An Acad Bras Cienc (2023) 95(Suppl. 2): e20220877 DOI 10.1590/0001-3765202320220877
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

CELLULAR AND MOLECULAR BIOLOGY

Resveratrol directly suppresses proteolysis possibly via PKA/CREB signaling in denervated rat skeletal muscle

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Abstract: Although there are reports that polyphenol resveratrol (Rsv) may cause muscle hypertrophy in basal conditions and attenuate muscle wasting in catabolic situations, its mechanism of action is still unclear. Our study evaluated the *ex vivo* effects of Rsv on protein metabolism and intracellular signaling in innervated (sham-operated; Sham) and 3-day sciatic denervated (Den) rat skeletal muscles. Rsv (10^{-4} M) reduced total proteolysis (40%) in sham muscles. Den increased total proteolysis (~40%) in muscle, which was accompanied by an increase in the activities of ubiquitin-proteasome (~3-fold) and lysosomal (100%) proteolytic systems. Rsv reduced total proteolysis (59%) in Den muscles by inhibiting the hyperactivation of ubiquitin-proteasome (50%) and lysosomal (~70%) systems. Neither Rsv nor Den altered calcium-dependent proteolysis in muscles. Mechanistically, Rsv stimulated PKA/CREB signaling in Den muscles, and PKA blockage by H89 (50μM) abolished the antiproteolytic action of the polyphenol. Rsv reduced FoxO4 phosphorylation (~60%) in both Sham and Den muscles and Akt phosphorylation (36%) in Den muscles. Rsv also caused a homeostatic effect in Den muscles by returning their protein synthesis rates to levels similar to Sham muscles. These data indicate that Rsv directly inhibits the proteolytic activity of lysosomal and ubiquitin-proteasome systems, mainly in Den muscles through, at least in part, the activation of PKA/CREB signaling.

Key words: Intracellular Signaling Pathways, Muscle atrophy and hypertrophy, Sciatic nerve resection, Polyphenol, Protein Metabolism.

INTRODUCTION

Skeletal muscle is the largest tissue in the body, accounts for 40-50% of body mass and plays a central role in whole-body metabolism, locomotion, thermoregulation, and other biological functions. Maintaining skeletal muscle mass and function is essential for human healthy. Muscle mass is regulated by dynamic protein turnover, which involves protein synthesis and breakdown. The imbalance between catabolic and anabolic processes may result in muscle hypertrophy (anabolic > catabolic) or atrophy (anabolic < catabolic), the latter being a common symptom of chronic diseases such as diabetes (Perry et al. 2016) and motor denervation (Den) (Furuno et al. 1990). Therefore, identifying therapeutic targets capable of inhibiting proteolysis is necessary for treating muscle loss and improve the disease prognosis.

Motor innervation is crucial for muscle physiology, including development, differentiation, metabolism, trophic status,

and force production (Das et al. 2020). On the other hand, Den results in muscle inactivity and, consequently, atrophy during the early stages (Tang et al. 1984, Adhihetty et al. 2007). Den-induced muscle loss may be due to an increase in the rate of proteolysis exceeding the rate of protein synthesis (Furuno et al. 1990). Autophagy/lysosomal and ubiquitin-proteasome (UPS) systems have been considered the critical regulators for the bulk of muscle proteolysis (Sartori et al. 2021). However, these proteolytic systems cannot degrade myofibrils attached to the sarcomere. It has been suggested that calpains, the proteases of calcium-dependent proteolytic system, may initially cleave proteins leading to sarcomere disassembly and myofibrils degradation by UPS (Tidball & Spencer 2002, Kachaeva & Shenkman 2012). The blockade of calpain via the PD150606, a calpain inhibitor, attenuates UPS activity and prevents unloading-induced skeletal muscle atrophy in rats (Shenkman et al. 2015).

In addition to calpains, the transcription of a common set of atrophy-related genes termed atrogenes is up- and down-regulated during muscle atrophy in different catabolic states (Sacheck et al. 2007, Lecker et al. 2004). Muscle RING finger 1 (*MuRF1* or *Trim63*) and Atrogin-1 (also known as *MAFbx* or *Fbxo32*) are two muscle-specific E3 ligases of UPS. These enzymes are up-regulated in several animal models of atrophy, such as Den and fasting (Sacheck et al. 2007). Autophagy-related genes, such as microtubule-associated protein 1 light chain 3 beta (*Map1lc3b*) and GABA type A receptor-associated protein (*Gabarap*), are also up-regulated in muscles from Den or fasted mice (Mammucari et al. 2007, Sartori et al. 2021). Most of these atrogenes are under transcriptional control of Forkhead box O (FoxO) factors, which play a critical role in muscle atrophy (Mammucari et al. 2007). FoxO, in turn, may be regulated by several intracellular pathways, including insulin/insulin-like growth factor-1 (IGF-1)/phosphatidylinositol 3-kinase (PI3K)/Akt signaling (Sandri et al. 2004, Milan et al. 2015, Mammucari et al. 2007). Both insulin and IGF-1 activate Akt that phosphorylates FoxO proteins exporting them from the nucleus to the cytoplasm and reducing their activity (Brunet et al. 1999, Sandri 2008) .

Resveratrol (Rsv; 3,5,4'-trihidroxistilbene), a natural antioxidant found in the skins of red grapes as well as other plants such as peanuts, has shown to induce *in vivo* both muscle hypertrophy in rest (Woodman et al. 2021) and exercise trained (Alway et al. 2017) conditions and muscle antiatrophic effect in several experimental catabolic situations, such as sarcopenic obesity (Huang et al. 2019) and Den (Asami et al. 2018). However, the mechanism underlying Rsv action in muscle protein metabolism and trophic status remains unclear. Moreover, whether Rsv acts directly or indirectly in skeletal muscle promoting its protein anabolic and anticatabolic effects has been poorly investigated. Rsv is a competitive and non-selective inhibitor of phosphodiesterases, a superfamily of enzymes responsible for the intracellular degradation of cyclic adenosine monophosphate (cAMP), thereby being able to elevate cAMP level as soon as 10 min after the incubation with Rsv (Park et al. 2012). Upon binding of cAMP to regulatory subunits of protein kinase A (PKA-R), their catalytic subunits (PKAcat) are activated and detach from regulatory subunits phosphorylating several proteins, including CREB transcription factor, the bona fide target of PKA (Silveira et al. 2020). Recently, it has been shown that the genetic gain-of-fucntion of PKA mitigates FoxO activity and atrogenes overexpression, and its genetic loss-of-function causes muscle loss in basal condition (Silveira et al. 2020).

Based on these findings, we hypothesized that Rsv could directly stimulate protein synthesis and inhibit protein degradation in innervated and denervated muscles via activation of the cAMP/PKA pathway. Therefore, this study aimed to evaluate the *ex vivo* effect of Rsv on protein metabolism and intracellular signaling pathways in muscles innervated (sham-operated rats; sham) and undergoing atrophy due to sciatic nerve resection (Den). Our data indicate that Rsv suppresses total proteolysis in both innervated and Den muscles, especially the lysosomal and UPS proteolytic systems in the latter condition. The pharmacological loss-of-function experiments suggest that these effects of Rsv are mediated by the activation of PKA/CREB signaling. Rsv also caused a homeostatic effect in Den muscles by returning their protein synthesis rates to levels similar to Sham muscles.

MATERIALS AND METHODS

Animals and sciatic denervation model

Wistar male rats (±80 g body mass; 4 weeks old; *n* = 4-6/group) were used in all experiments. 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 for at least 2 days before the beginning of the experiments, which were performed at 8:00 A.M. All animal experiments were conducted following with the guidelines established by the Animal Ethics Committee of Federal University of Pernambuco (CEUA/UFPE), which also approved the protocols (Approval No. 23076.01234/2012-79), and are in accordance with ethical principles in animal research.

The animals were anesthetized with ketamine hydrochloride solution (115 mg / Kg, i.p.) and xylazine (10 mg / Kg, i.p.) and underwent bilateral motor denervation (Den) caused by a surgical section of the sciatic nerve, with the removal of ~2 mm of nerve. 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 (Gonçalves et al. 2012). Sham (innervated) group was subjected to surgical stress, which included nerve visualization, but not its transection.

Isolated skeletal muscles and resveratrol incubation

Three days after Den (or sham operation), animals were euthanized by cervical dislocation, and *extensor digitorum longus* (EDL) muscles were carefully removed, avoiding damage to the muscles. EDL muscles were chosen due to their high proportion of fast glycolytic fibers that are more sensitive to the protein anabolic effects of cAMP enhancers such as rolipram (Lira et al. 2011) and formoterol (Gonçalves et al. 2019). The EDL muscle was rapidly macrodissected, weighed (innervated muscles: ~55g; denervated muscles: ~40g), and maintained at approximately its resting length by pinning their tendons on inert plastic supports. Tissues were incubated at 37°C in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 5 mM glucose equilibrated with 95% oxygen and 5% carbon dioxide. After 1h of preincubation in buffer medium, muscle from one limb was incubated for 2 h in the presence of resveratrol (Rsv, 10^{-5} or 10^{-4} M), while muscle from the contralateral limb was incubated with vehicle dimethylsulfoxide (DMSO, 1 %).

Determination of total proteolysis and the activity of proteolytic systems

Total proteolysis and the activity of proteolytic systems (ubiquitin-proteasome (UPS), lysosomal, and calcium-dependent) were measured using a method as described previously (Gonçalves et al. 2019). Briefly, proteolysis was measured by following the tyrosine released into the

medium in the presence of cycloheximide (0.5 mM), preventing protein synthesis and reincorporation of tyrosine back into proteins. After 1 h preincubation, muscles were incubated for 2 h in a fresh medium with an identical composition. For measurement of *UPS* activity, muscles from one limb were incubated with a calcium-free medium containing 25 µM E64, 50 µM leupeptin, 10 mM methylamine, 1 U/ml insulin, 170 µM leucine, 100 µM isoleucine, 200 µM valine to prevent activation of calcium-dependent and lysosomal systems. 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 presence of the inhibitors of lysosomal proteolysis, i.e., insulin, leucine, isoleucine, valine, and methylamine. Contralateral muscles were incubated in a "normal" buffer medium without these inhibitors. For measurement of *calcium-dependent* proteolytic activity, muscles from one limb were incubated in the presence of calcium and inhibitors of the lysosomal system. Contralateral muscles were incubated in a calcium-free medium containing lysosomal inhibitors and cysteine-protease inhibitors (E64 and leupeptin). UPS, lysosomal and calcium-dependent proteolytic activities were calculated from tyrosine release differences between the left and right muscles. Tyrosine release was assayed using the fluorometric method (Waalkes & Udenfriend 1957).

Determination of the rate of protein synthesis

The protein synthesis rate was measured using a method described previously (Gonçalves et al. 2012). Briefly, EDL muscles were incubated in a buffer containing all amino acids at concentrations similar to those of rat plasma. After a 1 h equilibration period, L-[U-^{14}C]tyrosine (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 tyrosine (intracellular tyrosine pool) in each muscle was estimated by measuring the radioactivity and the concentration of tyrosine in this pool, which was determined by the method of (Waalkes & Udenfriend 1957). After that, the rate of protein synthesis was calculated using the specific activity of the intracellular pool of tyrosine in each muscle.

Analysis of gene expression by real-time qPCR

Real-time qPCR was used to analyze the expression levels of the atrogenes *Fbxo32*, *Trim63,* and *Map1lc3b*. The analysis of qPCR was performed at the Laboratory of Metabolism Control from Ribeirão Preto Medical School (University of São Paulo). After the incubation procedure described above, EDL muscle was immediately frozen in liquid nitrogen and stored at -80°C for less than one month. Total RNA was extracted from muscle using TRIzol (50 mg of muscle was added to 0,5 ml of TRIzol, Invitrogen®). Samples were homogenized in tubes using a TissueLyser II (Qiagen®) with 5 mm stainless steel beads for 2×1 min cycles at 30 Hz, resting on ice in between. Homogenates were cleared by centrifugation at 10,000 x g for 5 min at 4 °C. RNA extraction was performed according to TRIzol manufacturer's instructions (Invitrogen®). RNA was eluted in 50 µL of RNase-free water and stored at −80 °C.

RNA samples were treated with DNase I, RNase-free (Thermo Fisher Scientific®), to remove genomic DNA contamination. RNA samples were quantitated using NanoDrop One spectrophotometer (Thermo Fisher Scientific®), following the manufacturer's instructions. The same device was used to assess the purity of RNA by measuring 260/280 and 260/230 ratios of absorbance values. Samples presenting a

260/280 ratio of ~2 and 260/230 ratio of 2 to 2.2 were accepted as "pure" for RNA.

According to the manufactures' protocols, one microgram of RNA was reverse transcribed into cDNA using 0,5 µL of SuperScript IV First-Strand Synthesis System (Invitrogen®). cDNA was diluted 25-fold with nuclease-free water. For qPCR, the total volume per reaction was 10 µL containing 5 µL of cDNA (2 ng/µL), 4.8 µL of PowerUp SYBR Green Master Mix (Thermo Fisher®), and 0.2 µL of primers (forward and reverse mixture; 50 µmol/L stock). qPCR run on Applied Biosystems™ 7500 Real-Time PCR System, using the recommended cycling conditions as follows: a pre-incubation of 2 min at 50 °C and 10 min at 95 °C, followed by a two-step amplification program of 40 cycles set at 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing + extension) and, finally, a dissociation stage set at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The last stage was performed to evaluate the quality of qPCR reactions regarding of nonspecific amplification and primer-dimer formation in a dissociation curve for each gene. The amplification specificity for each primer was confirmed by observing the single melt curve peak after the completion of qPCR.

Primers used were *Fbxo32* (forward 5'-CTT TCA ACA GAC TGG ACT TCT CGA-3' and reverse 5'-CAG CTC CAA CAG CCT TAC TAC GT-3'), *Trim63* (forward 5'-TCG ACA TCT ACA AGC AGG AA-3' and reverse 5'-CTG TCC TTG GAA GAT GCT TT-3'), *Map1lc3b* (forward 5'-TTT GTA AGG GCG GTT CTG AC-3' and reverse 5'-CAG GTA GCA GGA AGC AGA GG-3') and *Rpl39* (forward 5'-TCC TGG CAA AGA AAC AAA AGC-3' and reverse 5'-TAG ACC CAG CTT CGT TCT CCT-3'). Primer sequences were designed utilizing Primer3Plus (https://www.primer3plus.com/) in conjunction with OligoAnalyzer 3.1 (https://eu.idtdna.com/site) and cross-referenced using the Basic Local Alignment Search Tool program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A six-point relative standard curve was prepared for each gene by using five-fold serial dilutions of pooled cDNA samples in duplicate. No threshold cycle quantification value for the no template control was detected. The slope values and amplification efficiencies estimated from the standard curve, respectively, were as follows: *Fbxo32* (-3,3 and 99%), *Trim63* (-3,1 and 112%), *Map1lc3b* (-3,8 and 83%) and *Rpl39* (-3,1 and 108%).

The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak 2008). All reactions were carried out in four to five biological replicates and two technical replicates. Data from the target genes were normalized by the expression of *Rpl39*, which was used as a reference gene because it has been shown to be the most stable gene in Den muscles compared with other reference genes, such as *Gapdh*, *Actb* and, *Ppia* (unpublished data). Moreover, *Rpl39* has been previously used as a reference gene in other studies in skeletal muscle (Gonçalves et al. 2019, de O. Coelho et al. 2019).

Analysis of protein expression by Western Blot

After incubation, EDL muscle was quickly frozen in liquid nitrogen and stored at -80 °C. These muscles were collected and homogenized in buffer RIPA (Tris-HCl 50 mM, pH 7.4), containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 % sodium deoxycholate, 1% SDS, 10mM sodium pyrophosphate, 100mM sodium fluoride, 10mM sodium orthovanadate, 5g/ml aprotinin, 1mg/ml leupeptin, 1mM phenylmethylsulfonyl fluoride (PMSF), 50 µM MG132. The homogenate was centrifuged at 21,000 g at 4 °C. The supernatant was collected, and the amount of protein was determined by the method of (Lowry et al. 1951) using BSA (bovine serum albumin) as standard. An equal volume of sample buffer [20 % glycerol, 125 mM Tris-HCl, 4 % SDS, 100 mM dithiothreitol

(DTT), 0.02 % bromophenol blue, pH 6.8] was added to the supernatant, and the mixture was boiled at 70 °C for 10 min. 50 µg of total proteins were separated by SDS-PAGE acrylamide gel at 10-12 %, and after transferred to a nitrocellulose membrane, and blotted with anti-phospho (p)-Ser^{473} Akt (1:750, #9271, Cell Signaling Technology, Massachusetts, USA), anti-p-Ser$^{256/193}$ FoxO1/4 (1:750, #9461, Cell Signaling), anti-p-Ser^{133} CREB (1:750, #9198, Cell Signaling), anti-p-Thr^{172} AMPK (1:750, #2531, Cell Signaling), anti-p-Thr^{389} S6K1 (1:750, #9234, Cell Signaling) and anti-β-actin (1:2000; sc-81178; Santa Cruz Biotech, Texas, USA). Primary antibodies (Ab) were detected using peroxidase-conjugated secondary Ab (1:2000 for p-Ser$^{256/193}$-FoxO1/4, p- Ser^{473}-Akt, p-Ser^{133} CREB, anti-p-Thr^{172} AMPK, anti-p-Thr^{389} S6K1 and 1:5000 for other primary antibodies) and visualized using ECL method using a detection system ChemiDoc MP (Bio-Rad) and software ImageLab (version 5.2.1, Bio-Rad). Band intensities were quantified using ImageJ2 (version 1.53c, National Institutes of Health, USA).

Statistical analysis

The distribution and variance homogeneity were tested using Shapiro-Wilk test. Data were expressed as mean ± S.E.M. The one-way analysis of variance (ANOVA) followed by the Bonferroni test was employed to analyze total proteolysis in response to different Rsv concentrations. Two-way analysis of variance (ANOVA) followed by the Bonferroni test was employed to analyze total proteolysis (Rsv 10^{-4} M), proteolytic systems, atrogenes mRNA expression and protein synthesis. Paired test-t was employed to evaluate the phosphorylation levels of proteins because 1) the muscle from one limb was incubated with Rsv while the muscle from the contralateral limb (control muscle) was incubated with vehicle DMSO ("paired condition") in both Sham and Den conditions, and 2) samples from these conditions (i.e., Sham and Den) ran in different gels. $P < 0.05$ was considered statistically significant. GraphPad Prism 5.0 (San Diego, CA, USA) was used to perform the statical analysis and create the graphs.

RESULTS

Rsv reduces protein degradation in Sham and Den muscles

Sham (innervated) EDL muscles from rats were incubated with either 10^{-5} or 10^{-4} M. Figure 1a shows that total proteolysis was reduced by 25 % in muscles incubated with 10^{-4} M of Rsv but not with 10^{-5} M. For this reason, the following experiments were performed on Sham and 3-day Den EDL muscles incubated with the highest concentration of Rsv (i.e., 10^{-4} M) for evaluation of total proteolysis and activity of UPS, lysosomal and calcium-dependent proteolytic systems. Rsv reduced total proteolysis (40 %) in Sham muscles (Fig. 1b) but did not cause a significant reduction in the activity of UPS and lysosomal systems (Fig. 1c). As expected, Den up-regulated total proteolysis (~40 %, Fig. 1b), which was accompanied by an elevation in the activities of UPS (~3-fold) and lysosomal (100 %) systems (Fig. 1c). In contrast, Rsv induced a significant reduction in total proteolysis (~60 %, Fig. 1b) that were paralleled by an inhibition of UPS (50 %) and lysosomal (~70 %) proteolytic activities in Den muscles (Fig. 1c). Neither Rsv nor Den altered calcium-dependent proteolysis in muscles (Fig. 1c). To further investigate the expression of some essential components of the proteolytic systems inhibited by Rsv, the expression of UPS-related genes *Fbxo32* (Atrogin-1) *and Trim63* (MuRF1) and autophagy-related gene *Map1lc3b* (LC3b) was determined. Rsv tended to down-regulate (~50%; $P > 0.05$) the expression of *Fbxo32, Trim63, and Map1lc3b* in Sham and *Trim63 and Map1lc3b* in Den muscles (Fig. 1d). Den *per se* did not change

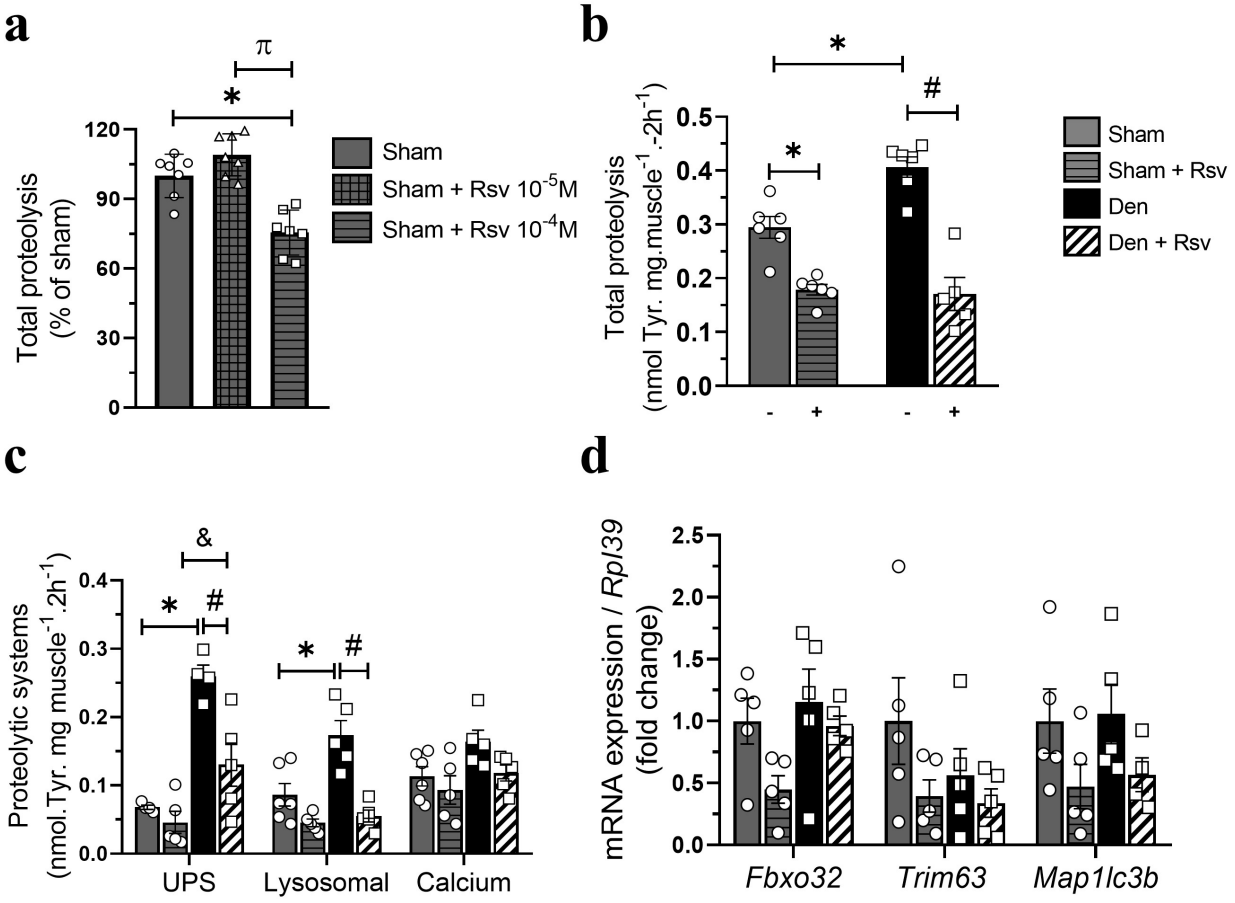

Figure 1. Effects of different concentrations (10^{-5} and 10^{-4} M) of Resveratrol (Rsv) on total proteolysis (a) in EDL muscle of Sham rats. Effects of Rsv (10^{-4} M) on total proteolysis (b), proteolytic activity of ubiquitin proteasome (UPS), lysosomal and calcium-dependent systems (c) and mRNA expression of *Fbxo32*, *Trim63* and *Map1lc3b* (d) in EDL muscle of Sham and Den rats. Values are presented as mean ± SEM of 5-6 rats. Rsv: Resveratrol; Sham: sham-operated rats; Den: denervated rats. *Significantly different ($P < 0.05$) from Sham. $^{\pi}$Significantly different ($P < 0.05$) from Sham + Rsv 10^{-5}M. #Significantly different ($P < 0.05$) from Den. &Significantly different ($P < 0.05$) from Sham + Rsv.

the expression of *Fbxo32*, *Trim63* and *Map1lc3b*. These results demonstrate that Rsv mitigates total proteolysis in both Sham and Den muscles, and this effect in atrophic muscles was due to the inhibition of the hyperactivation of UPS and lysosomal proteolytic systems.

Rsv reduces phosphorylation levels of Akt/FoxO4 in Den muscles

To further explore the signaling pathways involved in the Rsv-induced antiproteolytic effects, Akt/FoxO signaling was analyzed in Sham and Den muscles incubated with the polyphenol Rsv. Analyzing the ratio of phosphoproteins to the loading control β-actin, we observed that Rsv reduced the phosphorylation level of FoxO4 (~60 %) in both Sham and Den muscles without altering FoxO1 phosphorylation (Fig. 2b and d). This effect was associated with the down-regulation (36%) in Akt phosphorylation only in Den muscles (Fig. 2d), suggesting that Akt/FoxO signaling pathway does not mediate Rsv-induced inhibition of UPS and lysosomal systems and other intracellular mediators may be recruited by Rsv.

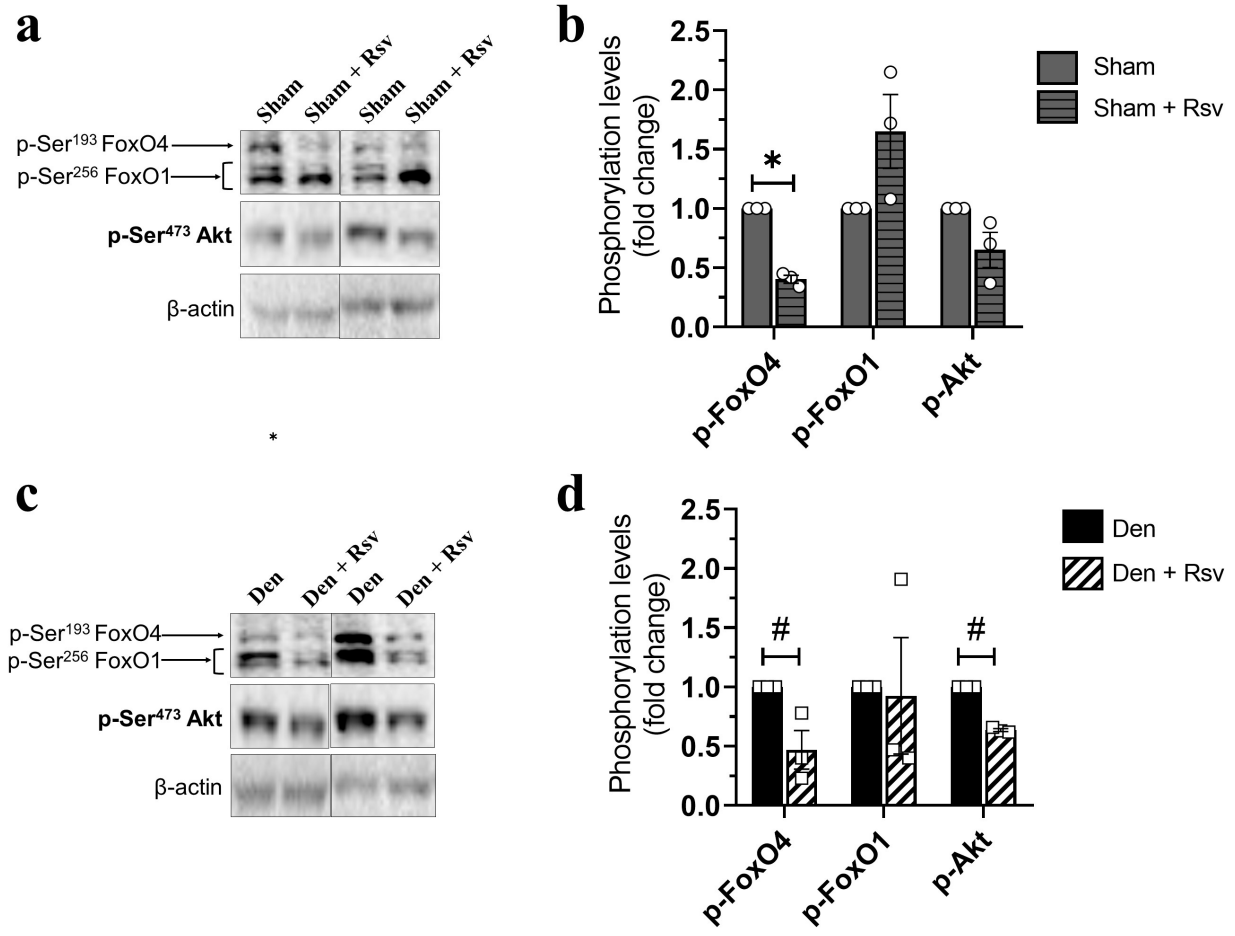

Figure 2. Effects of Rsv (10^{-4} M) on phosphorylation levels of p-Ser^{193} FoxO4, p-Ser^{256} FoxO4 and p-Ser^{473}Akt and total content of β-actin in EDL muscle of Sham (a and b) and Den (c and d) rats. Values are presented as mean ± SEM of 3 rats. Rsv: Resveratrol; Sham: sham-operated rats; Den: denervated rats. *Significantly different ($P < 0.05$) from Sham. #Significantly different ($P < 0.05$) from Den.

H89 abolishes the Rsv-induced antiproteolytic effects on Den muscles

In order to determine whether the Rsv-induced antiproteolytic effects are mediated by PKA/CREB pathway, we first evaluated the phosphorylation levels of CREB in Sham and Den muscles. Rsv tended to increase (~50%; $P \geq 0.05$) the phosphorylation levels of CREB in Sham muscles (Fig. 3b) and this effect was statistically significant (~40 %; $P < 0.05$) in Den muscles (Fig. 3d). These results suggest that PKA/CREB signaling pathway could mediate the antiproteolytic effects of Rsv.

In order to confirm the involvement of the PKA signaling in the negative regulation of total proteolysis by Rsv, we analyzed this metabolic parameter in Sham and Den muscles incubated in the absence or presence of Rsv and the PKA inhibitor, H89. Again, Rsv reduced total proteolysis in Sham (~40 %, Fig. 3e). H89 *per se* significantly reduced (73 %) total proteolysis in Sham muscles and the co-incubation of H89, and Rsv did not cause an additional effect on total proteolysis (Fig. 3e). In Den muscles, as previously demonstrated, Rsv also reduced total proteolysis (~35 %, Fig. 3f). In contrast to Sham muscles, H89 did not affect total proteolysis in

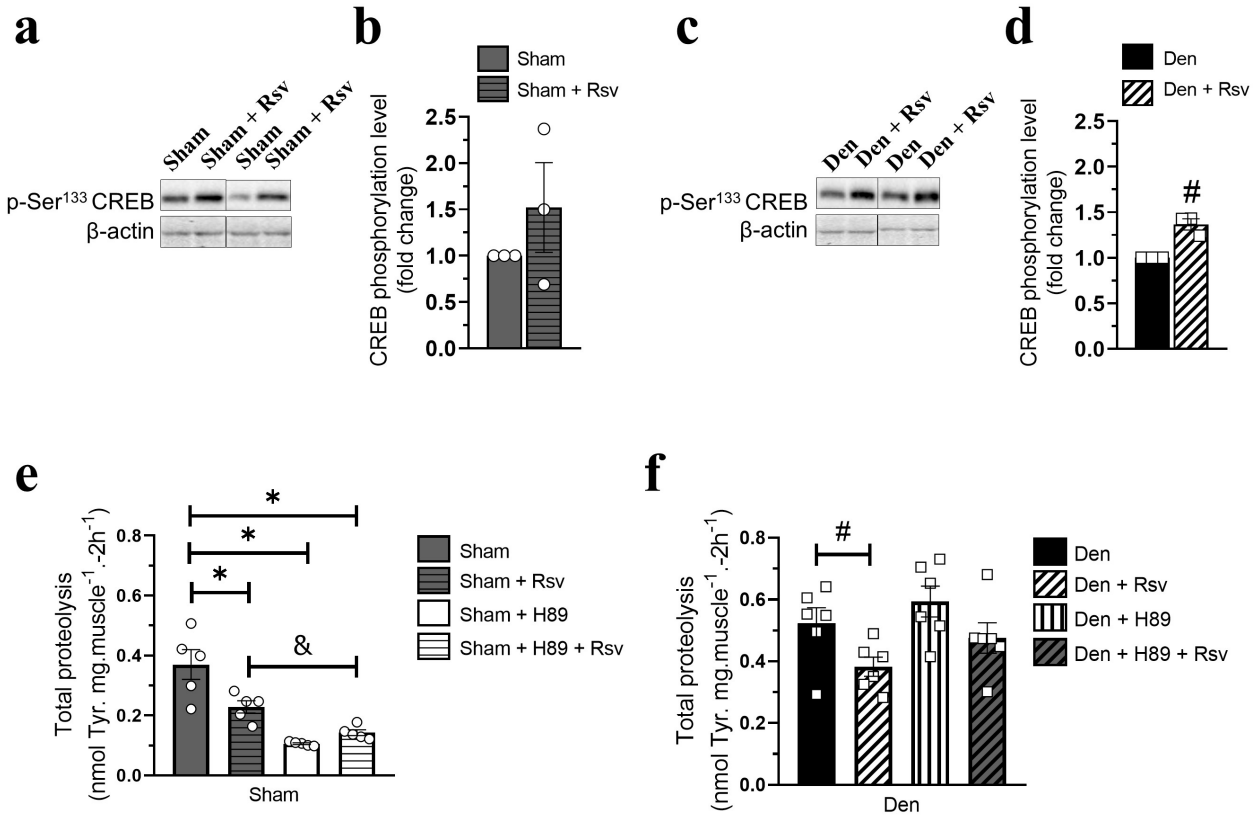

Figure 3. Effects of Rsv (10^{-4} M) on phosphorylation levels of Ser^{133} CREB (a-d) and total proteolysis (e and f) in EDL muscle of Sham (a, b and e) and Den (c, d and f) rats. Representative blots (a and c) and densitometric analysis (b and d) are shown for phosphorylation levels of Ser^{133} CREB. Values are presented as mean ± SEM of 3-6 rats. Rsv: Resveratrol; Sham: sham-operated rats; Den: denervated rats; H89: the PKA inhibitor. *Significantly different (P < 0.05) from Sham. #Significantly different (P < 0.05) from Den. &Significantly different (P < 0.05) from Sham + Rsv.

Den muscles (Fig. 3f). However, the inhibition of proteolysis induced by Rsv was abolished in muscles incubated with H89 (Fig. 3f), indicating that PKA mediates the antiproteolytic effects of Rsv on Den muscles and this effect may depend on muscle innervation.

Protein synthesis is reduced by Rsv in Den muscles

As shown in Fig. 4a, the ex vivo rate of protein synthesis was unaltered by Rsv in Sham muscles. Den *per se* tended to increase (~35 %, $P \geq 0.05$) muscle protein synthesis. On the other hand, Rsv reduced (~40 %) rates of protein synthesis in Den muscles to values similar to those in Sham muscles (Fig. 4a). We next analyzed the phosphorylation levels of protein kinases S6K, a downstream target of the major pathway (i.e., Akt/mTOR) that activates protein synthesis, and AMPK, a well-known target of Rsv that inhibits mTOR and protein synthesis (Sartori et al. 2021). As shown in Fig. 4c, analyzing the ratio of phosphoproteins to the loading control β-actin, Rsv did not alter S6K phosphorylation in Sham muscles but tended to increase ($P \geq 0.05$) AMPK phosphorylation. In Den muscles, S6K and AMPK phosphorylation was not significantly affected by Rsv (Fig. 4e). Our data demonstrate a possible homeostatic effect of Rsv by returning protein synthesis in Den muscles to levels similar to Sham muscles.

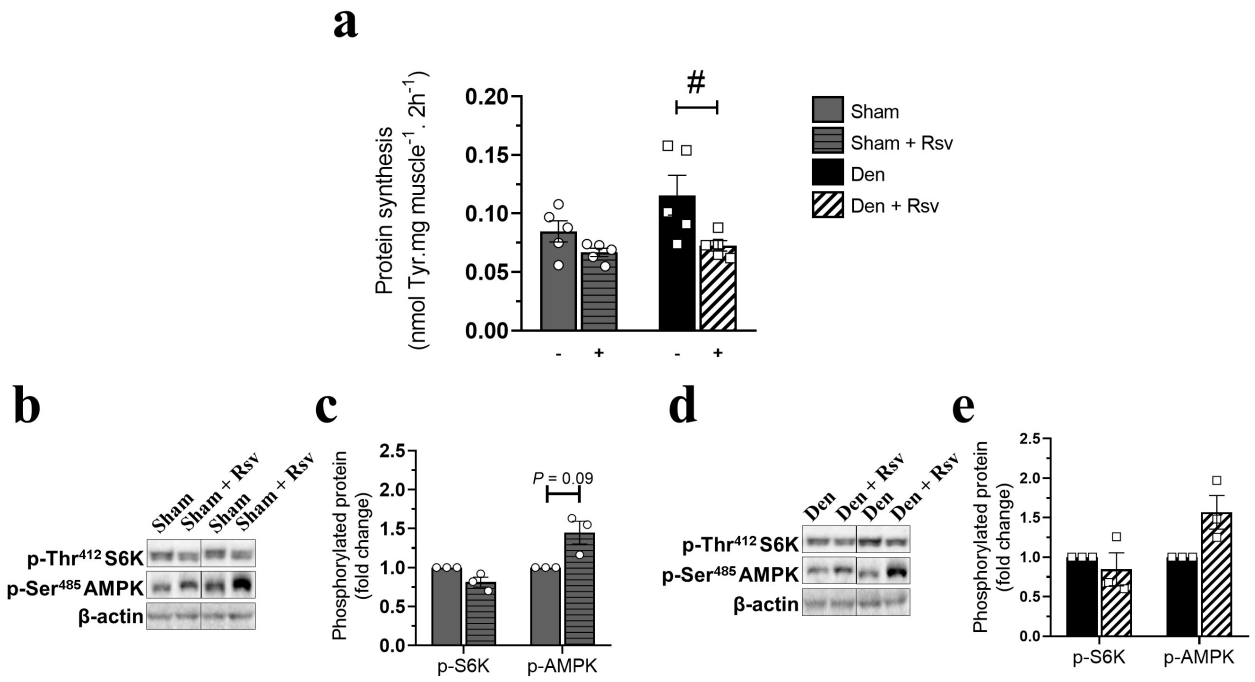

Figure 4. Effects of Rsv (10^{-4} M) on protein synthesis (a), phosphorylation levels of S6K and AMPK and total content of β-actin in EDL muscle of Sham (b and c) and Den (d and e) rats. Values are presented as mean ± SEM of 3-6 rats. Resveratrol; Sham: sham-operated rats; Den: denervated rats. #Significantly different ($P < 0.05$) from Den.

DISCUSSION

The present work sheds new light on intracellular mechanisms Rsv regulates protein metabolism in Sham (innervated) and Den rat skeletal muscles. It shows that Rsv directly inhibits lysosomal and UPS systems in Den muscles and suggests the involvement of PKA/CREB signaling in such effects. Prior studies have examined the effects of Rsv on muscle growth in cell cultures, animal models, and human beings (Wilson et al. 2015, Montesano et al. 2013, Woodman et al. 2021, Alway et al. 2017). Rsv *in vitro* has shown to induce cell cycle exit in C2C12 myoblasts, differentiation to myotubes, and hypertrophy in the late phase of the myogenic process (Montesano et al. 2013). *In vivo* experiments have demonstrated that a "low" dose of 5 mg of Rsv·kg body mass^{-1}·day^{-1} for 15 weeks promotes skeletal myofiber hypertrophy in sedentary mice (Woodman et al. 2021). Similarly, southern flounder fish fed with a diet supplemented with 600 µg Rsv·g of food^{-1} for 16 weeks had a greater length and body mass than control fish (Wilson et al. 2015). In humans, it has been shown that 500 mg of Rsv·day^{-1} for 12 weeks combined with aerobic and strength exercise programs in older men and women increased myofiber cross-section area, total myonuclei, markers of mitochondrial density, and muscle fatigue resistance (Alway et al. 2017). Altogether, these findings indicate a positive effect of Rsv on skeletal muscle growth; however, it has not been explored the underlying mechanism regulating protein metabolism and whether this is a direct or indirect effect on skeletal muscle. As far as we know, this is the first direct evidence showing that Rsv (10^{-4} M) *ex vivo* inhibits protein degradation rates in innervated skeletal muscles without altering protein synthesis rates. Although a previous study (Park et al. 2012) has shown that Rsv at low concentrations may activate intracellular signaling pathways in C_2C_{12} myotubes by causing

a significant increase in cAMP levels that reaches a plateau at 40 μM, we may not rule out the possibility that higher concentrations of Rsv (i.e., > 10^{-4} M or > 100 μM) could stimulate rates of protein synthesis in innervated muscles. More importantly, it is reasonable to speculate that antiproteolytic effect of Rsv may be responsible for stimulating muscle growth *in vivo* treatments previously reported above (Wilson et al. 2015, Woodman et al. 2021, Montesano et al. 2013, Alway et al. 2017), because muscle hypertrophy occurs when rates of protein synthesis exceed rates of degradation.

The protein metabolism imbalance may cause both muscle hypertrophy in "normal" (innervated) conditions and muscle wasting in catabolic conditions. Since protein synthesis in the Den muscle remains unchanged (Gonçalves et al. 2012, Furuno et al. 1990) or even risen (Furuno et al. 1990), excessive protein degradation has shown to essential for the development of skeletal muscle atrophy (Furuno et al. 1990, Gonçalves et al. 2012, Sartori et al. 2021). Accordingly, we also observed a tendency to increase protein synthesis and a significant increase in total proteolysis in 3-day Den muscles. Although calcium-dependent proteolysis has been related to muscle atrophy induced by unloading (Shenkman et al. 2015) and Den (Furuno et al. 1990), our data show no change in its activity and are in agreement with data from Gonçalves et al. (2012). UPS is responsible for degrading short- and long-lived proteins (e.g., myofibrillar proteins) and soluble misfolded proteins, whereas autophagy-lysosomal degrades long-lived proteins, insoluble protein aggregates, and even whole organelles (e.g., mitochondria). Excessive activation of the autophagy-lysosomal system and UPS during pathophysiological conditions contributes to the degradation of sarcomeric proteins and mitochondria loss resulting in energy imbalances, muscle wasting, strength loss, and fatigue (Sartori et al. 2021). In agreement with previously reported data (Gonçalves et al. 2012), our experiments demonstrated a dramatic activation of UPS and lysosomal proteolytic systems in Den muscles and implied a role for these processes in this atrophic model.

Rsv is a potential nutraceutical approach to counteract muscle wasting in a plethora of conditions, including sarcopenic obesity and Den (Huang et al. 2019, Asami et al. 2018). For example, Rsv supplementation (0.4 % in the diet) for 10 and 20 weeks prevented muscle loss and myofiber size decrease and improved grip strength in aged rats submitted to high-fat diet-induced sarcopenic obesity (Huang et al. 2019). Moreover, mice feeding a diet containing 0.5% of Rsv were partially protected from Den-induced muscle and myofiber atrophy (Asami et al. 2018). However, these studies did not evaluate in Den muscles: 1) the direct action of Rsv in tissue and 2) rates of protein metabolism, but only markers of protein degradation. Although the latter kind of analysis may help to identify molecular mechanisms regulated by Rsv, it may underestimate or overestimate the "real" changes in the rates of protein metabolism. Indeed, we demonstrated that Rsv suppressed rates of UPS and lysosomal proteolysis in rat Den muscles without any significant change in the gene expression of the Ub-ligases *Fbxo32* and *Trim63* and the autophagy gene *Map1lc3b*. Similarly, Asami et al. (2018) showed that Rsv did not alter the total amount of atrogin-1 protein in Den muscles, whereas reducing atrogin-1-positive nuclei in muscle cryosections, suggesting that subcellular localization and function of this Ub-ligase may be affected by Rsv rather than its content or expression. It is noteworthy that there are several other atrophy-related genes (i.e., atrogenes) belonging to UPS and autophagy-lysosomal systems, such as

the lysosomal protease cathepsin L (*Ctsl*) and the Ub-ligases *Musa1* and *Smart* (Sartori et al. 2021) that were not investigated in our study. Further experiments are needed to identify the atrogenes that Rsv may specifically regulate in Den muscles.

In skeletal muscle, FoxO proteins upregulate the transcription of several atrogenes, such as *Map1lc3b, Ctsl*, *Fbxo32,* and *Trim63*, boosting the activity of UPS and autophagy-lysosomal systems (Sartori et al. 2021). It is well known that Akt phosphorylates FoxO and blunts its transcriptional function by inducing FoxO nuclear export and keeping it away from its target genes (Sartori et al. 2021). Although FoxO1 phosphorylation was unaffected in any condition, unexpectedly, the Akt-induced phosphorylation of FoxO4 was reduced by Rsv in both Sham and Den muscles and Akt phosphorylation only in Den muscles, indicating that Rsv inactivates Akt/FoxO4 signaling. One limitation of our study was that the total protein levels of Akt and FoxOs were not evaluated, and their phosphorylation levels were normalized to the loading control β-actin. Therefore, we cannot determine whether the down-regulation of Akt/FoxO4 phosphorylation induced by Rsv was due to changes in total protein levels or modulation in the specific phosphorylation site. Independently of this limitation, the results obtained in our study should hold significance to suggest the inactivation of Akt/FoxO4 signaling by Rsv. These findings agree with the reduction in protein synthesis rates caused by Rsv in Den muscles. In sharp contrast, Montesano et al. (2013) reported that Rsv stimulates the IGF-1/Akt signaling pathway in C_2C_{12} myotubes. However, to the best of our knowledge, no study has analyzed Akt and FoxO4 phosphorylation in adult muscles incubated with Rsv *ex vivo*. One possible explanation for Rsv-induced repression of Akt phosphorylation in our study is that the antiproteolytic effect of Rsv could decrease the availability of free amino acids and, consequently, the stimulus to activate PI3K/Akt signaling (Tato et al. 2011). Although Akt does not seem to mediate the *ex vivo* effects of Rsv, we may not rule out the involvement of FoxO factors in the antiproteolytic action of Rsv because many different post-translational modifications, including phosphorylation, acetylation and ubiquitination may regulate FoxO (Sartori et al. 2021). In fact, Alamdari et al. (2012) have demonstrated that FoxO1 acetylation induced by the glucocorticoid dexamethasone mediates the upregulation of Atrogin-1 and MuRF1 expression and atrophy in cultured L6 myotubes and Rsv (10^{-4} M) is able to prevent these catabolic effects. Further experiments should be performed to test this hypothesis in *ex vivo* conditions of Den muscles.

The present study also shows that Rsv added to the incubation medium increased CREB phosphorylation, especially in Den muscles. These data suggest that cAMP/PKA signaling was stimulated by Rsv, because CREB is phosphorylated at Ser^{133} by PKA (Silveira et al. 2020). Indeed, Park et al. (2012) demonstrated that Rsv acts as a non-selective inhibitor of cAMP phosphodiesterases, particularly PDE 4, increasing cAMP levels as soon as 10 min after incubation in C_2C_{12} myotubes. Another study reported that bisphenols like curcumin also elevate cAMP and CREB phosphorylation (Ray Hamidie et al. 2015). Several studies have shown that distinct cAMP inducers may inhibit muscle proteolysis. For example, EDL muscles from diabetic rats incubated in the presence of pentoxifylline, a non-selective PDE inhibitor, showed lower calcium and UPS proteolytic activities than non-treated muscles (Baviera et al. 2007). Moreover, rat muscles incubated with rolipram, a selective PDE 4 inhibitor, attenuated UPS proteolytic activity and *Fbxo32* (Atrogin-1)

expression induced by fasting (Lira et al. 2011). *In vivo* treatment with rolipram mitigated the loss of muscle mass and force in Den muscles (Hinkle et al. 2005). Clenbuterol, a β_2-adrenergic agonist that elevates intracellular cAMP levels, has shown similar effects of Rsv on proteolysis in Den soleus muscles, i.e., clenbuterol ameliorated the hyperactivation of UPS and lysosomal systems (Gonçalves et al. 2012). More importantly, the addition of 6-BNZ-cAMP, a PKA activator, to the incubation medium of soleus muscle isolated from Sham and Den rats reduced total proteolysis (Gonçalves et al. 2012). Considering all these findings, we hypothesized that PKA could mediate Rsv-induced inhibition of muscle proteolysis. To address this issue, Sham and Den muscles were co-incubated with Rsv and/or H89, and total proteolysis was measured. Intriguingly, H89 alone decreased rates of proteolysis in innervated muscles but not in Den ones. This *ex vivo* effect of H89 was previously described and it was associated with a marked increase in Akt phosphorylation (Gonçalves et al. 2012), supporting the idea that PKA may inhibit Akt activation (Mei et al. 2002). In Den muscles, H89 *per se* did not cause any effect on proteolysis; however, it blocked the suppressive action of Rsv in total proteolysis. These findings indicates that PKA mediates, at least partially, the antiproteolytic effect of Rsv, especially in Den muscles. Because FoxO1 phosphorylation at Ser^{256}, a target of both Akt and PKA (Silveira et al. 2020), was unaffected by Rsv, it is possible to speculate that PKA is inhibiting proteolysis by indirectly regulating FoxO via phosphorylation-independent mechanisms or directly phosphorylating proteases or components of the proteolytic systems. We have recently shown that genetic activation of PKA inhibits FoxO transcriptional activity by multiple mechanisms *in vivo* (Silveira et al. 2020), such as phosphorylation, acetylation, downregulation of its contente, and inhibition by PGC-1α transcriptional coactivator, the latter has been considered a master regulator of mitochondrial biogenesis (Lin et al. 2002). Future experiments addressing this unresolved question will provide more insights into the signaling pathways and molecular mechanisms regulated by Rsv in atrophic conditions.

CONCLUSIONS

In summary, our findings show that Rsv inhibits total proteolysis in both Sham (innervated) and Den muscles and causes a marked suppression of the activities of UPS and lysosomal proteolytic systems in Den muscles. The antiproteolytic effects of Rsv in Den muscles seem to be mediated by PKA/CREB signaling. Moreover, Rsv reduces protein synthesis rates in Den muscles to values similar to those in Sham muscles. Our findings help identify novel mechanisms by which Rsv may attenuate muscle wasting in atrophic conditions and develop new therapeutic approaches based on these findings.

Acknowledgments

This work was funded by Fundação de Amparo à Pesquisa do Estado de Minas Gerais-FAPEMIG (APQ-01268-21 and APQ-02960-22 to DAG), Pró-Reitoria de Pesquisa da Universidade Federal de Minas Gerais (PRPq-UFMG; 27764 to DAG), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; 18/10089-2 to ICK/LCN), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; grant to ECL and scholarship to GOZ, finance code 001) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; 485835-8 to ECL). We are also indebted to Lilian do Carmo Heck, Neusa Maria Zanon, Elza Aparecida Filippin, and Victor Diaz Galban for their technical assistance at Ribeirão Preto Medical School-USP.

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How to cite

JÚNIOR IIS ET AL. 2023. Resveratrol directly suppresses proteolysis possibly via PKA/CREB signaling in denervated rat skeletal muscle. An Acad Bras Cienc 95: e20220877. DOI 10.1590/0001-3765202320220877.

Manuscript received on April 17, 2022;
accepted for publication on October 7, 2022

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All authors contributed extensively to the work presented in this paper and discussed the results. IISJ, DAPG, and ECL designed the study, researched data, and wrote the manuscript. TSV, FPA, and GOZ researched data and helped designing experiments and writing the manuscript. SPG, RRV, and FAG researched data. DAG, ICK, and LCCN helped designing experiments and writing the manuscript. ICK and LCCN provided critical reagents.