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Responses of skeletal muscle hypertrophy in Wistar rats to different resistance exercise models

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Running head: Progressive resistance exercise and mTOR.

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ABSTRACT

This study aimed to compare the effects of three different resistance exercise models on the quadriceps muscle cross-sectional area, as well as on mTOR phosphorylation and other pivotal molecules involved in the upstream regulation of mTOR. Twenty-four male Wistar rats were divided into untrained (control), endurance resistance training, strength resistance training, and hypertrophy resistance training (HRT) groups (n = 6). After 12 weeks of training, the red portion of the quadriceps was removed for histological and western blot analyses. The results showed that the quadriceps weight and cross-sectional areas in the exercised groups were higher than those of the untrained rats. However, the HRT group presented better results than the other two experimental groups. This same pattern was observed for mTOR phosphorylation and for the most pivotal molecules involved in the upstream control of mTOR (increase of PKB, 14-3-3, ERK, p38 MAPK, and 4E-BP1 phosphorylation, and reduction of tuberin, sestrin 2, REDD1, and phospho AMPK). In summary, our study showed that HRT leads to high levels of mTOR phosphorylation as well as of other proteins involved in the upstream regulation of mTOR.

Keywords: hypertrophy, mTOR phosphorylation, skeletal muscle, strength, hypertrophy training.

List of abbreviations

4E-BP1- 4E-binding protein-1

Akt- Protein Kinase B.

AMPK- AMP-activated Protein Kinase.

eIF4E- Eukaryotic Translation Initiation Factor 4E.

ERK- Extracellular Signal-Regulated Kinase.

Foxo1- Forkhead Box Protein O1.

HFES- High-frequency Electrical Stimulation.

LFES- Low-frequency Electrical Stimulation.

mTOR- Mammalian Target of Rapamycin.

p38MAPK- p38 Mitogen-Activated Protein Kinases.

p70S6k- 70-kDa ribosomal protein S6 kinase.

PKB- Protein Kinase B.

REDD1- Regulated in Development and DNA Damage Response 1.

SESN2- Sestrin 2.

INTRODUCTION

Physical exercise imparts many health benefits, including decreases of the body weight and fat pads, and improvements of total body strength and the cardiovascular system. It is well established that resistance exercise increases muscle mass, whereas endurance exercise is mainly associated with oxidative metabolism improvement (i.e. mitochondrial and capillary densities, and oxidative enzymes) and no hypertrophy (Van Wessel et al. 2010). However, the exact exercise load and intensity needed to achieve these objectives is unclear. Furthermore, the relationship of the different types of exercise with the molecular effects on muscle cells has not been completely elucidated.

Muscle cell hypertrophy is one of the effects of physical exercise and a key aim of the exercise practitioner. Although mechanistic target of rapamycin (mTOR)/protein kinase B (PKB) signaling has been suggested as a key factor in the process, the exact mechanism of how different exercise protocols affect cell hypertrophy remains unknown (Bodine et al. 2001). Muscle mass gain is associated with mechanical load-induced mTOR phosphorylation (Teleman et al. 2008). Bodine et al. (2001) have demonstrated that PKB activation and mTOR downstream targets such as 4E-binding protein-1 (4E-BP1) are involved in this process (Bodine et al. 2001). Once activated, the phosphorylated mTOR inhibits 4E-BP1, leading to the release of eukaryotic initiation factor 4E, which stimulates the initiation of protein synthesis.

The mTOR complex 1 (mTORC1) modulation can occur through various upstream proteins that converge at the tuberlin level (i.e., tuberous sclerosis complex 2 (TSC2); a negative regulator of mTOR activity), including AMP-activated kinase (AMPK), PKB/Akt, 14-3-3 (a family of conserved regulatory molecules with the ability to bind diverse signaling proteins, including kinases), p38 mitogen-activated protein kinases (p38 MAPK), extracellular signal-regulated kinase (ERK), regulated in development and DNA damage responses 1 (REDD1), and sestrin 2 (SESN2). PKB phosphorylates TSC2, thereby activating mTOR, at least in part by disrupting the TSC1/TSC2 complex (Inoki et al. 2002). Another upstream positive regulator of mTOR is ERK, a negative regulator of TSC2 (Miyazaki et al. 2011). In addition, mTOR can be activated via p38 MAPK, at least in part, through the p38 MAPK-induced inhibition of REDD1 (Hernández et al. 2011). Once inhibited, REDD1 leads to the dissociation of the mTOR inhibitory complex TSC1/TSC2 by enhancing the association of TSC2 with 14-3-3 family members (DeYoung et al. 2008). On the other hand, an increase in SESN2 leads to AMPK

phosphorylation (Budanov and Karin 2008), activating TSC2 and thereby inhibiting mTOR (Inoki et al. 2006).

Although different models of exercise are known to affect the hypertrophy mechanism, the exact molecular response of muscle cells to different exercise protocols remains unclear. Thus, the main aim of the present investigation was to compare the effects of hypertrophy resistance training (HRT), strength resistance training (SRT), and endurance resistance training (ERT) on the muscle fiber cross-sectional area, as well as on mTOR/PKB signaling and other proteins linked to mTOR regulation.

MATERIALS & METHODS

Animals

The study protocol was reviewed and approved by the Ethics Committee of the Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil (no. 174/2008). A total of 24 male Wistar rats (aged 3 months) were housed in cages in a room maintained at 23°C with a 12:12h light-dark cycle, and given access to water and food *ad libitum*. The animals were randomly distributed into four groups (n = 6): untrained (C), ERT, SRT, and HRT. All animals were familiarized with climbing a ladder (1.1 × 0.18 m, thirty 2-cm-high steps, 80° slope), as previously described (Watanabe et al. 2007; Scheffer et al. 2012). During the 1-week adaptation period, the animals carried a load of 5% of their individual body mass. The load was attached to the base of the rat's tail using plastic insulation tape. A repetition was deemed successful when the animal had climbed from the bottom of the rack to the top. After the adaptation period, the animals trained for 4 days/week (with a 48-h rest period between weeks) for a total of 12 weeks. The total time of each training session was approximately 30 min.

Progressive resistance exercise models

The progressive resistance exercise models (Fig. 1) used in the present investigation consisted of ERT, SRT, and HRT.

Endurance resistance training

ERT consisted of climbing the ladder carrying a load of 10% of body weight, which was increased progressively to 20%, 30%, 40%, and 50%, for 3–6 sets with 2-min breaks and 12–15 repetitions (Fig. 1a).

Strength resistance training

SRT consisted of climbing the ladder carrying a load of 25% of body weight, which was increased progressively to 50%, 100%, 125%, 150%, 175%, and 200%, for 3–6 sets with a 2-min break and 3–5 repetitions (Fig. 1b).

Hypertrophy resistance training

HRT consisted of climbing the ladder carrying a load of 25% of body weight, which was increased progressively to 50%, 75%, and 100%, for 3–6 sets with a 2-min break and 8–10 repetitions (Fig. 1c).

Protein analysis by immunoblotting

At 48 h after the last training session, the animals were killed by decapitation. The quadriceps (red portion) muscle was surgically removed and immediately homogenized in extraction buffer (1% Triton X-100 and 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM ethylenediaminetetraacetic acid, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, and 0.1 mg/mL of aprotinin) at 4°C with the use of an homogenizer (Polytron MR 2100; Kinematica, Luzern, Switzerland). The extracts were centrifuged at 11,000 rpm (5804R; Eppendorf AG, Hamburg, Germany) at 4°C for 40 min to remove insoluble material, and the supernatants of these tissues were used for protein quantification according to the Bradford method. The proteins were denatured by boiling in Laemmli sample buffer containing 100 mM dithiothreitol, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membranes. The membranes were blocked, probed, and blotted with the following primary antibodies: phospho mTOR^{ser2448}, phospho 4E-BP1^{Thr37/Ser46}, phospho-(Ser) 14-3-3 binding motif (Cell Signaling Technology, Beverly, MA, USA), phospho Akt^{ser473}, phospho ERK^{tyr204}, phospho p38 MAPK^{tyr182}, tuberin, REDD1, SESN2, phospho AMPK^{thr172}, and **β-actin** (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were stripped and then reblotted with their respective total proteins or with **β-actin** as the loading protein. Chemiluminescent detection was performed with horseradish-peroxidase-conjugated secondary antibodies (Thermo Scientific, Rockford, IL, USA). Radiographs of the membranes were taken for visualization of the protein bands. The results of the blots are presented as direct comparisons of the area

of the apparent bands, which were quantified by densitometry using the Scion Image software (Scion Corp., Frederick, MD, USA).

Histology

Skeletal muscle was prepared in paraffin blocks that were then sliced into 5- μm -thick histological sections for slide preparation. All slides were examined under an optical microscope by a researcher who was unaware of the origin of the material as well as of the objectives of the study. The analysis was performed with the computer-assisted image analysis program Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA). The results are presented as means \pm SEM of the cross-sectional area of muscle fiber (μm^2).

Statistical analysis

The molecular results are presented as representative bands. The cross-sectional area, body weight, and muscle morphometric parameters were expressed as the means \pm SEM. Differences between the groups were evaluated using one-way analysis of variance, followed by the Bonferroni *post-hoc* test. A *p* value of less than 0.05 was considered significant. The software used for analysis of the data was the Statistical Package for the Social Sciences (SPSS) version 17.0 for Windows.

RESULTS

Table 1 shows the body weight and skeletal muscle morphometric parameters. The body weight gain was similar among the experimental groups, whereas the quadriceps weight (mg of muscle/g of body weight) increased with increasing intensity of the exercise. The HRT group showed higher quadriceps weights than the other groups.

Figure 2a shows the fiber cross-sectional area. As expected, we observed a significant increase in the fiber cross-sectional area in all the exercised groups compared with that of the untrained group, with a higher increase of cross-sectional area seen in response to an increase of exercise intensity.

In order to evaluate whether the progressive increase of fiber cross-sectional area is related to mTOR activity, we analyzed the levels of mTOR phosphorylation in the groups. The exercised groups had higher levels of mTOR phosphorylation than the untrained group. In addition, the increase of

phospho mTOR in both the SRT and HRT groups was higher than that in the ERT group. mTOR phosphorylation is controlled by several molecules that can activate or inhibit its activity. We observed an increase in phosphorylation of the molecules that activate mTOR (i.e., PKB, 14-3-3 consensus binding sites, ERK, and p38 MAPK) in all three trained groups relative to that in the untrained group. Interestingly, these increases were more pronounced in the HRT group than in the SRT and ERT groups (Fig. 2b-f). The progressive resistance exercise models did not change the total protein levels of mTOR, PKB, 14-3-3, ERK, or p38 MAPK in the quadriceps (Fig. 2b-f, lower panels).

Next, we evaluated the proteins that inhibit mTOR (tuberin, REDD1, SESN2, and phospho AMPK). In all trained groups, the protein levels of tuberin, REDD1, SESN2, and phospho AMPK were lower than those in the untrained group. Interestingly, these reductions were higher in the SRT group than in the ERT group, and likewise in the HRT group compared with the other groups (Fig. 2g-j). The lower panels in Figures 2g-i represent the stripped tuberin, REDD1, and sestrin 2 membranes immunoblotted with anti- β -actin as the loading control. The progressive resistance exercise models did not change the total protein levels of AMPK (Fig. 2j, lower panels).

In order to reinforce that the mTOR phosphorylation increase is related to mTOR activity, we assayed for 4E-BP1 phosphorylation and obtained results similar to mTOR phosphorylation (Fig. 2k). The progressive resistance exercise models did not change the total protein levels of 4E-BP1 (Fig. 2k, lower panel).

DISCUSSION

The main finding of this work was that the increase of resistance training intensity led to an increase of mTOR/PKB signaling activation as well as of its associated proteins, and at the same time decreased the proteins that downregulate this intracellular signaling pathway. Moreover, the HRT model was more effective in increasing this intracellular signaling, resulting in increased muscle hypertrophy.

Our data demonstrated that both the fiber cross-sectional area and skeletal muscle weight increased in response to the three exercise protocols, with HRT having the largest effect compared with SRT and ERT. Several studies have demonstrated that muscle mass gain after resistance exercise is associated with mTOR activation (Miyazaki and Esser 2009; Philp et al. 2011). Indeed, mTOR phosphorylation was increased by all three training programs. Furthermore, HRT resulted in the highest activation of PKB, which is responsible for the phosphorylation of TSC2 at the Thr1462 site, resulting in

TSC2 inactivation and consequently mTOR activation (Inoki et al. 2002). It is known that this pathway is necessary for inducing muscle hypertrophy once the inhibition by rapamycin induces muscle atrophy (Bodine, 2001).

The role of the 14-3-3 family members on the protein synthesis mediated by different exercise protocols has not been completely elucidated (DeYoung, 2008). In response to increased training intensity, phosphorylation of 14-3-3 protein was increased, which likely induces activation of mTOR. The 4E-BP1 protein, another positive modulator of mTOR, presented the same pattern. Phosphorylation-dependent binding between tuberlin and 14-3-3 proteins (Nellist et al. 2003) indicates a relationship between 14-3-3 and mTOR activation. The 14-3-3 proteins interact with TSC1 and TSC2, negatively regulating their function, which can result in increased phosphorylation of mTOR (Shumway et al. 2003; Hernández et al. 2011). For example, Frøsig et al. (2010) showed that the 14-3-3 protein binding capacity was increased in human skeletal muscle in response to 0.5, 2, and 20 min of cycling exercise. Likewise, our results showed that the 14-3-3 protein was increased in response to an increase in training intensity, and this activated mTOR and induced cell hypertrophy.

ERK and p38 MAPK isoforms can also positively regulate the muscle size (Shi et al. 2009; Hulmi et al. 2012). The phosphorylation of these kinases, as well as of other proteins that activate mTOR, was increased by the exercise intensity. Hulmi et al. (2012) showed that hypertrophy training increased ERK and p38 MAPK phosphorylation levels relative to those in sedentary men. In addition, Nader and Esser (2001) showed that ERK and p38 MAPK were activated in response to electrical stimulation in rats. Finally, Miyazaki et al. (2011) concurred that the ERK pathway (through TSC2 phosphorylation at the Ser664 site) may contribute to mTORC1 activation during skeletal muscle hypertrophy. We demonstrated that both kinases are modulated by the level of exercise intensity.

In the present study, we also evaluated the proteins related to mTOR inhibition. Our results showed that the protein levels of tuberlin and REDD1 were decreased with increased exercise intensity. Moreover, SESN2 and phospho AMPK were decreased only in the HRT and SRT groups, showing an adaptive intracellular mechanism induced by the increase of training intensity. In addition, the lower level of REDD1 meant lower induction of TSC2, thus avoiding mTOR signaling inhibition by TSC2, which affects muscle hypertrophy (Atherton et al. 2005) by negatively regulating mTOR activity (Inoki et al. 2002). Recently, Joanne et al. (2012) showed that mice submitted to mechanical overload had decreased protein levels of REDD1. We hypothesized that the inhibition of REDD1 can be induced by p38 MAPK,

and leads to the dissociation of the mTOR-inhibiting complex TSC1/TSC2 via enhanced association of TSC2 with 14-3-3 family members (Hernández et al. 2011).

SESN2 and AMPK are also involved in tuberlin modulation. However, the exact mechanism by which hypertrophy training reduces SESN2 is unclear. In mammalian cells, sestrin proteins enhanced the AMPK-induced phosphorylation of TSC2 (Lee et al. 2010). In addition, Budanov and Karin (2008) demonstrated that the negative regulation of mTOR signaling by SESN2 was mediated by AMPK activation and TSC2 phosphorylation. Atherton et al. (2005) showed that AMPK phosphorylation was increased during low-frequency electrical stimulation (mimicking endurance training) and reduced during high-frequency electrical stimulation (mimicking resistance training).

In summary, our results showed that a higher training intensity and **number of repetitions** led to higher activation of mTOR/PKB intracellular signaling as well as of proteins that positively regulate this pathway, while at the same time inhibited the negative modulators of the pathway. Moreover, HRT resulted in the highest increase of muscle hypertrophy as well as of intracellular signaling activity.

Conflict of interest

The authors report no conflicts of interest.

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Table 1. Initial body weight (g), final body weight (g), and muscle quadriceps morphometric.

Analyzed parameters	C	ERT	SRT	HRT
Initial body weight (g)	311.9 ± 13.22	338 ± 10.09	327.8 ± 9.87	319.3 ± 12.14
Final body weight (g)	432.9 ± 11.06	447.4 ± 8.36	432.8 ± 7.01	434.4 ± 9.16
Quadriceps weight (mg of muscle/ g of bw)	13.3 ± 0.54	13.8 ± 0.84	14.7 ± 0.65 [#]	15.9 ± 0.75 ^S

Results of initial and final body weights are presented in grams (g). Results of quadriceps weights are presented in milligrams of muscle per grams of body weight. The quadriceps muscle weight was obtained by subtracting the weights of both right and left lower limbs and dividing by the total body weight of the rat. Results are expressed as the mean \pm SEM. [#] $p < 0.05$, significant difference versus C group; and [§] $p < 0.05$ significant difference versus C, ERT, and SRT groups.

Legends to figure

Figure 1. Distribution of the sets and loads of the endurance resistance training (**a**; ERT), strength resistance training (**b**; SRT), and hypertrophy resistance training (**c**; HRT) during the 12-week experimental period.

Figure 2. Effects of endurance resistance training (ERT), strength resistance training (SRT), and hypertrophy resistance training (HRT) on the muscle fiber cross-sectional area, as well as on mTOR phosphorylation and other pivotal molecules involved upstream of mTOR signaling. The graphic shows the cross-sectional area (μm^2) of the muscle fiber (**a**). Immunoblotting was performed for phospho mTOR (**b**; upper panel) and mTOR (lower panel); phospho Akt (**c**; upper panel) and Akt (lower panel); phospho 14-3-3 (**d**; upper panel) and 14-3-3 (lower panel); phospho ERK (**e**; upper panel) and ERK (lower panel); phospho p38 MAPK (**f**; upper panel) and p38 MAPK (lower panel); tuberin (**g**; upper panel) and β -actin (lower panel); REDD1 (**h**; upper panel) and β -actin (lower panel); SESN2 (**i**; upper panel) and β -actin (lower panel); phospho AMPK (**j**; upper panel) and AMPK (lower panel); and phospho 4E-BP1 (**k**; upper panel) and 4E-BP1 (lower panel). The results are presented as the mean \pm SEM of six rats per group, ^{*} $p < 0.05$, versus C group; [#] $p < 0.05$, versus ERT and C groups; and [§] $p < 0.05$, versus SRT, ERT, and C groups.

Figure 1

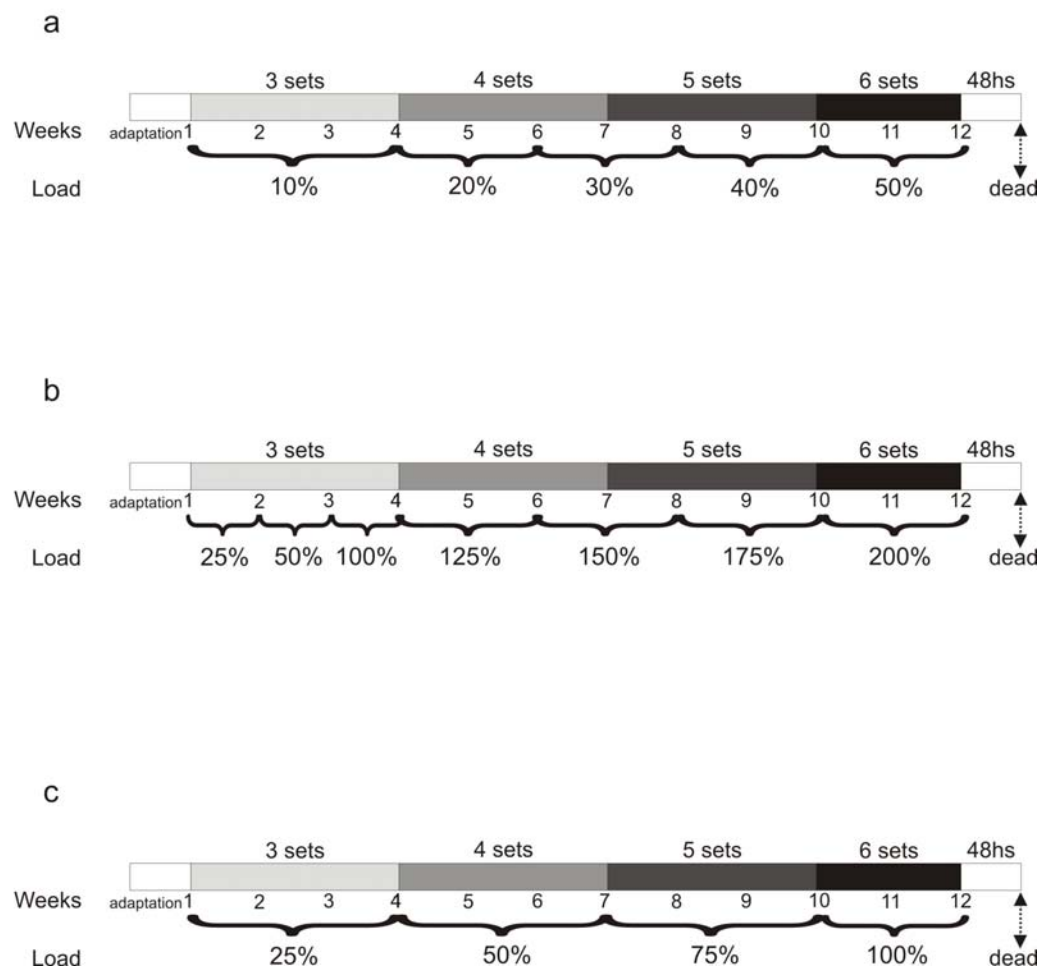


Figure 2

