Activation of mTORC1 signaling and protein synthesis in human muscle following blood flow restriction exercise is inhibited by rapamycin

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Gundermann DM, Walker DK, Reidy PT, Borack MS, Dickinson JM, Volpi E, Rasmussen BB. Activation of mTORC1 signaling and protein synthesis in human muscle following blood flow restriction exercise is inhibited by rapamycin. Am J Physiol Endocrinol Metab 306: E1198–E1204, 2014. First published April 1, 2014; doi:10.1152/ajpendo.00600.2013.—Restoration of blood flow to a contracting muscle during low-intensity resistance exercise (BFR exercise) stimulates mTORC1 signaling and protein synthesis in human muscle within 3 h postexercise. However, there is a lack of mechanistic data to provide a direct link between mTORC1 activation and protein synthesis in human skeletal muscle following BFR exercise. Therefore, the primary purpose of this study was to determine whether mTORC1 signaling is necessary for stimulating muscle protein synthesis after BFR exercise. A secondary aim was to describe the 24-h time course response in muscle protein synthesis and breakdown following BFR exercise. Sixteen healthy young men were randomized to one of two groups. Both the control (CON) and rapamycin (RAP) groups completed BFR exercise; however, RAP was administered 16 mg of the mTOR inhibitor rapamycin 1 h prior to BFR exercise. BFR exercise consisted of four sets of leg extension exercise at 20% of 1 RM. Muscle biopsies were collected from the vastus lateralis before exercise and at 3, 6, and 24 h after BFR exercise. Mixed-muscle protein fractional synthetic rate increased by 42% at 3 h postexercise and 69% at 24 h postexercise in CON, whereas this increase was inhibited in the RAP group. Phosphorylation of mTOR (Ser2448) and S6K1 (Thr389) was also increased in CON but inhibited in RAP. Mixed-muscle protein breakdown was not significantly different across time or groups. We conclude that activation of mTORC1 signaling and protein synthesis in human muscle following BFR exercise is inhibited in the presence of rapamycin.

blood flow restriction exercise; mammalian target of rapamycin; mammalian target of rapamycin complex 1; rapamycin; fractional synthetic rate

LOW-INTENSITY RESISTANCE EXERCISE with local vascular occlusion, termed “blood flow restriction (BFR) exercise,” has been identified for several years as a unique method to increase skeletal muscle size and strength. This method of exercise is attractive to populations with an increased susceptibility to muscle loss (e.g., aging) due to a compromised ability to lift heavy weight (17) and/or achieve maximal rates of muscle growth with traditional resistance exercise (15, 17). To date, researchers have observed clear benefits of BFR exercise and/or any potential changes in breakdown. In addition, we also sought to determine whether the muscle protein synthetic response persists for up to 24 h and whether muscle protein breakdown plays an acute role in the muscle metabolic response following BFR exercise. We hypothesized that mTORC1 signaling and human muscle protein synthesis would be inhibited by the administration of the mTORC1 inhibitor rapamycin. In addition, we proposed that the 24-h protein
synthesis time course would closely match the typical pattern observed with high-intensity exercise (15).

**EXPERIMENTAL PROCEDURES**

*Subjects.* Sixteen young, healthy, recreationally active males (25.5 ± 0.8 yr, 179.4 ± 1.7 cm, 82.4 ± 2.4 kg, and 24.8 ± 1.6% body fat) volunteered for this study. The inclusion criteria included nonsedentary and untrained, recreationally active males with a BMI between 20 and 30 and between 18 and 35 yr of age. Subjects were then randomly assigned to two groups and compared to verify that there were no significant differences between groups for age, weight, height, strength, BMI, and body fat percentage. Each subject gave written, informed consent before participating in the study, which was approved by the Institutional Review Board of the University of Texas Medical Branch. Screening was performed with clinical history and physical exams, electrocardiogram, and laboratory tests, including complete blood count with differential liver, kidney, and thyroid function tests, coagulation profile, fasting blood glucose, oral glucose tolerance test, hepatitis B and C screening, HIV test, urinalysis, and drug screening. A variety of anthropometric measurements were taken along with a dual-energy X-ray absorptiometry scan (Hologic DQR 4500W, Bedford, MA) and a 1-repetition maximum (1-RM) leg extension strength test. The strength test was performed on two occasions (>5 days apart) on a bilateral leg extension machine (Cybex-VR2, Medway, MA) located in the Institute for Translational Sciences Clinical Research Center (ITS-CRC) Exercise Laboratory. The strength test consisted of an initial warmup phase, with the subject performing 10 repetitions at a moderate weight to get accustomed to the machine. Following the warmup, subjects attempted to complete one repetition of the leg extension exercise at progressively greater resistance until a full repetition could no longer be performed. The weight of the last complete unassisted repetition was recorded as the 1-RM. The highest weight achieved from the two strength tests was deemed their 1-RM. The average 1-RM was (132.4 ± 3.4 kg).

*Study design.* Each subject was randomized into one of two treatments groups. Both treatments included an identical BFR exercise protocol (Fig. 1), with the exception that one group ingested 16 mg (1-mg tablets) of rapamycin (Rapamune/Sirolimus; Wyeth) (RAP), whereas the other group did not ingest rapamycin (CON).

The exercise study included 2 days of evaluation and lasted 11.5 h on the 1st day and 4.5 h on the morning of the 2nd day. The subjects were admitted to the ITS-CRC the evening before the exercise study and were fed a standard research dinner (10 kcal/kg of body wt; 60% carbohydrate, 20% fat, and 20% protein) and a snack before 2200 on the evening before each study day. Each day they were fasted overnight under basal conditions and given ad libitum access to water. On the morning of each day, a polyethylene catheter was inserted into an antecubital vein for tracer infusion, and another polyethylene catheter was inserted retrogradely into a hand vein of the opposite arm and kept under a heating pad for arterialized blood sampling. After a background blood sample was drawn, a primed continuous infusion of L-[ring-13C6] phenylalanine and [15N]phenylalanine (Isotec; Sigma-Aldrich, Miamisburg, OH) was begun and maintained at a constant rate. The L-[ring-13C6]phenylalanine was maintained throughout for the measurement of mixed-muscle fractional synthetic rate, whereas the [15N]phenylalanine infusion was arrested at specific periods where the plasma decay was used to measure the fractional breakdown rates at specific periods (40). The priming dose for each labeled phenylalanine was 2 μmol/kg, and the infusion rate was 0.05 μmol·kg⁻¹·min⁻¹.

On the morning of the 1st day of the experimental trial, subjects randomly assigned to the RAP group ingested 16 mg of rapamycin 1 h prior to exercise (Fig. 1). Previously, this dosage has been validated to appear at peak concentrations in the bloodstream after 1 h and to blunt the increase in mTORC1 activation in humans in response to amino acids or high-intensity resistance exercise (8, 11) while not affecting basal muscle metabolism or basal mTORC1 signaling (7). Subjects randomly assigned to the CON group did not ingest rapamycin yet still remained at rest for an additional hour. As shown in Fig. 1, the tracer infusion was stopped at 6 h postexercise, and subjects were fed lunch and a few hours later a research dinner with the identical nutrient composition, as provided the evening before. Subjects then spent a second night in the ITS-CRC.

*Exercise.* Subjects were transported from the bed to the leg extension exercise machine to complete BFR exercise. Immediately prior to the commencement of exercise, subjects were fitted with 11-cm-wide pressure cuffs (Hokanson SC10, Bellevue, WA) placed on the most proximal portion of the upper thighs and attached to a Hokanson E20 Rapid Cuff Inflater and AG101 Air Source. Subjects were gradually acclimated to the pressure cuff in increments of 20 mmHg starting at 120 mmHg to a final pressure of 200 mmHg. During the acclimation phase, each increment consisted of 30 s of pressure followed by 10 s...
of no pressure. Exercise commenced once the final pressure of 200 mmHg was reached. The exercise consisted of four sets of bilateral leg extensions to 20% of their 1-RM performing 30, 15, 15, and 15 repetitions, respectively, with 30 s of rest between sets. Verbal encouragement was used to ensure that each subject completed the full exercise protocol. The pressure was not released until the completion of the final set of exercise.

Muscle sampling. Seven muscle biopsies were performed over the 2-day trial with a 5-mm Bergström biopsy needle utilizing sterile procedures and local anesthesia (1% lidocaine). Prior to exercise on the 1st day, two muscle biopsies were obtained from the lateral portion of the vastus lateralis muscle between 15 and 25 cm superior to the midpatella. The first biopsy was collected 2 h after the initiation of the tracer infusion to allow for steady-state enrichments and the second biopsy 2.5 h later for the measurement of basal muscle metabolism. Both biopsies were sampled from a common incision on the left leg, although the orientation of the needle was angled so that samples were collected ~5 cm from each other. It has been demonstrated that sequential biopsies from the same incision do not interfere with muscle protein synthesis (MPS) measurements (32). The third and fourth biopsies were collected at 0 and 3 h postexercise from the right leg. A fifth muscle biopsy was collected at 6 h postexercise in a new incision on the right leg made proximal to the first incision site. Finally, the sixth and seventh muscle biopsies were collected at 22 and 24 h postexercise, respectively, the following morning from a common incision on the left leg made proximal to the first incision site. All muscle tissue was immediately blotted, frozen in liquid nitrogen, and stored at −80°C until analysis.

Blood sampling. Arterialized blood was collected from the retrograde hand catheter 26 times during the study (Fig. 1). Each time, 2 ml was aliquoted for the determination of the enrichment of labeled phenylalanine in blood using gas chromatography-mass spectrometry (GC-MS). Phenylalanine concentration was calculated using an internal standard approach, as described by Wolfe and Chinkes (37).

Mixed-muscle protein fractional synthetic and breakdown rates. Muscle intracellular free amino acids and mixed-muscle proteins were extracted as described previously (33, 34). Enrichment of intracellular free phenylalanine was determined by GC-MS (6890 Plus GC, 5973N MSD, 7683 autosampler; Agilent Technologies, Palo Alto, CA) and used with an appropriate internal standard ([1-13C6]phenylalanine) (33, 34) to determine intracellular free phenylalanine concentration. Mixed-muscle protein-bound phenylalanine enrichment was analyzed by GC-MS after protein hydrolysis and amino acid extraction with the external standard curve approach (6).

The fractional synthetic rate (FSR) of mixed-muscle proteins was calculated by measuring the incorporation rate of the phenylalanine tracer into the proteins (ΔEp/t) and using the precursor-product principle to calculate the synthesis rate:

\[ FSR = \frac{\Delta E_p}{t} \left( \frac{E_{M1} + E_{M2}}{2} \right) \cdot 100 \]

where \( \Delta E_p \) is the increment in protein-bound [ring-13C6]phenylalanine enrichment between two biopsies, \( t \) is the time between the two biopsies, and \( E_{M1} \) and \( E_{M2} \) are the phenylalanine enrichments in the free intracellular pool in the two biopsies. Data are expressed as percent per hour.

The fractional breakdown rate (FBR) of mixed-muscle proteins was calculated with the [1-15N]phenylalanine tracer using the trace release method (40). This method requires the intracellular free phenylalanine enrichment to reach steady state prior to the [1-15N]phenylalanine tracer being stopped. The muscle intracellular enrichment decay can be determined by the arterial 1-h plasma tracer decay with frequent arterialized blood sampling as well as the free and bound muscle intracellular phenylalanine content. FBR was calculated using the formula

\[ FBR = \frac{E_{M(t)} - E_{M(t-t)}}{p \cdot \int_{t_1}^{t_2} E_{M(t)} \cdot dt} - \frac{Q_M}{T} \]

where \( E_{M(t)} \) and \( E_{M(t-t)} \) are the arterialized and muscle free enrichments at time \( t \) and \( t-t \) are two time points, \( P = E_{FA}/(E_{FA} - E_{MB}) \) at plateau, \( E_A \) and \( E_M \) are enrichments in the arterial pool and muscle intracellular pool, respectively, and \( Q_M/T \) is the ratio of free to bound phenylalanine in muscle. Data are expressed as percent per hour.

Net balance was calculated from the subtraction of FBR from FSR. Data are expressed as percent per hour.

**Results**

Our primary objective was to compare FSR responses following BFR exercise between groups and over a 24-h time course. Basal values for mixed-muscle protein FSR were similar between groups (P > 0.05; Fig. 2). FSR increased significantly by 41.5% (P < 0.05) in the CON group at 3 h postexercise and returned to baseline at 6 h postexercise. However, FSR measured across the entire 6-h period postexercise was significantly elevated by 30% compared with baseline (P < 0.05). At 24 h postexercise, FSR was elevated by 69.4% (P < 0.05) compared with basal values. The FSR in the RAP group was unchanged at all time points (P > 0.05; Fig. 2). Specifically, there was a <1% increase in FSR at 3 or 6 h and a nonsignificant 25% increase at 24 h postexercise in the RAP group. Because the RAP group had on average a slightly higher resting FSR and a small but insignificant increase in
FSR at 24 h postexercise, we were unable to detect group differences during postexercise recovery ($P > 0.05$; Fig. 2).

The differences in the protein-synthetic response between groups showed a similar pattern when compared with mTORC1 signaling. Phosphorylation of mTOR at Ser$^{2448}$ increased in the CON group at all time points postexercise and was elevated above the RAP group at 6 and 24 h postexercise ($P < 0.05$; Figs. 3 and 4). Conversely, mTOR phosphorylation remained at basal levels in the RAP group at all time points postexercise ($P > 0.05$). Phosphorylation of S6K1 at Thr$^{389}$ increased at all time points in the CON group postexercise ($P < 0.05$), whereas S6K1 phosphorylation did not increase at any time points postexercise in the RAP group ($P > 0.05$) and in fact decreased at 6 and 24 h postexercise ($P < 0.05$; Figs. 3 and 4). Because of the established correlation between mTORC1 and MAPK signaling following BFR exercise (17, 20), the phosphorylation status of the ERK1/2 and Mnk1 signaling proteins were also examined. The phosphorylation of ERK1/2 trended only to increase at each time point postexercise in the CON group ($P > 0.05$). Phosphorylation of ERK1/2 remained at basal values in the RAP group ($P > 0.05$). Phosphorylation of Mnk1 also trended to increase in the CON group and was significantly elevated above basal values at 24 h postexercise ($P < 0.05$; Figs. 3 and 4). Phosphorylation of Mnk1 remained at basal values in the RAP group ($P > 0.05$).

A secondary objective was to uncover the role of proteolysis from BFR exercise. The FBR remained unchanged throughout the study, with no significant differences between groups or time ($P > 0.05$; Fig. 5). Thus, the net balance was improved significantly from baseline to 24 h postexercise in the CON group due to the larger increase in FSR ($P < 0.05$; Fig. 6). On the contrary, net protein balance was unchanged from baseline to 24 h postexercise in the RAP group ($P > 0.05$; Fig. 6).

DISCUSSION

The primary purpose of this study was to determine whether mTORC1 activation is necessary to increase muscle protein synthesis following acute BFR exercise. Using the mTOR inhibitor rapamycin, we show for the first time that activation of mTORC1 signaling and muscle protein synthesis following BFR exercise is impaired when rapamycin is administered to human subjects prior to exercise.

The use of rapamycin in human clinical trials has been employed previously to investigate the necessity of mTORC1 activity in the regulation of muscle protein synthesis following high-intensity resistance exercise (11) and following the ingestion of essential amino acids (8). The phosphorylation of mTOR and its primary downstream effector S6K1 was quantified to verify the effectiveness of rapamycin inhibiting mTORC1 signaling. In agreement with previous studies, 16 mg of rapamycin, as used in this study, was sufficient to block the increase in the phosphorylation of mTOR and S6K1 at all time points within the RAP group. Accordingly, the concomitant increase in muscle protein synthesis following BFR exercise was also inhibited by rapamycin at all time points postexercise, indicating that the muscle protein-synthetic response is at least partially dependent on a rapamycin-sensitive pathway.

Because of the low-load, short-duration nature of BFR exercise, it is tempting to speculate that the mechanism through which BFR exercise confers the hypertrophic response is more complex than the mechanotransduction that is thought to confer muscle hypertrophy in traditional high-resistance exercise (14, 21). However, it appears that such a complex system may still require mTORC1 to increase muscle protein synthesis. Despite the progressive decline in S6K1 phosphorylation throughout the 24-h period in the rapamycin group, there was a nonsignificant trend for an elevated FSR in the RAP group at 24 h postexercise. No direct conclusions can be made from this observation; however, mTORC1-independent mechanisms may also be involved in the regulation of muscle protein synthesis following BFR exercise. For instance, previous studies have shown that MAPK signaling is increased with BFR exercise, including the phosphorylation of ERK1/2 and Mnk1 (17, 20), and activation of this pathway is known to enhance protein synthesis through an mTORC1-independent mechanism (10). The phosphorylation of ERK1/2 in the current study, although elevated, never increased significantly above basal values at 3, 6, or 24 h postexercise. This is not surprising since changes in ERK1/2 are reported to be rapid and transient in response to resistance exercise (22). Accordingly, the BFR exercise literature has detected a significant elevation only in ERK1/2 during the 1st hour of postexercise recovery (17). Mnk1, on the other hand, acting downstream of ERK1/2, is thought to produce a more sustained effect, and our data indicate a significant increase at 24 h postexercise. Interestingly, this response is blunted in the RAP group despite the accepted idea that ERK/Mnk signaling occurs independent of mTORC1 signaling. Although this is perplexing, Drummond et al. (11) also observed this phenomenon with resistance exercise and concluded that rapamycin may indirectly inhibit ERK/Mnk signaling through an unidentified mechanism. Nonetheless, data from the current study indicate that signaling through mTORC1 is important for the BFR exercise-induced increase in muscle protein synthesis. Concurrently, it may also be possible that mTORC1 indirectly contributes to ERK/Mnk signaling, and both mTORC1 and ERK1/2 signaling are necessary for the resulting increase in muscle protein synthesis. Our data suggest that mTOR-independent mechanisms have a minimal or possibly only a permissive role in the increase in muscle protein synthesis following BFR exercise.
The time course of postexercise muscle protein synthesis from a single bout of BFR exercise compared with previously published studies of traditional high-resistance exercise appears to follow a unique pattern. Both Fry et al. (15) and Phillips et al. (30) observed a sustained elevation in mixed-muscle protein synthesis for up to 24 h following a bout of resistance exercise. The increase in FSR at 3 h postexercise is consistent with data from previous BFR exercise studies conducted in our laboratory (17, 18, 20). However, muscle protein synthesis returned to basal levels 6 h postexercise, unlike with high-intensity exercise, which remained elevated at 6 h (15). The phosphorylation of S6K1 followed a similar trend as FSR, although it did not return to baseline at 6 h postexercise. This response pattern may suggest that the postexercise muscle protein synthesis response occurs in phases such as an immediate acute response and a delayed longer lasting response, which may also suggest responses from multiple stimuli (i.e., mechanical and metabolic). For example, several investigators studying BFR exercise have measured a similar increase in myofiber recruitment compared with high-intensity exercise (26, 31, 36). It is possible that the low mechanical stress works in concert with metabolic stress, resulting ultimately in a full hypertrophic response. An alternative explanation to the biphasic response is that BFR exercise increases skeletal muscle sensitization to ingested nutrients, and the 24-h increase in MPS persists from the last meal prior to the overnight fast. In either case, further research is necessary to investigate the upstream stimuli involved in BFR exercise to grasp a better understanding of this biphasic response.

Considering that muscle hypertrophy can be regulated by alterations in the rates of protein synthesis or degradation, this study was designed to also investigate muscle protein breakdown (MPB). A limited number of studies have investigated the role of MPB following resistance exercise, with conflicting results. Research by Phillips et al. (30) indicated that the rate of MPB increases 3 h postexercise and remains elevated for 24 h, similar to the rise in FSR. On the contrary, Fry et al. (16) did not observe any differences in the rates of MPB 24 h post-exercise but concluded that the rise in breakdown rates may have occurred during time points not measured.

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These two studies were marginally different in that Fry et al. (16) investigated an exercise intensity of 70% 1-RM, whereas Phillips et al. (30) investigated 80% 1-RM. It is possible that MPB is sensitive only to extremely high-intensity exercise. Results from the current study indicate that the rate of MPB after BFR exercise does not change at 6 or 24 h postexercise compared with basal values. However, if the rate of protein breakdown truly follows the time course for the initial rise in FSR, then it is possible that this study also missed the increase in MPB, which may have been elevated at 3 h postexercise and returned to baseline at 6 h. On the other hand, it is possible that MPB is not increased in association with the increase in FSR following BFR exercise, as opposed to traditional resistance exercise, and instead follows a pattern unique to BFR exercise. Markers of proteolysis following BFR exercise have been reported in the literature in a variety of ways with unique results. For example, the mRNA expression of muscle RING finger 1 (MuRF1), but not atrogin-1, has been shown to be increased 3 h post-BFR exercise in the fasted state (12, 20). On the other hand, in a postprandial state, MuRF1 mRNA expression decreases at 8 h post-BFR exercise (26). Regardless, these transient changes in MuRF1 mRNA expression seem to have little impact on the overall MPB response. The role of exercise-induced increases in MPB is less understood than muscle protein synthesis. With that in mind, MPB may play a partial role in the hypertrophic response with BFR training if the low-intensity nature of BFR exercise is not sufficient to stimulate a large increase or greater duration of MPB compared with traditional resistance exercise.

One potential limitation of administering rapamycin to human volunteers is that we are unable to provide a dosage large enough to guarantee complete inhibition of mTORC1. In fact, the rapamycin dose used in our study is much less than that commonly used in rodent experiments. Another consideration is that there are important limitations of rapamycin and its ability to completely inhibit mTORC1 signaling. A thorough assessment of this topic can be found in a recent and comprehensive review (24). Those authors highlighted several limitations, including partial inhibition of mTORC1 signaling, as demonstrated by a differential regulation of S6K1 (rapamycin sensitive) and 4E-BP1 (rapamycin insensitive) and the presence of several compensatory mechanisms and negative feedback loops that can alter the activation status of the mTORC1-signaling pathway. Future work examining the effectiveness of improved or more specific mTORC1 inhibitors will be useful in identifying the precise role of mTORC1 signaling in the regulation of muscle protein synthesis following exposure to anabolic stimuli.

In summary, our research demonstrates that the activation of mTORC1 signaling and muscle protein synthesis response following BFR exercise is impaired in the presence of rapamycin. Furthermore, MPB appears to play a minimal role, if any, in the muscle metabolic response to BFR exercise. Finally, the rise in muscle protein synthesis appears to be biphasic and is elevated 24 h postexercise, suggesting the possibility of multiple stimuli or enhanced anabolic sensitivity. Further research is needed to clarify other cellular mechanisms that may be involved in the muscle growth response following both acute BFR exercise and BFR exercise training.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

REFERENCES


