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administration of clenbuterol to rodents suppressed the gene expression of atrogin-1 and MuRF-1 in fast-twitch muscles upon motor denervation (26) and fasting (20). These effects are probably mediated by cAMP, as drugs that induce an increase in the intracellular concentrations of cAMP, such as the selective and nonselective cAMP-PDE inhibitors, have also been employed in preventing muscle atrophy in rodents (22). Indeed, nonselective PDE inhibitors or rolipram, a selective PDE-4 inhibitor, reduce the UPS activity and the atrogin-1 mRNA in isolated skeletal muscles from normal rats (30) and in cultured muscle cells incubated with dexamethasone (20). Although this evidence indicates that the cAMP signaling in skeletal muscles is the mechanism by which catecholamines suppress the Ub-ligases and the UPS, the physiological role of muscle sympathetic innervation, and more specifically, NE, in the control of this proteolytic process, has not been hitherto investigated.

Previous work from our laboratory has shown that the chemical sympathectomy with guanethidine for 2 days increased the Ca\(^{2+}\)-dependent proteolysis in soleus, suggesting that the SNS through catecholamines exerts an inhibitory adrenergic tonus that restrains Ca\(^{2+}\)-dependent protein degradation in oxidative muscles of normal rats (36). On the other hand, it was also observed that the UPS activity in the extensor digitorum longus (EDL) muscle was reduced after 2 days of guanethidine treatment (36). This was interpreted as an anti-proteolytic response to the adrenergic supersensitivity of the EDL muscles following guanethidine treatment. Because in that study both plasma and muscle catecholamine levels were reduced by chemical sympathectomy, we could not dissociate if the observed effects were due to plasma catecholamines or NE released directly by noradrenergic innervation (36). To investigate the specific role of NE in the control of skeletal muscle protein synthesis in rodents, we have been using a model of surgical sympathectomy, NE (data not shown). For overall proteolysis and lysosomal and UPS activities, we have used both muscles (left and right) from the same animal. All rats were killed by cervical dislocation. The protocol studies were approved by Ethical Commission of Ethics in Animal Research from School of Medicine of Ribeirão Preto (017/2009) and agree with Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation.

**SDEN.** The sympathetic denervation surgery was performed with the help of a dissection microscope. For this, the animals were anesthetized with ketamine and xylazine (85 and 10 mg/kg body wt, respectively) and kept warm at 37°C on the heating bed during the surgery and allowed to recover from anesthesia (~30 min) before being returned to their normal environment.

The SDEN consisted of the surgical excision of the bilateral second and third lumbar ganglia of the paravertebral sympathetic chain, from which arises the sympathetic fibers to the skeletal muscles of the rat hindlimb (6). The lumbar sympathetic chain on both sides lies embedded in connective tissue in front of the vertebral column, behind the aorta and vena cava. Sham-operated animals were used as controls (CON). Food consumption was not altered after 2, 3, 4, and 7 days of surgery.

**NE measurements.** For the determination of muscle NE content, a group of rats was killed 2, 3, 4, and 7 days after SDEN or sham operation, and the EDL and soleus muscles from both hindlimbs (left and right) were harvested and immediately frozen in liquid nitrogen and then stored at −80°C until assayed. NE was assayed as previously described (18) using HPLC (LC-7A, Shimadzu Instruments) with a Spherisorb ODS-2 (5 μm; Sigma-Aldrich) reversed-phase column.

**Incubation procedure.** The EDL and soleus were rapidly dissected, with care being taken to avoid damaging the muscles. Soleus were maintained at approximately resting length by pinching them on inert plastic supports. Tissues were incubated at 37°C in Krebs-Ringer bicarbonate buffer, pH 7.4, equilibrated with 95% O\(_2\)/5% CO\(_2\), containing glucose (5 mM).

**Rates of protein degradation.** For measurement of the rate of overall protein degradation and proteolytic activities, skeletal muscles (EDL and soleus) from CON and SDEN rats for 2, 3, 4, and 7 days were incubated as described above. Briefly, after a 2-h incubation period, the overall proteolysis and lysosomal and UPS activities were determined by measuring the rate of tyrosine release in the incubation medium in the presence of cycloheximide (0.5 mM). Since muscle cannot synthesize or degrade tyrosine, its release reflects the rate of protein breakdown. Preliminary experiments showed that, as previously reported for normal animals (4), the intracellular pools of tyrosine of the denervated rats were not significantly affected by all of the incubation conditions used here. Therefore, rates of amino acid release into the medium reflect rates of protein degradation.

For measurement of UPS activity, muscles from one limb were incubated under conditions that prevent activation of the lysosomal (10 mM methylamine and 1 U/ml insulin), branched-chain amino-acid-dependent (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. Muscles from the contralateral limb were incubated in the presence of both lysosomal and Ca\(^{2+}\)-dependent proteolytic systems inhibitors, plus the proteasome inhibitor MG132 (20 μM).

For measurement of lysosomal activity, muscles from one limb were incubated in the absence of methylamine, 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 U/ml), leucine (170 μM), isoleucine (100 μM), valine (200 μM), and methylamine (10 mM), a weak base that increases intramyosomal pH and inhibits lysosomal proteolysis. UPS and lysosomal activities were calculated from the difference in tyrosine release between the left and right muscles. Tyrosine release was assayed using the fluorometric method (46).

**Determination of muscle cAMP levels.** The intracellular levels of cAMP were measured in EDL and soleus muscles from CON and 3- and 7-day SDEN rats by using a method based on a competitive enzyme immunoassay system (GE Healthcare). Briefly, muscles were homogenized in 6% trichloroacetic acid, and, after extraction of lipid content with diethyl ether, the aqueous phase was lyophilized and resuspended in the assay buffer.

**AC activity assay.** The AC activity was determined by measuring the formation of cAMP in membrane fractions in EDL and soleus muscles from CON and 3- and 7-day SDEN rats. Muscles were homogenized in 10 volumes (weight/volume) 12.5 mM Tris, pH 7.4 (containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM pepstatin A), and the samples were centrifuged at 20,000 g for 10 min at 4°C. The pellet was suspended in the same buffer and centrifuged two times at 20,000 g for 10 min at 4°C. The final pellet was suspended in five volumes of the same buffer, and the protein concentration was determined according to the method described by Bradford (11) using bovine serum albumin (BSA) as standard. For the AC activity assay, the membrane pellet containing 10 μg of protein were incubated at 30°C with 12.5 mM Tris, pH 7.4 (containing 0.6 mM ATP, 1 μM GTP, 10 mM MgCl₂, 1 mM IBMX, and 1 mg/ml BSA) in the presence of 10 μM forskolin (FSK) for 30 min. The reaction was stopped by boiling the samples for 10 min, and the samples were centrifuged for 15 min at 20,000 g. Cyclic AMP from the supernatant was determined using the cAMP kit [³²P]-assay system (GE Healthcare, LifeSciences, Fairfield, CT) and expressed as picomoles of cAMP formed per hour per milligram of protein.

**³²S/GTPγS-binding assay for G protein activation.** The basal and activated G protein levels in EDL muscles from CON and 3-day SDEN rats were determined using the procedure described previously (2). Briefly, muscles were homogenized in 100 mg/ml (weight/volume) 50 mM Tris-HCl buffer (pH 7.4) containing 3 mM MgCl₂, 100 mM NaCl (reaction buffer), and 1 mM EDTA plus 0.12 M sucrose. The samples were centrifuged two times at 1,000 g for 15 min at 4°C; the supernatant was mixed and centrifuged at 20,000 g for 30 min at 4°C. The final pellet was resuspended in five volumes of the same buffer, and the protein concentration was determined according to the method described by Bradford (11).

For the functional binding of [³²S]GTPγS, membranes were incubated with reaction buffer plus 0.2 mM EGTA at 37°C in 200 μl final volume. To analyze the effect of G protein-coupled receptor-mediated G protein activation on [³²S]GTPγS binding, membranes were incubated with TPA (0–100 μM) in the presence of 30 μM GDP, at 37°C, in 200-μl final volume. Nonspecific binding was obtained in the presence of 50 μM GTPγS. Samples were centrifuged for 20 min at 15,000 g at 4°C. To remove the free radioligand, the pellets were rinsed with 1.0 ml reaction buffer and centrifuged for 20 min at 15,000 g at 4°C twice. The final pellet was suspended in 200 μl of 1% SDS, and the radioactivity was determined by scintillation counting (Micro Beta Jet 1450, Perkin Elmer).

**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 total RNA with 20 pmol oligo(dT) primer (Invitrogen), and Advantage ImProm-II reverse transcriptase (Promega, Madison, WI). Real-time PCR was carried out using an ABI7000 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 GTG GAT GGT CGT GAG-3'), MuRF1 (forward 5'-TCG ACA TCT ACA AGG AA-3' and reverse 5'-CTG TTC TTG GAA GAT GCT TT-3'), and cyclophilin B (forward 5'-GCA TAC AGG TCC 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 respective 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 (14).

**Western blotting analysis.** CREB, Akt, and Foxo1 phosphorylation levels were measured in EDL muscles from CON and 3- and 7-day SDEN rats. 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 of aprotinin, 1 mg/ml of leupeptin, and 1 mM phenylmethylsulfonyl fluoride at 4°C. The homogenate was centrifuged at 21,000 g at 4°C for 20 min, retaining the supernatant, and protein content was determined using BSA as a standard (32). An equal volume of sample buffer (20% glycerol, 125 mM Tris-HCl, 4% SDS, 100 mM dithiothreitol, 0.02% bromophenol blue, pH 6.8) was added to the supernatant, and the mixture was boiled. Thirty to one hundred micrograms of total proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-Akt (1:750), anti-phospho (p)-Ser⁴⁷³-Akt (1:750), anti-Foxo1 (1:1,000), anti-p-Ser³⁸⁶-Foxo1 (1:750), anti-p-Ser³⁸⁶-CREB (1:750), and anti-β-actin (1:2,000). Primary antibodies (Ab) were detected using peroxidase-conjugated secondary Ab (1:1,000 for Foxo1, p-Ser³⁸⁶ Foxo1, p-Ser³⁸⁶ CREB, and 1:5,000 for the other primary Abs) and visualized using ECL reagents by an ImageQuant 350 detection system (GE Healthcare, Piscataway, NJ). Band intensities were quantified using ImageJ (version 1.43u, National Institutes of Health).

**NE and 6-BNZ-cAMP in isolated skeletal muscles.** To investigate the in vitro effect of NE (10⁻⁴ M) and 6-BNZ-cAMP (500 μM), the cAMP analog that activates PKA, on the UPS activity, cAMP levels, Ub-ligases mRNA expression (atrogin-1 and MuRF1), and protein levels (CREB, Akt, and Foxo1), EDL muscles from normal rats were incubated at different times in the presence or absence, of each compound using the same procedure described above. Contralateral muscles were incubated with vehicle and used as CON.

Abs, drugs, and reagents. Rabbit polyclonal anti-p-Ser⁴⁷³-Akt, anti-Akt, anti-p-Ser³⁸⁶-Foxo1, anti-Foxo1 and anti-p-Ser³⁸⁶-CREB were purchased from Cell Signaling Technology (Danvers, MA). Mouse anti-β-actin Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 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).

**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 one-way ANOVA followed by a Student-Newman-Keuls post hoc test. *P ≤ 0.05 was taken as the criterion for significance.

**RESULTS**

Effect of sympathetic denervation on muscle NE. SDEN from 2 to 7 days induced a significant reduction (~85%) in NE content of both EDL and soleus muscles (Fig. 1, A and B, respectively). As previously shown (35), plasma levels of catecholamines were not altered in this experimental model of sympathectomy (Fig. 1, C and D). The surgery did not affect body weight gain or skeletal muscle weight at any of the experimental periods (data not shown). Averaged values for EDL from CON and SDEN were, respectively (in mg/100 g): at day 3 (40.8 ± 1.3 and 42.4 ± 0.8); at day 7 (45.0 ± 1.0 and
45.3 ± 2.3); and at day 15 (46.8 ± 1.1 and 46.8 ± 0.8). For soleus, averaged mass values from CON and SDEN were, respectively (in mg/100 g body wt): at day 3 (49.7 ± 0.7 and 49.8 ± 1.0); at day 7 (45.5 ± 2.2 and 46.6 ± 2.1); and at day 15 (48.1 ± 1.2 and 50.1 ± 0.8).

Effect of sympathetic denervation on rates of protein degradation. As shown in Fig. 2A, SDEN induced a 10–18% decrease in overall proteolysis at 2, 3, and 4 days, but not at 7 days, in both EDL and soleus muscles. In parallel, an ～25% decrease in the UPS activity was observed in denervated EDL muscle after 2 and 3 days of surgery (Fig. 2B). However, after 4 and 7 days, UPS activity in EDL reverted to control levels. In soleus muscle, the UPS activity was significantly decreased by ～20% at the three first experimental intervals (2, 3, and 4 days), but not at 7th day (Fig. 2B). Because the lysosomal pathway is one of the major protein degradation systems in muscle cells, we investigated the effect of SDEN on lysosomal proteolytic activity. In contrast to UPS, the proteolytic activity of lysosomal pathway in both soleus and EDL muscles was not altered by SDEN in any time studied (Fig. 2C). These findings suggest that noradrenergic innervation modulates the UPS activity in rat skeletal muscles under control resting conditions.

Effect of sympathetic denervation on cAMP signaling. Because the majority of the intracellular effects of catecholamines in skeletal muscle are mediated by cAMP, we investigated the effects of SDEN on different components of the cAMP/PKA signaling in EDL and soleus muscles of rats. As shown in Fig. 3A, SDEN did not alter the EDL basal active pool of G proteins, determined in the absence of isoproterenol. Also, the total amount of G protein associated to EDL membranes was not affected by denervation (data not shown). The addition of isoproterenol to the incubation medium, at different concentrations, was able to activate G proteins by up to 137% in EDL membranes of both CON and 3-day SDEN rats (Fig. 3A). However, the cAMP levels was 191% higher in muscles of SDEN compared with those of CON rats (Fig. 3C), which was consistent with a 112% increase in AC activity induced by SDEN (Fig. 3B). Furthermore, SDEN increased by 32% the Ser phosphorylation levels of CREB, a well-known target of PKA (Figs. 3, D and E). Figure 3D also shows that these effects were not associated with phosphorylation of Akt and its downstream target Foxo1, an effect that could prevent Foxo translocation to the nucleus and UPS activation. The 3-day SDEN-induced increase in AC activity and cAMP levels were reverted to basal values 7 days after surgery (Fig. 3, B and C). In soleus, 3-day SDEN also increased the AC activity (in pmol cAMP/mg protein) in 334% (1,060.2 ± 77.1 vs 244.0 ± 14.7 in CON rats), an effect that was abolished 7 days after surgery (366.9 ± 47.3 vs 341.2 ± 18.7 in CON rats). Taken together, these data suggest that SDEN leads to a transitory adrenergic supersensitivity for cAMP, secondary to the depletion of muscle NE content.

In vitro effect of NE on UPS and cAMP/PKA and Akt/Foxo signaling in isolated muscle. NE, at the concentration of 10^{-4} M, was added to the incubation medium of EDL muscle isolated from normal rats. This concentration was used because our laboratory has previously shown that it causes a statistically significant reduction in the overall proteolysis in EDL muscle (37). As shown in Fig. 4A, NE reduced the UPS activity by 21%. Additionally, NE reduced basal atrogin-1 (64%) and MuRF1 (43%) mRNA levels (Fig. 4B). Similar effects were observed in soleus muscle (data not shown). To verify whether the observed effects of NE were mediated via activation of cAMP/PKA signaling pathway, muscle cAMP levels and the phosphorylation levels of CREB were assessed. NE increased muscle cAMP levels by 115% (Fig. 4C) and the phosphorylation levels of CREB (2.7-fold) in EDL muscle (Fig. 4, D and E), indicating increased PKA activity. As Akt/Foxo signaling

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Fig. 1. Effect of surgical sympathetic denervation (SDEN) on norepinephrine (NE) content in rat extensor digitorum longus (EDL; A) and soleus muscles (B) and on plasma catecholamines (C and D) after 3 and 7 days. Values are means ± SE of 7 muscles. EPI, epinephrine. *P ≤ 0.05 vs. control (CON).

Fig. 2. Effect of SDEN on overall proteolysis (A), ubiquitin-proteasome system (UPS) activity (B), and lysosomal activity (C) in rat EDL and soleus muscles after 2, 3, 4, and 7 days (%CON values). Values are means ± SE of 5–7 muscles. *P ≤ 0.05 vs. CON.
DISCUSSION

The present data show that the SDEN induced by surgical excision at L2 and L3 levels of the sympathetic chain, which innervates rat hindlimb skeletal muscles (6) transiently, decreased rates of total protein degradation in both fast- and slow-twitch skeletal muscles. The lysosomal proteolytic system did not participate in any of the changes observed, as the activity of this system remained unchanged in soleus and EDL throughout the experimental period (Fig. 2C). The present data also show that the decrease in the rate of overall proteolysis (in both soleus and EDL muscle, during the first 3 days) was accompanied by a parallel decrease in the activity of UPS, occurred without any change in plasma levels of catecholamines (Fig. 1, C and D), and it was probably a direct consequence of the depletion of muscle NE. This view is consistent with a similar inhibition of the UPS, but not lysosomal activity, that was observed after a short-term adrenergic blockade induced by guanethidine (36), a model of chemical sympathectomy in which both plasma catecholamines and muscle NE are reduced. This antiproteolytic response was interpreted as an adaptive mechanism of the muscle to limit protein breakdown. The present study reinforces this hypothesis and shows, for the first time, that the inhibition of overall protein breakdown and UPS was clearly associated with an increase in the activity of AC and, as consequence, in the levels of cAMP, which in turn induced CREB activation, possibly via PKA (Figs. 2 and 3). It is interesting to note that the biphasic pattern of proteolysis observed in muscles from denervated rats, with an initial decrease during the first 3 days, followed by a return to the CON values after 7 days (Fig. 2), has also been observed for cAMP and the activity of AC in both EDL and soleus muscles (Fig. 3, B and C). Taken together, these data strongly suggest that SDEN induces skeletal muscle adrenergic supersensitivity for cAMP, leading to the suppression of UPS activity under normal conditions, when circulating catecholamines can be regulated in EDL muscle by EPI in vitro (7), we investigated the phosphorylation levels of Akt and Foxo1 in isolated EDL muscles from normal rats in the presence of NE. Interestingly, NE increased the Ser phosphorylation of Foxo1 (~6-fold), but did not alter the Ser phosphorylation of Akt (Fig. 4, D and E). Similar results were obtained in serum-deprived 

In vitro effect of 6-BNZ-cAMP on UPS and Akt/Foxo signaling in isolated muscles. To investigate further the role of PKA in the inhibitory effect of NE on UPS, EDL muscles were incubated in the presence of 6-BNZ-cAMP (at the concentration of 500 µM), a specific PKA activator. As shown in Fig. 6, A and B, 6-BNZ-cAMP significantly reduced the activity of UPS (45%) and basal MuRF1 mRNA levels (40%), but did not alter the atrogin-1 mRNA. Moreover, 6-BNZ-cAMP increased the Ser phosphorylation levels of CREB (64%) after 30 min, and the Ser phosphorylation levels of Foxo1 (~3-fold) after 120 min of incubation (Fig. 6, C and D). Interestingly, the Ser phosphorylation levels of Akt were not altered by 6-BNZ-cAMP at any time interval (Fig. 6, C and D). Collectively, these data suggest that the suppressive effect of PKA on UPS activity and atrophy-related Ub-ligases expression is mediated by the inhibition of Foxo1 and independently of Akt.
are at normal levels. These data confirm previous investigations demonstrating that chemical and/or surgical sympathectomy induce the development of adaptive supersensitivity in a number of tissues, including cerebral cortex and cardiac muscle (43, 45). Indeed, the mechanisms underlying sympathectomy-induced adrenergic supersensitivity may include multiple steps of action. The depletion of NE in different tissues leads to an increased number of β-adrenergic receptors (45) and/or an alteration in the intracellular transduction processes responsible for the action of an agonist following receptor occupation by that agent (43). The present finding that the increases in AC activation were not correlated with any detectable change in the levels or activity of G protein (Fig. 3A) suggests that the cellular mechanism that underlies the development of adaptive supersensitivity in the rat skeletal muscle may involve a modification of AC. The subsequent decrease to basal levels in the AC activity observed after 7 days of denervation can be interpreted as a restoration of muscle capacity to limit the rate of muscle protein degradation. The mechanism of the temporary nature of the increased adrenergic sensitivity in denervated skeletal muscles cannot be explained on the basis of the present data.

Although our in vivo data suggest that the adrenergic supersensitivity induced by SDEN inhibited the UPS activity, it could be argued that this response was a consequence of the lack of NE itself, which would imply that this neurotransmitter acts as a potential activator of UPS system. Alternatively, decreased muscle NE content by lumbar surgical excision could act indirectly by changing insulin responsiveness (24), or regional blood flow. In fact, a significant increase in muscle blood flow following chronic lumbar sympathectomy has been reported in dogs (28). However, these possibilities seem unlikely, since previous studies from our laboratory have convincingly shown that both catecholamines, EPI and NE, exert a direct inhibitory control of proteolysis in rat skeletal muscle, with the participation of a CAMP-dependent pathway (37, 38).

In agreement, the present data show that NE in vitro increased UPS activity (A), mRNA expression of atrogin-1 and muscle RING finger 1 (MuRF1; B), cAMP content (C), and phosphorylation levels of CREB (p-Ser133), Akt (p-Ser473), and Foxo1 (p-Ser256) (D and E) in EDL muscles from normal rats. Muscles were isolated and incubated for 120 min in the presence, or absence, of 10−4 M NE. Gene expression levels were analyzed by using cyclophilin B and β-actin as endogenous control. Phosphorylated proteins were normalized to respective total proteins, and CREB (p-Ser133) was normalized to β-actin. Membranes were stripped and reprobed for β-actin as a loading control. Values are means ± SE of 5–7 muscles. *P ≤ 0.05 vs. CON.

**Fig. 4.** In vitro effect of NE (10−4 M) on UPS activity (A), mRNA expression of atrogin-1 and muscle RING finger 1 (MuRF1; B), cAMP content (C), and phosphorylation levels of CREB (p-Ser133), Akt (p-Ser473), and Foxo1 (p-Ser256) (D and E) in EDL muscles from normal rats. Muscles were isolated and incubated for 120 min in the presence, or absence, of 10−4 M NE. Gene expression levels were analyzed by using cyclophilin B and β-actin as endogenous control. Phosphorylated proteins were normalized to respective total proteins, and CREB (p-Ser133) was normalized to β-actin. Membranes were stripped and reprobed for β-actin as a loading control. Values are means ± SE of 5–7 muscles. *P ≤ 0.05 vs. CON.

**Fig. 5.** In vitro effect of AC activator [forskolin (FSK); 10 μM] on phosphorylation levels of CREB (p-Ser133), Akt (p-Ser473), and Foxo1 (p-Ser256) in 6-h serum-deprived C2C12 myoblasts. Muscle cells were incubated for 15 min in the presence, or absence, of FSK and insulin (INS). Phosphorylated proteins were normalized to β-actin.
activity of UPS (Fig. 4A) and the gene expression of the atrogin-1 and MuRF1 (Fig. 4B). These adrenergic effects are probably mediated by β2-adrenoceptors, given that clenbuterol, and drugs that induce an increase in the muscle cAMP levels, such as the selective and nonselective cAMP-PDE inhibitors, decreased the basal activity of UPS, levels of Ub-protein conjugates, and expression of atrogin-1 mRNA (20, 21, 30). Accordingly, previous studies have shown that sympathetic....

Fig. 6. In vitro effect of PKA activator [6-BNZ-cAMP (6-BNZ); 500 μM] on UPS activity (A), mRNA expression of atrogin-1 and MuRF1 (B), and phosphorylation levels of CREB (p-Ser133), Akt (p-Ser473), and Foxo1 (p-Ser256) (C and D) in EDL muscles from normal rats. Muscles were isolated and incubated for 120 (or 180) min in the presence, or absence, of 6-BNZ. Gene expression levels were analyzed by using cyclophilin B gene as endogenous control. Phosphorylated proteins were normalized to respective total proteins, and CREB (p-Ser133) was normalized to β-actin. Membranes were stripped and re-probed for β-actin as a loading control. Values are means ± SE of 5–7 muscles. *P ≤ 0.05 vs. CON.

Fig. 7. A proposed scheme of the mechanisms involved in the inhibition of UPS activity by muscle sympathetic innervation in rat. IML, intermediolateral nucleus; PKA, protein kinase A.
Akt phosphorylation (Fig. 6, phosphorylation levels of CREB and Foxo1, without altering A) and clearly show that the direct activation of PKA in vitro induced muscle from normal rats were incubated in the presence of effects on UPS through a PKA-dependent signaling, EDL 5). To further support the notion that NE exerts inhibitory diate the inhibitory actions of NE onUb-ligases in vitro (Fig. AC activator), suggesting that cAMP and probably PKA me-

Furthermore, we investigated whether or not the in vitro effects of NE on UPS could involve the activation of the canonical cAMP/PKA signaling pathway. It is well-established that the expression of Ub-ligases and the consequent activation of the proteasome are inhibited by the activation of phosphatidylinositol 3-kinase/Akt signaling pathway through the phosphorylation and nuclear exclusion of Foxo transcription factors (41, 44). Because the production of cAMP onEPI stimulation in isolated muscles may activate Akt/Foxo signaling and inhibit proteolysis (7, 12), we first postulated that NE would affect this signaling pathway. Interestingly, the data show that NE in vitro increased the Ser phosphorylation levels of CREB and Foxo1, but did not alter the Ser phosphorylation of Akt (Fig. 4, D and E). Similar effects were obtained in serum-deprived C2C12 muscle cells incubated with FSK (a specific AC activator), suggesting that cAMP and probably PKA mediate the inhibitory actions of NE on Ub-ligases in vitro (Fig. 5). To further support the notion that NE exerts inhibitory effects on UPS through a PKA-dependent signaling, EDL muscle from normal rats were incubated in the presence of 6-BNZ-cAMP, a specific PKA activator. The present data clearly show that the direct activation of PKA in vitro induced by 6-BNZ-cAMP decreased the UPS activity (Fig. 6A) and MuRF1 mRNA (Fig. 6B) and in parallel increased the Ser phosphorylation levels of CREB and Foxo1, without altering Akt phosphorylation (Fig. 6, C and D). In contrast to the effect of NE in isolated muscle, the 6-BNZ-cAMP did not alter the expression of atrogin-1 mRNA (Fig. 6B). On the other hand, 6-BNZ-cAMP reduced the mRNA levels of atrogin-1 in C2C12 muscle cells under nutritional stress (data not shown). In agreement with these findings, we have recently found that 6-BNZ-cAMP in vitro significantly suppressed the expression of MuRF1 mRNA in normal and atrophying soleus muscles, but had no effect on atrogin-1 mRNA (21). The available data do not allow any conclusion about the reason for these differences between MuRF1 and atrogin-1, but the lack of responsiveness of the atrogin-1 mRNA to the 6-BNZ-cAMP could be explained, at least in part, by the fact that the stimulatory effect of the 6-BNZ-cAMP on the phosphorylation levels of Foxo1 and CREB was less potent compared with NE (Figs. 4 and 6).

To our knowledge, this constitutes the first report that Foxo1 may be a direct substrate for PKA in skeletal muscle cells, a finding that strongly supports similar conclusions in vascular endothelial cells (29). Further experiments are needed to certify if PKA may directly phosphorylate and inhibit transcriptional activity of Foxo1 under physiological conditions in intact skeletal muscles.

In summary, the present data show that the surgical SDEN induces the phenomenon of adrenergic supersensitivity for cAMP that is associated with suppression of the basal activity of UPS in EDL and soleus muscles. Furthermore, as shown in Fig. 7, the inhibitory effect of NE on UPS observed in vitro may be mediated through cAMP/PKA activation, leading to the inhibition of Foxo1 transcription factor by phosphorylation and a consequent suppression of atrophy-related Ub-ligases. Based on these data and assuming that the NE released by the sympathetic innervation acts on skeletal muscle through the same mechanisms, the antiproteolytic effects of sympathetic denervation here observed might be an important adaptive effect for preservation of proteins. Finally, these results represent further evidence on the antiproteolytic role of SNS in skeletal muscle and suggest that modulating the cAMP/PKA signaling may serve as a potential target for muscle-wasting conditions in populations characterized by sympathetic dysfunction, such as spinal cord-injured adults.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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