 Articles in PresS. J Appl Physiol (June 14, 2007). doi:10.1152/japplphysiol.00195.2007

Blood Flow Restriction during Low-Intensity Resistance Exercise Increases S6K1 Phosphorylation and Muscle Protein Synthesis

Satoshi Fujita[1,4], Takashi Abe[4], Micah J. Drummond[2,3], Jerson G. Cadenas[1], Hans C. Dreyer[2,3], Yoshiaki Sato[4], Elena Volpi[1], and Blake B. Rasmussen[2,3]

[1]Departments of Internal Medicine and [2]Physical Therapy

[3]Division of Rehabilitation Sciences

University of Texas Medical Branch

[4]Department of Human and Engineered Environment, Graduate School of Frontier Sciences, University of Tokyo, Japan

Running title: Blood Flow Restriction and Muscle Protein Synthesis

Address for correspondence: Satoshi Fujita, Ph.D.

University of Tokyo

Graduate School of Frontier Sciences

5-1-5, Kashiwanoha, Kashiwa-shi

Chiba, 277-8563

Phone: +81-4-7136-4665

Fax: +81-4-7136-4665

E-mail: fujita@k.u-tokyo.ac.jp

Copyright © 2007 by the American Physiological Society.

Abstract

Low-intensity resistance exercise training combined with blood flow restriction (REFR) increases muscle size and strength as much as conventional resistance exercise with high-loads. However, the cellular mechanism(s) underlying the hypertrophy and strength gains induced by REFR are unknown. We have recently shown that both the mTOR signaling pathway and muscle protein synthesis were stimulated after an acute bout of high-intensity resistance exercise in humans. Therefore, we hypothesized that an acute bout of REFR would enhance mTOR signaling and stimulate muscle protein synthesis (MPS). We measured MPS and phosphorylation status of mTOR-associated signaling proteins in 6 young male subjects. Subjects were studied once during blood flow restriction (REFR, bilateral leg extension exercise at 20% of 1-RM while a pressure cuff was placed on the proximal end of both thighs and inflated at 200mmHg) and a second time using the same exercise protocol but without the pressure cuff (CTRL). MPS in the vastus lateralis muscle was measured by using stable isotope techniques and the phosphorylation status of signaling proteins were determined by immunoblotting. Blood lactate, cortisol, and growth hormone were higher following REFR as compared to CTRL ($P<0.05$). S6K1 phosphorylation, a downstream target of mTOR, increased concurrently with a decreased eEF2 phosphorylation and a 46% increase in MPS following REFR ($P<0.05$). MPS and S6K1 phosphorylation were unchanged in the CTRL group post-exercise. We conclude that the activation of the mTOR signaling pathway appears to be an important cellular mechanism which may help explain the enhanced muscle protein synthesis during REFR.

Introduction

Resistance exercise is a potent stimulus for an increase in muscle protein synthesis and subsequent muscular hypertrophy. After an acute bout of high-intensity resistance exercise, muscle protein synthesis increases significantly within 1 to 2 hours and remains elevated for up to 48 hours (34; 36; 37). The mammalian target of rapamycin (mTOR) signaling pathway plays a significant role in stimulating translation initiation and muscle protein synthesis (50). Recent studies have shown that the activation of mTOR signaling pathway is gradually activated during the recovery phase of resistance exercise (5; 9; 20). In fact, mTOR signaling to its downstream effector, ribosomal S6 kinase 1 (S6K1), is involved in the regulation of mRNA translation initiation and appears to be a critical regulator of exercise induced muscle protein synthesis and training induced hypertrophy (3; 4; 39). More recently, we have shown that following an acute bout of resistance exercise the mTOR signaling pathway is activated in association with an increase in protein synthesis in human skeletal muscle (9). Furthermore, the phosphorylation of Akt/PKB increased and the phosphorylation of eEF2 decreased during post-exercise recovery (9). Akt activation promotes mTOR phosphorylation and signaling (50). Additionally, mTOR signaling to S6K1 inhibits eEF2 kinase which reduces eEF2 phospohrylation and thus promotes translation elongation (50).

According to the size principle (9; 14; 15), motor units with a smaller cell size, i.e. slow twitch type I muscle fibers, are recruited first at lower exercise intensities, whereas at higher exercise intensities, larger motor units and their associated type II muscle fibers are recruited. These fast twitch type II fibers respond to high-intensity resistance exercise with a greater amount of hypertrophy than type I fibers (28; 31). Therefore, the American College of Sports Medicine (22) recommends that resistance exercise be

performed at an intensity of at least 70% of an individuals one repetition maximum (1-RM) in order to achieve the maximal hypertrophy. Although these guidelines are optimal for healthy people, there are numerous circumstances in which it would be extremely difficult to achieve such a high exercise intensity level in populations such as the frail elderly, in patients with osteoarthritis, or in patients undergoing the immediate rehabilitation phase following surgery. Therefore, interventions designed to prevent muscle atrophy and/or enhance muscle hypertrophy using exercise protocols consisting of lower intensities may be useful for counteracting a variety of muscle wasting conditions such as sarcopenia, cancer cachexia, AIDS, COPD, stroke, trauma/surgery, etc. Low-intensity resistance exercise during blood flow restriction may be a useful interventions to enhance muscle growth in these clinical conditions.

A series of recent studies have shown that a low-intensity (20~50% of 1-RM) resistance exercise training combined with a moderate reduction of blood flow to the working muscle (REFR) produces similar increases in muscle size and strength as compared traditional high-intensity resistance training (1; 42; 46; 47). However, the metabolic and molecular mechanisms responsible for inducing the increase in muscle hypertrophy during REFR are currently unknown.

Therefore, the purpose of the current study was to determine the effect of an acute bout of low-intensity resistance exercise (with or without blood flow restriction) on the muscle mTOR signaling pathway (an important regulator of translation initiation, elongation, muscle protein synthesis, and muscle cell growth) and muscle protein synthesis. We hypothesized that an acute bout of REFR would enhance mTOR signaling and stimulate muscle protein synthesis.

Experimental Procedures

Subjects

We studied 6 young male subjects on two separate occasions. All subjects were healthy and physically active, but were not currently engaged in an exercise training program. All subjects gave informed written consent before participating in the study, which was approved by the Institutional Review Board of the University of Texas Medical Branch. Screening of subjects were performed with clinical history, physical exam, and laboratory tests including complete blood count with differential, liver and kidney function tests, coagulation profile, fasting blood glucose and oral glucose tolerance test (OGTT), hepatitis B and C screening, HIV test, TSH, lipid profile, urinalysis, drug screening, and ECG. The subjects' characteristics are summarized in **Table 1**. The subjects were initially randomized to an infusion study in which they performed resistance exercise during blood flow restriction (REFR) or a control group in which the subjects performed resistance exercise with no restriction of blood flow (CTRL).

Study design

Each subject was admitted to the GCRC of the University of Texas Medical Branch the day prior to the exercise study, and a Dual-energy X-ray absorptiometry (DEXA) scan (Hologic QDR 4500W, Bedford, MA) was performed to measure body composition and lean mass. The subjects were then fed a standard dinner, and a snack was given at 2200 hr. The subjects were studied following an overnight fast under basal conditions and refrained from exercise for 24 hours prior to study participation. The morning of the infusion study, at 0600 hr an 18-gauge polyethylene catheter was inserted into an antecubital vein for tracer infusion. Another 18-gauge polyethylene catheter was inserted retrogradely in a hand vein of the opposite arm, which was kept in a heated pad for arterialized blood sampling. After

drawing a background blood sample, a primed continuous infusion of L-[ring-$^{13}C_6$] phenylalanine (Cambridge Isotope Laboratories, Andover, MA) was begun (time = 0 hr at 0800 hr) and maintained at a constant rate until the end of the experiment (**Figure 1**). The priming dose for the labeled phenylalanine was 2 μmol· kg^{-1} and the infusion rate was 0.05 μmol · kg^{-1}· min^{-1}.

Two hours following the initiation of the tracer infusion, the 1st muscle biopsy was obtained from the lateral portion of the *vastus lateralis* of the leg with the biopsy site between 15 and 25 cm from the mid-patella. The biopsy was performed using a 5 mm Bergström biopsy needle, under sterile procedure and local anesthesia (1% lidocaine). Muscle tissue was immediately blotted and frozen in liquid nitrogen and stored at -80°C until analysis.

Resistance Exercise with Blood Flow Restriction Group (REFR): After the second biopsy was obtained, a lower extremity pressure cuff (Kaatsu-Master Mini, Sato Sports Plaza, Tokyo, Japan) was placed around the most proximal portion of each leg. While the subject was seated on a chair, the pressure cuff was increased to 120 mmHg for 30 seconds, and the air pressure was released. The pressure cuff was then inflated four more times with each period being increased by 20 mmHg. Each period lasted 30 seconds and then the cuff was released for a 10 seconds between periods until a final pressure of 200 mmHg was reached. With the pressure maintained at 200 mmHg, the subjects then performed a set of 30 repetitions of bilateral leg extension exercise at 20% of 1-RM, followed by a 30 second rest period. Subsequently subjects then performed three more sets of 15 repetitions with 30 second rest intervals for a total of 4 sets and 75 repetitions. Immediately after the fourth set, the pressure was released from the cuff. Total time for the exercise period was approximately 4 to 5 minutes.

Resistance Exercise without Blood Flow Restriction Control (CTRL) group: The subjects in the CTRL group performed the identical exercise protocol as the REFR group except that the cuff was not inflated and no pressure was applied to the legs.

Infusion Study #2: Three weeks after the first visit, subjects assigned to the REFR group during infusion study #1 repeated the protocol without blood flow restriction (CTRL). Subjects assigned to the CTRL group during infusion study #1 then completed the blood flow restricted exercise protocol (REFR). Subjects were initially randomized to either the CTRL or the REFR group and therefore 6 subjects were studied for both groups.

Hormones and Glucose/Lactate

Serum concentrations of growth hormone, IGF-1, total testosterone, and cortisol were determined by chemiluminescent enzyme immunoassay using the Immunolite Automated Analyzer (Diagnostic Products Corporations, Los Angeles, CA). Plasma glucose and lactate concentration was measured using an automated glucose and lactate analyzer (YSI, Yellow Springs, OH).

SDS PAGE and Western Blot Analysis

Details of the immunoblotting procedures have been previously published (9). Briefly, ~30-50 mg of frozen tissue was homogenized (1:9, wt/vol), centrifuged for 10 min at 4° C followed by the removal of the supernatant. Total protein concentrations were determined by using the Bradford assay (BioRad, Smartspec plus spectrophotometer). The supernatant was diluted (1:1) in a sample buffer mixture containing 125 mM Tris, pH 6.8; 25% glycerol; 2.5% SDS; 2.5% B-mercaptoethanol and 0.002% bromophenol blue then boiled for 3 min at 100° C. Fifty micrograms of total protein was loaded into each lane and the samples were

separated by electrophoresis (100 V for 60 min) on a 7.5% polyacrylamide gel (BioRad, Criterion),. A molecular weight ladder (BioRad, Precision Plus protein standard) was also included on each gel. Following electrophoresis, protein was transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA) at 50V for 60 min. Blots were incubated in a single primary antibody overnight at 4° C (antibody concentrations are described below). The next morning, blots were incubated in secondary antibody for 1 h at room temperature. Chemiluminescent solution (ECL plus, Amersham BioSciences, Piscataway, NJ) was applied to each blot. After a 5 min incubation, optical density measurements were obtained with a phosphoimager (BioRad, Hercules, CA) and densitometric analysis was performed using Quantity One software (Ver 4.5.2) (BioRad, Hercules, CA). Preliminary experiments were performed to assess if protein abundance changed over the 7 hr of the experiment. We found that protein abundance did not change over the short time frame of the study; therefore, the data were expressed as the change in phosphorylation (in arbitrary units) normalized to a rodent standard.

Antibodies

The primary antibodies used were all purchased from Cell Signaling (Beverly, MA): phospho-mTOR (Ser2448; 1:1000); phospho-p70 S6K1 (Thr389; 1:500); phospho-Akt (Ser473; 1:500); phospho-eEF2 (Thr56; 1:1000). Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from Amersham Bioscience (1:2000).

Muscle fractional synthetic rate

Muscle intracellular free amino acids and muscle proteins were extracted as previously described (51; 52). Muscle intracellular free concentration and enrichment of phenylalanine was determined by gas chromatography-mass spectrometry (GCMS, 6890 Plus GC, 5973N

MSD, 7683 autosampler, Agilent Technologies, Palo Alto, CA) using appropriate internal standard (L-[^{15}N] phenylalanine) (51; 52). Mixed muscle protein-bound phenylalanine enrichment was analyzed by GCMS after protein hydrolysis and amino acid extraction (51; 52), using the external standard curve approach (6). We calculated the fractional synthetic rate of mixed muscle proteins (FSR) by measuring the incorporation rate of the phenylalanine tracer into the proteins ($\Delta E_p/t$) and using the precursor-product model to calculate the synthesis rate:

$$FSR = (\Delta E_p/t)/[E_{M(1)} + E_{M(2)}/2] \bullet 60 \bullet 100$$

where ΔE_p is the increment in protein-bound phenylalanine enrichment between two sequential biopsies, t is the time between the two sequential biopsies, and $E_{M(1)} + E_{M(2)}$ are the phenylalanine enrichments in the free intracellular pool in the two sequential biopsies. Data are expressed as percent per hour.

Statistical Analysis

All values are expressed as mean ± SEM. Comparisons were performed using analysis of variance with repeated measures, the effects being group (REFR, Control) and time (Baseline, Post-exercise). Post-hoc testing was performed using Tukey-Kramer when appropriate. Significance was set at $P<0.05$.

Results

Blood pH and Lactate

There were no significant differences in blood pH and plasma lactate between groups prior to performing the bout of resistance exercise. However, peripheral blood pH decreased immediately after exercise in both groups ($P<0.05$, **Figure 2A**) and gradually returned to baseline levels. There were no differences between groups in the blood pH

response to exercise, however, blood pH tended to return to the baseline value more slowly in the REFR group (P=0.10).

Plasma lactate concentration increased immediately after exercise and stayed high for 40min after exercise in the REFR group (P<0.05, **Figure 2B**). Similarly plasma lactate increased significantly after exercise in the Control group, but the values were significantly lower than those of REFR group (P<0.05, **Figure 2B**).

Hormonal Response

There were no significant differences in serum growth hormone, cortisol, IGF-1 or total testosterone between groups at baseline (P>0.05, **Figure 3**).

Serum growth hormone concentration significantly increased at 10 min post-exercise and remained elevated for 40 min post-exercise in the REFR group as compared to both baseline and CTRL concentrations (P<0.05, **Figure 3A**). Growth hormone concentration did not change following exercise in the CTRL group (P>0.05).

Serum cortisol concentration increased at 10 min post-exercise and remained elevated (as compared to baseline and CTRL) for 30 min post-exercise in the REFR group (P<0.05, **Figure 3B**). Cortisol concentrations remained significantly higher than CTRL at 40, 50, and 60 min post-exercise (P<0.05). Serum cortisol concentration did not change during or following exercise in the CTRL group.

No significant changes in IGF-1 were observed in either the REFR or the CTRL group (P>0.05). Similarly, serum total testosterone concentration did not change in either group following the bout of resistance exercise (P>0.05, **Figure 3D**).

Phosphorylation of Translation Initiation and Elongation Regulatory Proteins

Phosphorylation status of Akt, mTOR, S6K1, or eEF2 were not different between groups prior to performing the bout of resistance exercise (P>0.05). Therefore, the data in **Figure 4** are expressed as the percent change in phosphorylation status 3 hours post-exercise as compared to baseline values for both groups.

Phosphorylation of Akt at Ser473 tended to increase in both the REFR and CTRL group (time effect = 0.054, **Figure 4A**), however, no group differences were observed (P>0.05).

The phosphorylation status of mTOR at Ser2448 did not change significantly 3 hours post-exercise in either group (**Figure 4B**).

Phosphorylation of S6K1 at Thr389 was increased by 3-fold post-exercise in the REFR group (P<0.05, **Figure 4C**). S6K1 phosphorylation at 3 hours post-exercise was also significantly higher in the REFR group as compared to the CTRL group (P<0.05). S6K1 phosphorylation status in the CTRL group was not different from baseline 3 hours post-exercise (P>0.05).

Interestingly, phosphorylation of eEF2 at Thr56 decreased to a similar extent in both the REFR and CTRL group (P<0.05, **Figure 4D**) in response to exercise.

Phenylalanine Concentration and Enrichment

Phenylalanine concentration within the muscle free pool was similar between groups at baseline without a significant group difference (P>0.05, **Table 2**). In addition, muscle phenylalanine concentration at 3 hours post-exercise did not change in either group (P>0.05). Muscle intracellular enrichment of phenylalanine was at a steady state throughout the infusion study with no significant differences between groups (P>0.05, **Table 2**).

Muscle Protein Synthesis

Whereas the phosphorylation status of signaling proteins are indicators of enhanced translation initiation and elongation, we also directly measured muscle protein synthesis, the end product of translation initiation and elongation. We found that the mixed muscle protein fractional synthetic rate (FSR, a direct measure of the incorporation of amino acids into protein) was significantly increased 3 hours post-exercise in the REFR group as compared to baseline and the CTRL group ($P<0.05$, **Figure 5**). Muscle FSR did not change following exercise in the CTRL group ($P>0.05$).

Discussion

The major and novel finding from our study was that a key downstream effector of the mTOR signaling pathway, S6K1, became phosphorylated and muscle protein synthesis was stimulated following an acute bout of low-intensity (20% 1-RM) resistance exercise combined with blood flow restriction (REFR). Specifically, muscle fractional synthetic rate increased by 46% ($P<0.05$) 3 hours after a bout of exercise in REFR while muscle protein synthesis was not increased in the control-exercise (CTRL) group without the blood flow restriction. Furthermore, the increased muscle protein synthesis in REFR group was associated with a significant increase in the phosphorylation of S6K1 and a significant decrease in eEF2 phosphorylation, suggesting enhanced translation initiation and elongation after REFR. Therefore, enhanced mTOR signaling may be an important cellular mechanism which may in part explain the hypertrophy induced by low-intensity resistance exercise during blood flow restriction.

High-intensity resistance exercise is a potent stimulus for muscle protein synthesis and muscular hypertrophy (9; 21; 37; 54). Muscle fibers respond to overload stress with an increase in the cross-sectional area of muscle fibers and an increase in force generating capacity. In general, a training intensity of over 70% of 1-RM is required to achieve a substantial muscle hypertrophy. On the contrary, exercise with an intensity of <65% of 1-RM generally induces an improvement of muscular endurance with no substantial increases in muscular size or the strength (24). The basis of this adaptive response is supported by a number of acute studies which reported a significant increase in muscle protein synthesis after a bout of resistance exercise (9; 34; 41). We have recently shown that a single bout of resistance exercise with 70% 1-RM significantly increased muscle protein synthesis within 1 to 2 hours in combination with an increase in the

phosphorylation of mTOR, Akt/PKB, and S6K1 and a reduced phosphorylation of eEF2 (9). A recent *in vivo* study has shown that during high intensity concentric contraction, there is a significant increase in the phosphorylation of S6K1 whereas no significant change was observed with submaximal contraction (10). It appears that during the recovery phase following high-intensity resistance exercise, an increase in translation initiation and elongation lead to the muscle anabolic response, however, there seems to be a threshold for the exercise intensity below which the anabolic response is not properly triggered.

Recent studies have shown that low-intensity (approximately 20-50% of 1-RM) resistance training combined with reduced muscular blood flow (REFR) leads to significant elevations in muscle strength and muscle fiber cross-sectional area (1; 42; 46; 47), with the increases comparable to those with high intensity resistance training. Our current study indicates for the first time that low-intensity resistance exercise acutely stimulates muscle protein synthesis when combined with blood flow restriction. Furthermore, the increase in the phosphorylation of S6K1 associated with REFR suggests an upregulation of translational efficiency allowing for the synthesis of specific mRNAs essential for the muscle growth. There is a direct correlation between the increase in S6K1 measured at 6 hr after an acute exercise bout and the percent change in muscle mass measured after 6 weeks of training (3), suggesting the phosphorylation of S6K1 could be a marker for the long-term increase in muscle mass. Although the mechanism(s) of how mTOR signaling to S6K1 is enhanced during REFR was not identified in the current study, it is possible that either changes in the hormonal response, metabolic stress, and/or mechanotransduction signaling may be involved.

An interesting finding was that eEF2 phosphorylation decreased in the CTRL group during low-intensity exercise despite the fact that the phosphorylation status of Akt, mTOR,

and S6K1 was unchanged. Since eEF2 lies downstream of mTOR we are not completely sure how to explain the disconnect between mTOR and eEF2 phosphorylation. However, it is possible that Akt/mTOR/S6K1 phosphorylation may have transiently increased in the CTRL group prior to obtaining the 3 hr post-exercise biopsy. This may have occurred since it appears that muscle protein synthesis (although not significant) was slightly elevated in the CTRL group. Another possibility may be that other regulatory proteins influenced eEF2 kinase during the low-intensity control trial.

It has been suggested the acute change in anabolic hormones such as testosterone and growth hormones (GH) after resistance exercise are critical for skeletal muscle growth (25). In response to an acute bout of high-intensity resistance exercise, GH concentration increases significantly above resting values (17; 23; 49). Similarly, REFR has been shown to induce a significant GH response (35; 38; 44; 45). GH has been shown to be a positive regulator of cellular differentiation in a variety of cells, including muscle and liver cells (11; 33). In animal and *in vitro* studies, GH treatment is associated with increased muscle protein synthesis rates and decreased muscle protein breakdown (40). Although the signaling pathways involved in the GH stimulation of muscle protein synthesis are unclear, GH has been shown to activate phosphatidylinositol 3-kinase and S6K1 in 3T3 fibroblasts (29). More recently, Hayashi and Proud have shown that GH stimulates protein synthesis in H4IIE hepatoma cells through mTOR signaling (13). Thus, it is plausible that there may be a link between GH signaling and translational control in our study. However, in humans there is no evidence that growth hormone enhances muscle protein synthesis when combined with traditional resistance exercise training (53). In our study, the peak growth response after REFR was 10-fold higher in the REFR group compared to the control-exercise group and was similar to values reported following

high-intensity resistance exercise (23). Circulating IGF-1 concentrations were also elevated immediately post-exercise and serum cortisol concentrations were increased in the REFR group during post-exercise recovery. Thus, blood flow restriction during low-intensity exercise produced a hormonal response similar to high-intensity exercise without vascular occlusion (25). Although the acute increase in GH following high-intensity resistance exercise has been thought to be a key player in producing the muscle anabolic response, it is not clear if the acute increase in GH concentration after REFR in our study plays a role in the activation of mTOR signaling.

Another possibility relates to the increased muscle fiber recruitment associated with REFR. Two previous studies have shown that the integrated EMG values during REFR were significantly higher than a control group (32; 47), suggesting that a greater number of normally inactive muscle fibers are recruited to lift the a similar load when the blood flow is restricted. Although the mechanism of enhanced muscle fiber recruitment during REFR is not clear, it may involve a premature fatigue of active muscle fibers and resultant recruitment of normally inactive muscle fibers. Although in the current study, the magnitude of decrease in blood pH after exercise was not different between groups, the increase in plasma lactate concentration was significantly higher in REFR as compared the control group suggesting a pooling and local accumulation of lactate. A recent study has shown that the phosphorylation of S6K1 increased to a greater extent in the type II fibers compared with type I fibers after an acute bout of high-intensity resistance exercise (20). If the higher proportion of type II muscle fibers are activated during REFR, similar to the condition during high-intensity resistance exercise, it may explain the substantial increase in the phosphorylation of S6K1 in our current study. Thus, metabolic stress may also be playing a role in stimulating Type II fiber recruitment.

Blood flow restriction induced by the pressure cuff during exercise may have caused a mild ischemia and with the release of the pressure cuff a subsequent hyperemia could have occurred (i.e., an ischemia-reperfusion condition). Although prolonged hypoxia has been shown to decrease mTOR signaling and muscle protein synthesis (2; 16; 27), ischemic preconditioning of the heart has been shown to protect against myocardial infarction (8; 30), and this ischemic preconditioning may involve the cell survival and cell growth control through the mTOR signaling pathway (7; 12; 18; 19). On the contrary, Reeves et al. has shown that the low-intensity resistance exercise combined with blood flow restriction resulted in a significant growth hormone response whereas blood flow restriction alone did not induce such an effect (38). Furthermore, Takarada et al. demonstrated that 8 weeks of low-intensity resistance training (twice a week) caused a significant increase in muscle cross sectional area of knee extensors and muscular strength whereas no such response was observed with blood flow restriction alone (48). Therefore, blood flow restriction alone apparently is not an adequate stimulus to induce an acute muscle anabolic response; however, future work is required to determine if hypoxia/occlusion (without exercise) can also alter mTOR signaling and protein synthesis in human muscle. Although we did not directly measure whether an ischemic-reperfusion condition was present in our subjects, it is interesting to speculate that muscular contraction and ischemia-reperfusion stress, when combined, additively stimulate the mTOR signaling pathway and muscle protein synthesis by promoting an upregulation of cell survival mechanisms.

In summary, we have shown that an acute bout of low-intensity resistance exercise combined with blood flow restriction stimulates S6K1 phosphorylation (a downstream component of the mTOR signaling pathway and a key regulator of translation initiation)

and protein synthesis in human skeletal muscle. The mTOR signaling pathway and muscle protein synthesis did not change in the low-intensity resistance exercise group (without blood flow restriction). Therefore, we conclude that the activation of the mTOR signaling pathway appears to be an important cellular mechanism which may help to explain the enhanced muscle protein synthesis during low-intensity resistance exercise with blood flow restriction. Future studies are required to determine the cellular mechanism(s) responsible for activating S6K1 and whether the stimulation of the mTOR signaling pathway is the primary mechanism for inducing muscle hypertrophy during low-intensity resistance exercise with blood flow restriction.

Acknowledgements

We wish to thank the nurses and personnel of the General Clinical Research Center of the University of Texas Medical Branch for their help with the conduct of the clinical portion of this study and Shanon Casperson, Dr. Melinda Sheffield-Moore, Ming Zheng, and Shelley Medina for technical assistance.

This study was supported by a research grant from the Japan Kaatsu Training Society, grant R01 AR049877 from the National Institute for Arthritis and Musculoskeletal and Skin Diseases, grant S10 RR16650 from the Shared Instrumentation Grant Program, grant P30 AG024832 from the National Institutes on Aging, and grant M01 RR00073 from the General Clinical Research Branch, National Center for Research Resources.

References

1. **Abe T, Kearns CF and Sato Y**. Muscle size and strength are increased following walk training with restricted venous blood flow from the leg muscle, Kaatsu-walk training. *J Appl Physiol* 100: 1460-1466, 2006.

2. **Arsham AM, Howell JJ and Simon MC**. A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. *J Biol Chem* 278: 29655-29660, 2003.

3. **Baar K and Esser K**. Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise. *Am J Physiol* 276: C120-C127, 1999.

4. **Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ and Yancopoulos GD**. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol* 3: 1014-1019, 2001.

5. **Bolster DR, Kubica N, Crozier SJ, Williamson DL, Farrell PA, Kimball SR and Jefferson LS**. Immediate response of mammalian target of rapamycin (mTOR)-mediated signalling following acute resistance exercise in rat skeletal muscle.[see comment]. *Journal of Physiology 553(Pt 1):213-20,* 2003.

6. **Calder AG, Anderson SE, Grant I, McNurlan MA and Garlick PJ**. The determination of low d5-phenylalanine enrichment (0.002-0.09 atom percent excess),

after conversion to phenylethylamine, in relation to protein turnover studies by gas chromatography/electron ionization mass spectrometry. *Rapid Commun Mass Sp* 6: 421-424, 1992.

7. **Crozier SJ, Zhang X, Wang J, Cheung J, Kimball SR and Jefferson LS**. Activation of signaling pathways and regulatory mechanisms of mRNA translation following myocardial ischemia-reperfusion. *J Appl Physiol* 101: 576-582, 2006.

8. **Downey JM and Cohen MV**. Reducing infarct size in the setting of acute myocardial infarction. *Prog Cardiovasc Dis* 48: 363-371, 2006.

9. **Dreyer HC, Fujita S, Cadenas JG, Chinkes DL, Volpi E and Rasmussen BB**. Resistance exercise increases AMPK activity and reduces 4E-BP1 phosphorylation and protein synthesis in human skeletal muscle. *J Physiol* 576: 613-624, 2006.

10. **Eliasson J, Elfegoun T, Nilsson J, Kohnke R, Ekblom B and Blomstrand E**. Maximal lengthening contractions increase p70 S6 kinase phosphorylation in human skeletal muscle in the absence of nutritional supply. *Am J Physiol Endocrinol Metab* 291: E1197-E1205, 2006.

11. **Ewton DZ and Florini JR**. Relative effects of the somatomedins, multiplication-stimulating activity, and growth hormone on myoblasts and myotubes in culture. *Endocrinology* 106: 577-583, 1980.

12. **Harada H, Andersen JS, Mann M, Terada N and Korsmeyer SJ**. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. *Proc Natl Acad Sci U S A* 98: 9666-9670, 2001.

13. **Hayashi AA and Proud CG**. The rapid activation of protein synthesis by growth hormone requires signaling through the mammalian target of rapamycin, mTOR. *Am J Physiol Endocrinol Metab* 2007.

14. **Henneman E**. The size principle and its relation to transmission failure in Ia projections to spinal motoneurons. *Ann N Y Acad Sci* 627: 165-168, 1991.

15. **Henneman E, Somjen G and Carpenter DO**. FUNCTIONAL SIGNIFICANCE OF CELL SIZE IN SPINAL MOTONEURONS. *J Neurophysiol* 28: 560-580, 1965.

16. **Hui AS, Bauer AL, Striet JB, Schnell PO and Czyzyk-Krzeska MF**. Calcium signaling stimulates translation of HIF-alpha during hypoxia. *FASEB J* 20: 466-475, 2006.

17. **Hymer WC, Kraemer WJ, Nindl BC, Marx JO, Benson DE, Welsch JR, Mazzetti SA, Volek JS and Deaver DR**. Characteristics of circulating growth hormone in women after acute heavy resistance exercise. *Am J Physiol Endocrinol Metab* 281: E878-E887, 2001.

18. **Jonassen AK, Sack MN, Mjos OD and Yellon DM**. Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. *Circ Res* 89: 1191-1198, 2001.

19. **Kis A, Yellon DM and Baxter GF**. Second window of protection following myocardial preconditioning: an essential role for PI3 kinase and p70S6 kinase. *J Mol Cell Cardiol* 35: 1063-1071, 2003.

20. **Koopman R, Zorenc AH, Gransier RJ, Cameron-Smith D and van Loon LJ**. Increase in S6K1 phosphorylation in human skeletal muscle following resistance exercise occurs mainly in type II muscle fibers. *Am J Physiol Endocrinol Metab* 290: E1245-E1252, 2006.

21. **Kosek DJ, Kim JS, Petrella JK, Cross JM and Bamman MM**. Efficacy of 3 days/wk resistance training on myofiber hypertrophy and myogenic mechanisms in young vs. older adults. *J Appl Physiol* 101: 531-544, 2006.

22. **Kraemer WJ, Adams K, Cafarelli E, Dudley GA, Dooly C, Feigenbaum MS, Fleck SJ, Franklin B, Fry AC, Hoffman JR, Newton RU, Potteiger J, Stone MH, Ratamess NA and Triplett-McBride T**. American College of Sports Medicine position stand. Progression models in resistance training for healthy adults. *Med Sci Sports Exerc* 34: 364-380, 2002.

23. **Kraemer WJ, Marchitelli L, Gordon SE, Harman E, Dziados JE, Mello R, Frykman P, McCurry D and Fleck SJ**. Hormonal and growth factor responses to heavy resistance exercise protocols. *J Appl Physiol* 69: 1442-1450, 1990.

24. **Kraemer WJ and Ratamess NA**. Fundamentals of resistance training: progression and exercise prescription. *Med Sci Sports Exerc* 36: 674-688, 2004.

25. **Kraemer WJ and Ratamess NA**. Hormonal responses and adaptations to resistance exercise and training. *Sports Med* 35: 339-361, 2005.

26. **Lamb GD and Stephenson DG**. Point: lactic acid accumulation is an advantage during muscle activity. *J Appl Physiol* 100: 1410-1412, 2006.

27. **Liu L, Cash TP, Jones RG, Keith B, Thompson CB and Simon MC**. Hypoxia-induced energy stress regulates mRNA translation and cell growth. *Mol Cell* 21: 521-531, 2006.

28. **MacDougall JD, Elder GC, Sale DG, Moroz JR and Sutton JR**. Effects of strength training and immobilization on human muscle fibres. *European Journal of Applied Physiology & Occupational Physiology 43(1):25-34,* 1980.

29. **MacKenzie SJ, Yarwood SJ, Peden AH, Bolger GB, Vernon RG and Houslay MD**. Stimulation of p70S6 kinase via a growth hormone-controlled phosphatidylinositol 3-kinase pathway leads to the activation of a PDE4A cyclic

AMP-specific phosphodiesterase in 3T3-F442A preadipocytes. *Proc Natl Acad Sci U S A* 95: 3549-3554, 1998.

30. **Marber MS, Latchman DS, Walker JM and Yellon DM**. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 88: 1264-1272, 1993.

31. **McCall GE, Byrnes WC, Dickinson A, Pattany PM and Fleck SJ**. Muscle fiber hypertrophy, hyperplasia, and capillary density in college men after resistance training. *J Appl Physiol* 81: 2004-2012, 1996.

32. **Moore DR, Burgomaster KA, Schofield LM, Gibala MJ, Sale DG and Phillips SM**. Neuromuscular adaptations in human muscle following low intensity resistance training with vascular occlusion. *Eur J Appl Physiol* 92: 399-406, 2004.

33. **Norstedt G and Palmiter R**. Secretory rhythm of growth hormone regulates sexual differentiation of mouse liver. *Cell* 36: 805-812, 1984.

34. **Phillips SM, Tipton KD, Aarsland A, Wolf SE and Wolfe RR**. Mixed muscle protein synthesis and breakdown after resistance exercise in humans. *Am J Physiol* 273: E99-107, 1997.

35. **Pierce JR, Clark BC, Ploutz-Snyder LL and Kanaley JA**. Growth hormone and muscle function responses to skeletal muscle ischemia. *J Appl Physiol* 101: 1588-1595, 2006.

36. **Rasmussen BB and Phillips SM**. Contractile and nutritional regulation of human muscle growth. *Exerc Sport Sci Rev* 31: 127-131, 2003.

37. **Rasmussen BB, Tipton KD, Miller SL, Wolf SE and Wolfe RR**. An oral essential amino acid-carbohydrate supplement enhances muscle protein anabolism after resistance exercise. *J Appl Physiol* 88: 386-392, 2000.

38. **Reeves GV, Kraemer RR, Hollander DB, Clavier J, Thomas C, Francois M and Castracane VD**. Comparison of hormone responses following light resistance exercise with partial vascular occlusion and moderately difficult resistance exercise without occlusion. *J Appl Physiol* 101: 1616-1622, 2006.

39. **Reynolds TH, Bodine SC and Lawrence JC, Jr.** Control of Ser2448 phosphorylation in the mammalian target of rapamycin by insulin and skeletal muscle load. *J Biol Chem* 277: 17657-17662, 2002.

40. **Rooyackers OE and Nair KS**. Hormonal regulation of human muscle protein metabolism. *Annu Rev Nutr* 17: 457-485, 1997.

41. **Sheffield-Moore M, Paddon-Jones D, Sanford AP, Rosenblatt JI, Matlock AG, Cree MG and Wolfe RR**. Mixed muscle and hepatic derived plasma protein metabolism is differentially regulated in older and younger men following resistance exercise. *Am J Physiol Endocrinol Metab* 288: E922-E929, 2005.

42. **Shinohara M, Kouzaki M, Yoshihisa T and Fukunaga T**. Efficacy of tourniquet ischemia for strength training with low resistance. *Eur J Appl Physiol Occup Physiol* 77: 189-191, 1998.

43. **Stephenson DG, Lamb GD and Stephenson GM**. Events of the excitation-contraction-relaxation (E-C-R) cycle in fast- and slow-twitch mammalian muscle fibres relevant to muscle fatigue. *Acta Physiol Scand* 162: 229-245, 1998.

44. **Takano H, Morita T, Iida H, Asada K, Kato M, Uno K, Hirose K, Matsumoto A, Takenaka K, Hirata Y, Eto F, Nagai R, Sato Y and Nakajima T**. Hemodynamic and hormonal responses to a short-term low-intensity resistance exercise with the reduction of muscle blood flow. *Eur J Appl Physiol* 95: 65-73, 2005.

45. **Takarada Y, Nakamura Y, Aruga S, Onda T, Miyazaki S and Ishii N**. Rapid increase in plasma growth hormone after low-intensity resistance exercise with vascular occlusion. *J Appl Physiol* 88: 61-65, 2000.

46. **Takarada Y, Sato Y and Ishii N**. Effects of resistance exercise combined with vascular occlusion on muscle function in athletes. *Eur J Appl Physiol* 86: 308-314, 2002.

47. **Takarada Y, Takazawa H, Sato Y, Takebayashi S, Tanaka Y and Ishii N**. Effects of resistance exercise combined with moderate vascular occlusion on muscular function in humans. *J Appl Physiol* 88: 2097-2106, 2000.

48. **Takarada Y, Tsuruta T and Ishii N**. Cooperative effects of exercise and occlusive stimuli on muscular function in low-intensity resistance exercise with moderate vascular occlusion. *Jpn J Physiol* 54: 585-592, 2004.

49. **Vanhelder WP, Radomski MW and Goode RC**. Growth hormone responses during intermittent weight lifting exercise in men. *Eur J Appl Physiol Occup Physiol* 53: 31-34, 1984.

50. **Wang X and Proud CG**. The mTOR pathway in the control of protein synthesis. *Physiology (Bethesda)* 21: 362-369, 2006.

51. **Wolfe RR**. *Radioactive and stable isotope tracers in biomedicine. Principles and practice of kinetic analysis.* New York: Wiley-Liss, 1992.

52. **Wolfe RR and Chinkes DL**. *Isotope Tracers in Metabolic Research: Principles and Practice of Kinetic Analysis*. Hoboken, New Jersey: Wiley-Liss, 2005.

53. **Yarasheski KE, Campbell JA, Smith K, Rennie MJ, Holloszy JO and Bier DM**. Effect of growth hormone and resistance exercise on muscle growth in young men. *Am J Physiol Endocrinol Metab* 262: E261-E267, 1992.

54. **Yarasheski KE, Pak-Loduca J, Hasten DL, Obert KA, Brown, MB and Sinacore DR**. Resistance exercise training increases mixed muscle protein synthesis rate in frail women and men >/=76 yr old. *Am J Physiol Endocrinol Metab* 277: E118-E125, 1999.

Figure Legends

Figure 1. Study design. Blood and muscle samples were taken at the times indicated by the arrows. Exercise was performed immediately after the second biopsy.

Figure 2. A) Peripheral vein blood pH response in REFR and CTRL subjects before, immediately after, and 1-3 hours post-exericse. B) Plasma lactate response in REFR and CTRL subjects before, immediately after, and 1-3 hours post-exericse.
* Significantly different from baseline (P<0.05). # Significantly different from CTRL (P<0.05).

Figure 3. Peripheral vein serum hormone concentrations in REFR and CTRL subjects before, immediately after, and for1-3 hours post-exercise, A) Serum growth hormone concentration B) Serum cortisol concentration, C) Serum IGF-1 concentration, and D) Serum total testosterone concentration.
Significantly different from CTRL (P<0.05).

Figure 4. Phosphorylation status of human muscle mTOR-associated signaling proteins in REFR and CTRL subjects before and after 3 hours post-exercise. A) Akt/PKB phosphorylation at Ser473, B) mTOR phosphorylation at Ser2448, C) S6K1 phosphorylation at Thr389, and D) eEF2 phosphorylation at Thr56.
Data are expressed in arbitrary units and presented as the percent of baseline values.
* Significantly different from baseline (P<0.05). # Significantly different from CTRL (P<0.05).

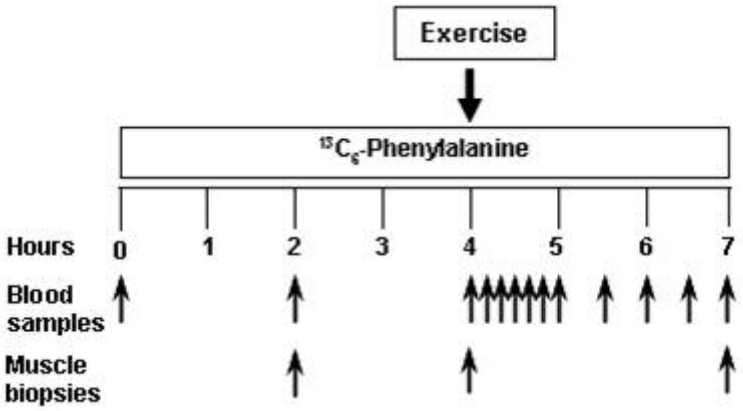
Figure 5. Muscle protein synthesis as expressed by the mixed muscle fractional synthetic rate (FSR) in REFR and CTRL subjects before and after 3 hours post-exercise. * Significantly different from baseline ($P<0.05$). # Significantly different from CTRL ($P<0.05$).

Table 1. Subject Characteristics (N=6). Each subject was tested once during blood flow restriction (REFR) and a second time without the pressure cuff (CTRL).

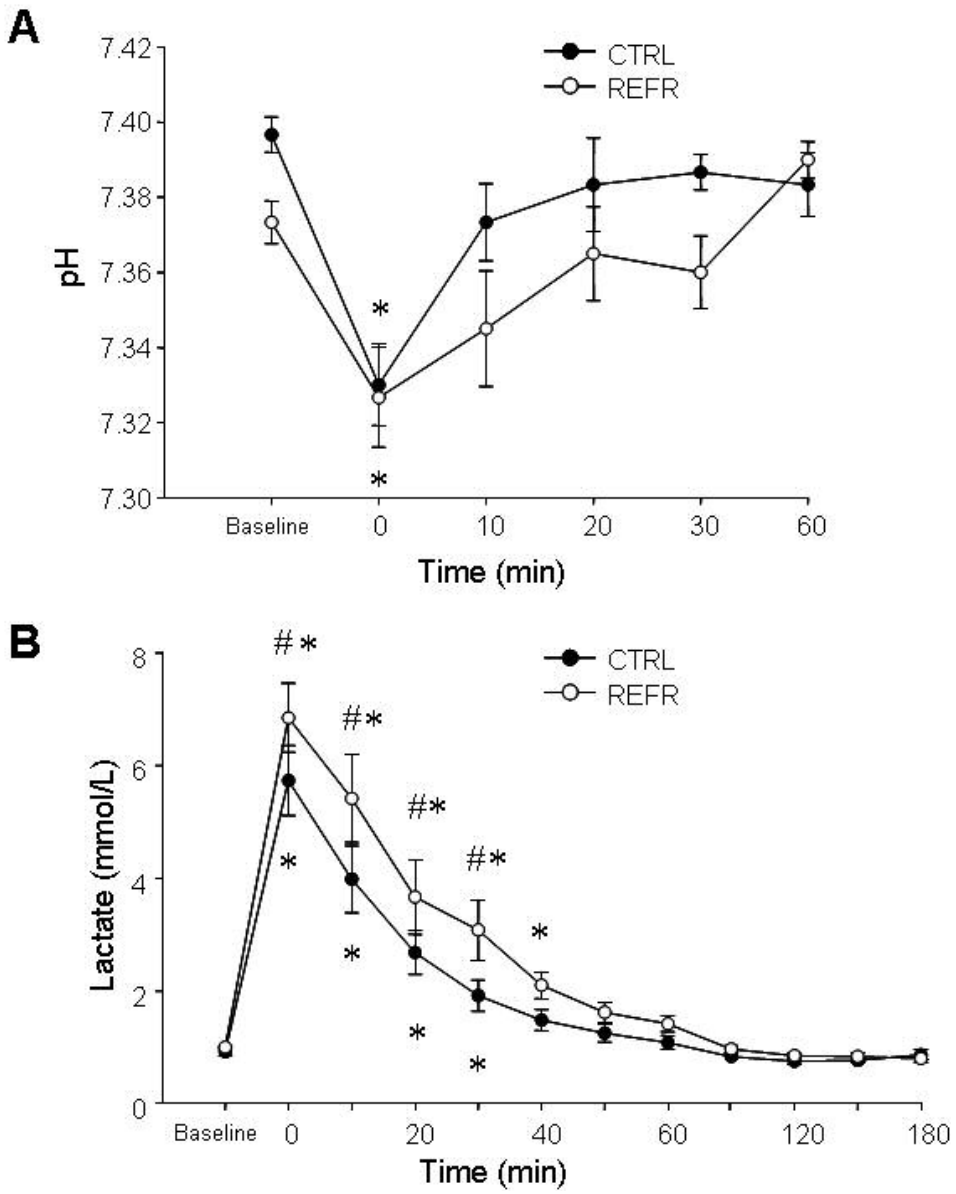
Age (yrs)	32	±	2
Weight (kg)	84	±	4
Height (m)	1.70	±	0.02
BMI (kg/m²)	29	±	1
Leg lean mass (g)			
Left leg	9840	±	447
Right leg	9974	±	541

Table 2. Muscle free phenylalanine enrichment and concentration. IC = intracellular.

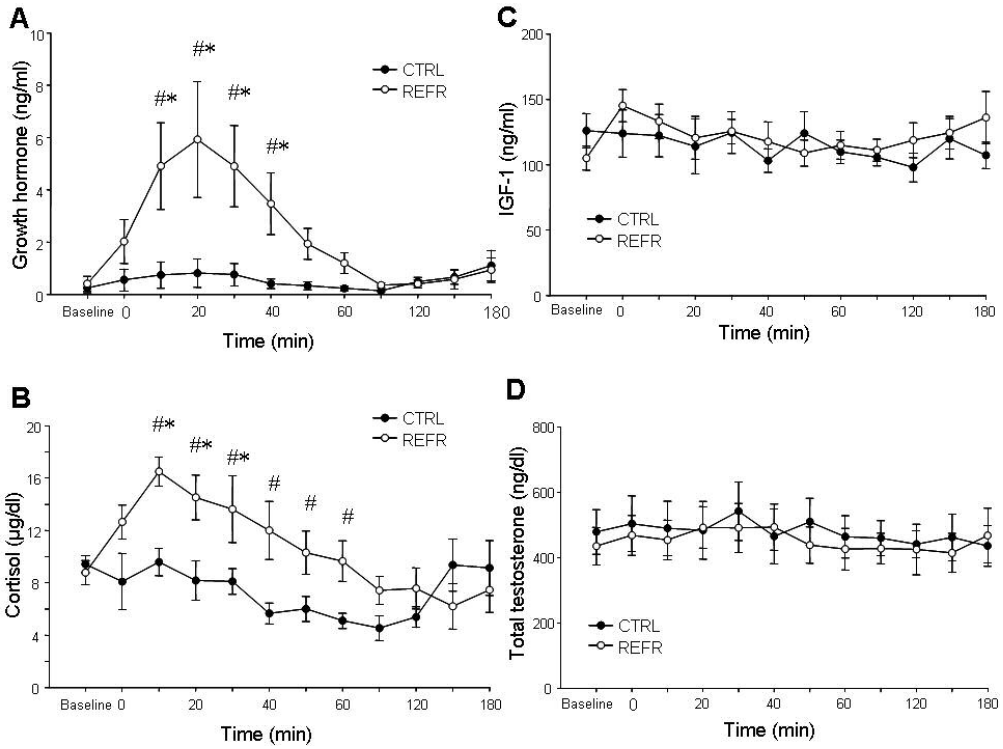
		CTRL	REFR
IC phenylalanine concentration (μmol/L)			
	Baseline	95.4 ± 4.7	95.5 ± 6.8
	Exercise	99.8 ± 8.1	97.0 ± 7.4
IC phenylalanine enrichment (%)			
	120 min	5.6 ± 1.0	5.4 ± 1.2
	240 min	6.7 ± 0.7	5.5 ± 0.3
	420 min	5.8 ± 0.4	5.4 ± 0.5



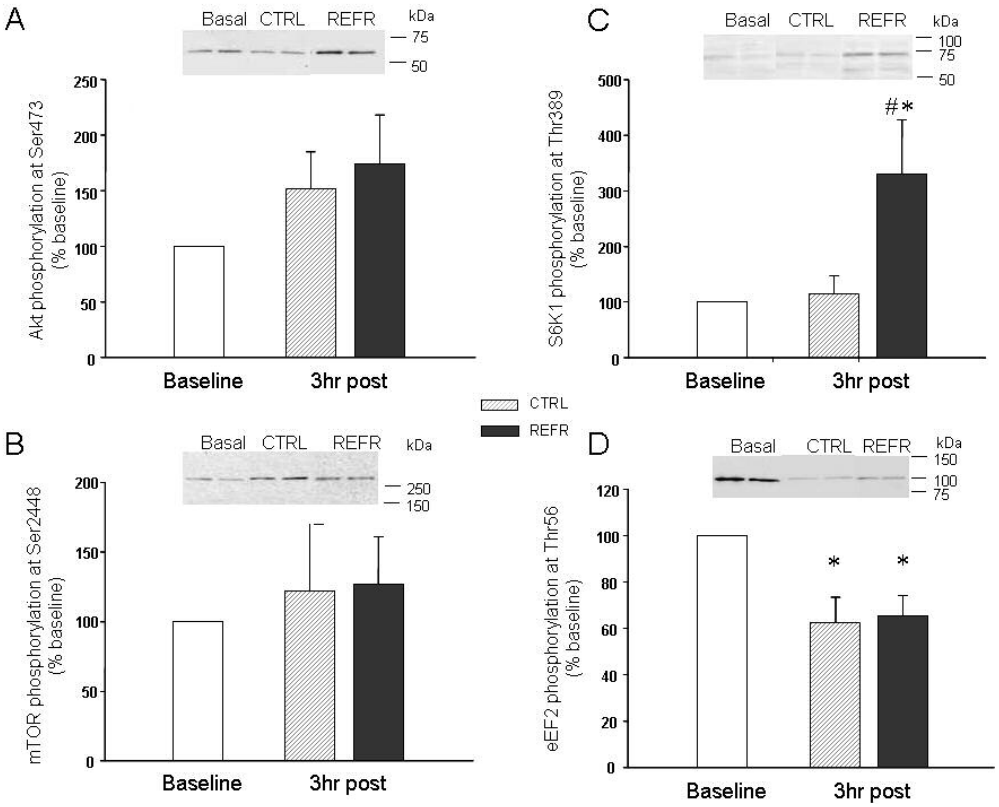
15x9mm (600 x 600 DPI)



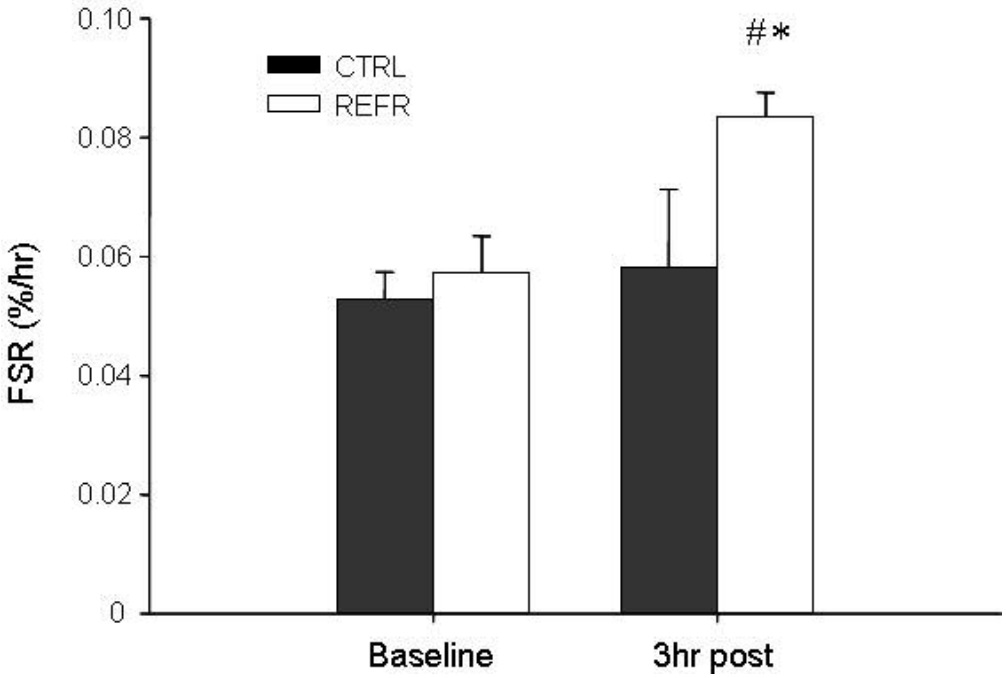
24x30mm (600 x 600 DPI)



40x30mm (600 x 600 DPI)



37x30mm (600 x 600 DPI)



23x16mm (600 x 600 DPI)