The order of concurrent endurance and resistance exercise modifies mTOR signaling and protein synthesis in rat skeletal muscle

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Ogasawara R, Sato K, Matsutani K, Nakazato K, Fujita S. The order of concurrent endurance and resistance exercise modifies mTOR signaling and protein synthesis in rat skeletal muscle. Am J Physiol Endocrinol Metab 306: E1155–E1162, 2014. First published April 1, 2014; doi:10.1152/ajpendo.00647.2013.—Concurrent training, a combination of endurance (EE) and resistance exercise (RE) performed in succession, may compromise the muscle hypertrophic adaptations induced by RE alone. However, little is known about the molecular signaling interactions underlying the changes in skeletal muscle adaptation during concurrent training. Here, we used an animal model to investigate whether EE before or after RE affects the molecular signaling associated with muscle protein synthesis, specifically the interaction between EE-induced mammalian target of rapamycin complex 1 (mTORC1) signaling and EE-induced AMP-activated protein kinase (AMPK) signaling. Male Sprague-Dawley rats were divided into five groups: an EE group (treadmill, 25 m/min, 60 min), an RE group (maximum isometric contraction via percutaneous electrical stimulation for 3 × 10 s, 5 sets), an EE before RE group, an EE after RE group, and a nonexercise control group. Phosphorylation of p70S6K, a marker of mTORC1 activity, was significantly increased 3 h after RE in both the EE before RE and EE after RE groups, but the increase was smaller in latter. Furthermore, protein synthesis was greatly increased 6 h after RE in the EE before RE group. Increases in the phosphorylation of AMPK and Raptor were observed only in the EE after RE group.Akt and mTOR phosphorylation were increased in both groups, with no between-group differences. Our results suggest that the last bout of exercise dictates the molecular responses and that mTORC1 signaling induced by any prior bout of RE may be downregulated by a subsequent bout of EE.

Concurrent exercise; mammalian target of rapamycin complex 1; adenosine 5′-monophosphate-activated protein kinase; resistance exercise; endurance exercise

SKELETAL MUSCLE IS A HIGHLY PLASTIC TISSUE that adapts differently to different stimuli, including various types of muscle contractions. Chronic muscle contraction induces diverse metabolic and morphological adaptations because of the cumulative effects of repeated bouts of exercise, and certain molecular and cellular responses lead to specific adaptations (3, 10, 47). The well-known adaptive features of chronic muscle contraction include muscle hypertrophy and increased strength. These features are induced by high-intensity muscle contraction, such as that involved in resistance exercises (RE) (50, 52). Other typical adaptations are increases in oxidative capacity and glucose uptake, which are induced by low-intensity, high-volume muscle contraction, such as that observed during endurance exercises (EE) (5, 28, 53).

Concurrent training, a combination of EE and RE in succession, is used widely in exercise prescription to simultaneously improve muscular strength and cardiovascular function. Interestingly, accumulating evidence indicates that this combination of the two exercise modes may hamper strength and muscle hypertrophic adaptations to RE (27, 35, 46, 64). However, studies on the effects of physiological adaptations of RE on RE-induced muscle hypertrophy have shown mixed results (43, 64). Intriguingly, a recent study in humans reported that EE performed prior to RE increased muscle mass to a greater extent than long-term RE alone (42), suggesting that EE may not always attenuate RE-induced muscle hypertrophy.

The mammalian target of rapamycin complex 1 (mTORC1) is known to play a critical role in regulating muscle mass in response to a wide range of stimuli, including mechanical stress, nutrients, growth factors, and hormones, mainly by regulating translation initiation and muscle protein synthesis (32, 56, 60). Numerous studies have reported an increase in mTORC1 signaling (e.g., an increase in phosphorylation of downstream targets of mTORC1 such as p70S6K) after RE in both animal and human models (4, 15, 16, 20, 22, 49, 51), and some studies have reported that muscle contraction-induced increases in muscle protein synthesis and subsequent muscle hypertrophy are almost fully dependent on the activation of mTORC1 within the skeletal muscle (17, 23). On the other hand, AMP-activated protein kinase (AMPK), which is a key sensor of cellular energy status, is known to be partly responsible for skeletal muscle adaptations to EE training (31, 38, 66, 67). The upstream mechanisms regulating mTORC1 activation are complex and have not been fully characterized. However, previous studies have reported that 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR)-induced AMPK activation suppressed muscle protein synthesis and mTORC1 signaling (6, 62). Furthermore, AICAR-induced AMPK activation also attenuated a high-intensity muscle contraction-induced mTORC1 signaling response (57), indicating antagonistic interactions between the mTORC1 and AMPK signaling pathways. EE-induced AMPK activation is known to negatively affect mTORC1 activation (3, 63). Therefore, the attenuation of RE-induced muscle hypertrophy by EE may be triggered by EE-induced AMPK activation. However, although AICAR-induced AMPK activation is relatively prolonged after AICAR injection (57), exercise-induced AMPK activation lasts a short time, although it is stimulated immediately after exercise (54, 65). Therefore, during concurrent training, EE-
induced AMPK activation may differentially affect RE-induced mTORC1 activation, depending on the exercise order.

Therefore, the purpose of the present study was to examine whether EE before or after RE may differentially affect mTORC1 activation in a basic animal model. The purpose of the present study was to examine whether EE before or after RE differentially affects mTORC1 activation in a basic animal model. To this end, we induced muscle contraction via electrical stimulation and assessed the subsequent mTORC1 activation. Furthermore, we used treadmill running to simulate EE and activate AMPK signaling. We hypothesized that EE-induced AMPK activation after RE would attenuate mTORC1 activation, whereas that before RE would not.

MATERIALS AND METHODS

Animals

Ten-week-old male Sprague-Dawley rats, weighing 310–340 g, were obtained from CLEA Japan (Tokyo, Japan). All of the rats were housed for 1 wk in an environment maintained at 22–24°C in a 12-h light-dark cycle, and were allowed food (CE2; CLEA Japan) and water ad libitum. The animal protocol and facilities were reviewed and approved by the Ethics Committee for Animal Experiments at Ritsumeikan University.

Experiment 1

We evaluated the time course of signaling protein responses to EE (EE group) or RE (RE group) alone following a 12-h overnight fast, as detailed below. The animals were euthanized at 0, 1, or 3 h after completion of the exercise routine. Nonexercise control animals (CON group) were euthanized at a basal state. Tissues were rapidly frozen in liquid nitrogen and stored at −80°C until use.

Experiment 2

We evaluated whether EE performed before or after RE would differentially affect mTORC1 activation. After a 12-h overnight fast, the rats were randomly assigned to either the EE before RE group or the EE after RE group. A 1-h rest period was provided between exercises. The animals in both groups were euthanized at 3 and 6 h after the completion of the exercise. A CON group was included in this experiment as well. Tissues were rapidly frozen in liquid nitrogen and stored at −80°C until use.

RE Protocol

Under isoflurane anesthesia, the hair was shaved off from the right lower leg of each rat, and the shaved legs were cleaned with alcohol wipes. The rats were then positioned with their right foot on a footplate (the ankle joint angle was positioned at 90°) in the prone posture. The triceps surae muscle was stimulated percutaneously with electrodes (Vitrode V, Ag/AgCl; Nihon Kohden, Tokyo, Japan), which were cut into 10 × 5-mm sections and connected to an electric stimulator and isolator (SS-104J; Nihon Kohden). The right gastrocnemius muscle was cut into 10 contractions, with a 7-s interval between contractions, for 5 sets with 3-min rest intervals, whereas the left gastrocnemius muscle served as an internal control. The voltage (~30 V) and stimulation frequency (100 Hz) were adjusted to produce maximal isometric tension. In a previous study, we demonstrated that this exercise protocol induces significant muscle hypertrophy, simulating long-term training (50).

The high-frequency electrical stimulation model has the advantage of ensuring highly controlled muscle activation and loading parameters compared with volitional muscle activation models such as the squat and ladder training models. Furthermore, although volitional muscle activation models are known to increase anabolic signaling activity, they have often failed to increase muscle weight after repeated bouts of exercise, which is due probably to the insufficient exercise load or the exercise volume added to the muscle (reviewed in Ref. 1).

EE Protocol

The rats were familiarized with running on a rodent treadmill at 10–20 m/min for 3 days prior to the experiment. Subsequently, they were placed on a flat treadmill and made to run for 60 min at a speed of 25 m/min, which corresponds approximately to the lactate threshold (36).

Regarding the choice of EE model, the low-frequency electrical stimulation is often used to mimic the EE training. However, typical adaptations require repeated bouts of 3 h or more low-frequency electrical stimulation (40), and an in situ model is often preferred (3). Furthermore, our recent study indicated that high-frequency electrical stimulation triggered similar muscle anabolic effects as high-frequency electrical stimulation when stimulation parameters, including contraction time and voltage, were identical (58). Therefore, we chose the treadmill model to simulate the physiological response to a bout of EE.

Measurement of Serum Insulin-Like Growth Factor I and Insulin Concentrations

Insulin-like growth factor I (IGF-I) levels in the serum and insulin levels in the plasma were determined using the mouse/rat IGF-I Quantikine ELISA kit (R & D Systems) and rat insulin ELISA kit (Shibayagi, Japan), respectively, according to the manufacturer’s instructions.

Western Blotting Analysis

Western blotting analysis was performed as reported previously (50). In brief, muscle samples were homogenized with a Polytron homogenizer in a homogenization buffer containing 20 mM Tris-HCl (pH 7.5), 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, and protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The homogenates were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected, and the protein concentration of each sample was determined using a protein concentration determination kit (Protein Assay Rapid kit; WAKO). The samples were diluted in 3x sample buffer and boiled at 95°C for 5 min. Using 5–20% sodium dodecyl sulfate-polyacrylamide gradient gels, 50 μg of protein was separated using electrophoresis and subsequently transferred to polyvinylidene difluoride membranes. After transfer, the membranes were washed in Tris-buffered saline containing 0.1% Tween-20 (TBST) and blocked with 5% powdered milk in TBST for 1 h at room temperature. Next, the membranes were washed and incubated overnight at 4°C with primary antibodies, namely phosphorylated (phospho) Akt (Thr308, cat. no. 9278), phosphorylated Akt (Ser473, cat. no. 9272), total Akt (cat. no. 9272), phospho-Raptor (Ser2483), total Raptor (cat. no. 2280), phospho-mTOR (Ser2483), total mTOR (cat. no. 9271), total p70S6K (Ser389, cat. no. 9205), total p70S6K (cat. no. 2708), phospho-AMPK (Thr172, cat. no. 2535), and total AMPK (cat. no. 2532) (Cell Signaling Technology). The membranes were then washed again in TBST and incubated for 1 h at room temperature with the appropriate secondary antibodies. Chemiluminescent reagents (ECL Plus; GE Healthcare) were used to facilitate the detection of protein bands. The images were scanned using a chemiluminescence detector (ImageQuant LAS 4000; GE Healthcare). After the scan, the membranes were stained with Coomassie blue to verify equal loading in all lanes (61). Band intensities were quantified using Image J 1.46 software (National Institutes of Health).
Muscle Protein Synthesis

Muscle protein synthesis was measured using the in vivo surface sensing of translation or SUnSET method (24, 29, 33). Briefly, 0.04 μmol puromycin/g body wt (Calbiochem) diluted using a 0.02 mol/l PBS stock solution was injected into the rats intraperitoneally after 5 min of anesthesia, and muscle was removed exactly 15 min after puromycin administration. Following homogenization and centrifugation at 2,000 g for 3 min at 4°C, the supernatant was collected and processed for Western blotting. A mouse monoclonal anti-puromycin antibody (KeraFAST) was used to detect puromycin incorporation, which was evaluated as the sum of the intensity of all protein bands in the Western blot.

Statistical Analyses

One-way analysis of variance was used to evaluate changes in the phosphorylation of signaling proteins. Post hoc analyses were performed using t-tests, with a Benjamini and Hochberg false discovery rate correction for multiple comparisons when appropriate. All values were expressed as means ± SE. The level of significance was set at P < 0.05.

RESULTS

Experiment 1

Effect of EE. The phosphorylation status of signaling proteins in response to EE alone is shown in Figs. 1 and 2. EE increased AMPK phosphorylation immediately after it was performed, but the phosphorylation returned to the basal levels within 1 h postexercise. Similarly, Raptor phosphorylation was significantly increased immediately after exercise but returned to the control level at 1 h postexercise. Additionally, Akt (both Thr308 and Ser473), mTOR, and p70S6K showed the same trend.

Effect of RE. The phosphorylation status of signaling proteins in response to RE alone is shown in Figs. 3 and 4. AMPK and Raptor phosphorylation were increased immediately after RE but returned to the control levels within 3 h. A gradual increase in p70S6K phosphorylation was seen after RE. Akt (both Thr308 and Ser473) and mTOR phosphorylation were significantly increased immediately after RE and remained high for ≥ 3 h after RE, except for Akt Thr308 at 3 h.

Experiment 2

The serum IGF-I and plasma insulin concentrations did not change in either the EE before RE or EE after RE group, and these parameters did not differ between the groups (Table 1). The phosphorylation status of signaling proteins 3 h after the completion of exercise is shown in Figs. 5 and 6. Although no changes were observed in AMPK or Raptor phosphorylation in the EE before RE group, the phosphorylation of these signaling proteins increased immediately after exercise and returned to the control level at 1 h postexercise. Additionally, Akt (both Thr308 and Ser473), mTOR, and p70S6K showed the same trend.
components was significantly increased in the EE after RE group. The phosphorylation of p70S6K increased regardless of the exercise order. However, this increase was significantly lower in the EE after RE group than in the EE before RE group. In contrast, although phosphorylation of Akt Ser^{473} and mTOR was increased following exercise, no significant difference was found between the exercise order groups. No significant change in Akt Thr^{308} phosphorylation with exercise was observed.

Muscle protein synthesis was increased threefold in the EE before RE group, whereas it was significantly blunted in the EE after RE group (Fig. 7).

DISCUSSION

In this study, we investigated whether the order of concurrent exercise (i.e., EE before or after RE) differentially affects mTORC1 activation. To the best of our knowledge, this is the first study to show in an animal model that the order of EE and RE in concurrent training affects RE-induced p70S6K phosphorylation, which is a marker of mTORC1 activation as well as muscle protein synthesis. In other words, EE performed after RE resulted in attenuated p70S6K phosphorylation and muscle protein anabolism, unlike the opposite exercise order. These data provide important new information regarding the interaction between RE- and EE-induced molecular and protein metabolic responses.

Although no prior animal studies have examined the effect of concurrent exercise on mTORC1 signaling responses, a few human studies have investigated this topic. However, most of these human studies found no significant increase in p70S6K phosphorylation in response to either RE alone or concurrent training (14, 41). In addition, these previous human studies have compared either concurrent training to RE alone (2, 14, 41) or mTORC1 signaling after a second bout of concurrent exercise, which was either EE or RE (12). Thus, these studies could not distinguish the cumulative effect of combined exercise bouts or the time course of mTORC1 activation following the last exercise bout.

Our study model was designed specifically to investigate the changes in RE-induced upregulation of mTORC1 signaling when EE was performed before or after RE. Hence, in all animal groups, the muscle samples were collected 3 h after high-intensity muscle contraction. We used electrical stimulation to simulate RE and induce maximal muscle contraction and activate mTORC1 signaling. Previous studies using electrical stimulation reported elevated phosphorylation of mTORC1 signaling proteins for more than 24 h after exercise (4, 26).
results were in agreement with these results in that the RE model stimulated the mTORC1 signaling pathway, as indicated by the gradual but significant increase in p70S6K phosphorylation. Thus, we believe that our animal model is suitable for examining the effect of EE on muscle contraction (RE)-induced mTORC1 signaling responses.

AMPK is known to act as a negative regulator of muscle protein anabolism mainly via inhibition of mTORC1 (37, 45). Numerous studies have examined the effect of EE on AMPK activity (or phosphorylation) and have reported that AMPK activity is increased during and immediately after exercise, but it returns to the baseline levels within a few hours after exercise (21, 59). A recent study investigated the effect of EE after RE on mTORC1 signaling activation induced by RE and reported no inhibitory effect (2). However, the EE in this study did not induce significant AMPK activation. The other above-mentioned human studies on concurrent training were also unable to demonstrate significant changes in AMPK phosphorylation with EE alone or in combination with RE (2, 12, 14). In contrast, the EE protocol in the present study (25 m/min for 60 min) significantly upregulated AMPK and Raptor, a downstream target of AMPK and thereby a marker of AMPK activation, immediately after EE, and the levels of these markers returned to the baseline levels within 1 h after exercise. Therefore, EE-induced AMPK activation did not coincide with the mTORC1 activation induced by RE when EE was performed after RE. In contrast, EE-induced AMPK activation may have occurred simultaneously with RE-induced mTORC1 activation when EE was performed after RE.

Unlike p70S6K, Akt phosphorylation at both Thr308 and Ser473 and mTOR phosphorylation did not differ between the EE before RE and EE after RE groups in the present study, indicating that the exercise order affects the p70S6K phosphorylation response independent of the Akt/mTOR signaling pathway. One of the possible mechanisms underlying the different p70S6K phosphorylation responses may be the inhibition of mTORC1 activity via the AMPK/Raptor signaling pathway. Raptor is known to be a direct AMPK substrate, and Raptor phosphorylation at Ser792 by AMPK leads to suppression of mTORC1 activity (8, 25). Phosphorylation of AMPK at Thr172 and Raptor at Ser792 was significantly elevated at 3 h after RE when EE was performed after RE. These findings suggest that EE after RE may downregulate mTORC1 activity via activation of AMPK/Raptor signaling, whereas prior activation of AMPK/Raptor signaling by EE before RE does not affect mTORC1 activation. However, in a previous study, although EE increased AMPK and repressed mTORC1 signaling, there was also an increased association of Raptor with mTOR, possibly via ERK signaling activation (63). It has been shown previously that mTORC1 activity is regulated either positively or negatively by site-specific Raptor phosphorylation via various signaling pathways (19, 25). Furthermore, the same study also reported an increased association of tuberous sclerosis (TSC) with mTOR (63). AMPK is known to activate the TSC complex via TSC2 phosphorylation, thereby diminishing mTORC1 activity (30). Therefore, it appears that the different mTORC1 signaling responses are mediated by AMPK/TSC signaling rather than AMPK/Raptor signaling. Future studies should examine how RE-induced mTORC1 activation is differentially regulated by EE.

Interestingly, two previous studies conducted by the same research group have found that RE-induced immediate (15 min postexercise) mTORC1 activation was attenuated by prior maximal sprints (6 bouts of 6 s each) (11) but not by continuous EE (12). However, the prior maximal sprint did not alter RE-induced AMPK activation. Therefore, unlike EE, moderate-intensity EE, maximal sprint-type exercise may differentially attenuate RE-induced immediate mTORC1 activation via an AMPK-independent pathway. We hypothesize that maximal sprint-type exercise-induced metabolic acidosis, which is known to decrease muscle protein syn-

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Table 1. Serum IGF-I and plasma insulin concentrations

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<th>CON</th>
<th>EE before RE</th>
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<tr>
<td>Insulin, μIU/ml</td>
<td>5.5 ± 1.0</td>
<td>6.5 ± 0.8</td>
<td>5.9 ± 1.5</td>
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<tr>
<td>IGF-I, ng/ml</td>
<td>1,840 ± 150</td>
<td>1,803 ± 158</td>
<td>1,898 ± 166</td>
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Values are means ± SE. CON, controls; EE, endurance exercise; RE, resistance exercise.

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Fig. 5. Phospho-AMPK Thr172 (A) and Raptor Ser792 (B) after concurrent exercise. EE before RE, endurance exercise (EE) bout prior to resistance exercise (RE); EE after RE, endurance exercise bout after resistance exercise. Values are means ± SE. *P < 0.05 vs. CON.
thesis (9, 34), is at least partly responsible for the inhibition of mTORC1 activation. It should be noted that in the current study, a significant but temporal increase in mTORC1 activity was observed in response to EE alone. Immediate mTORC1 signaling response is not RE specific, and EE also activates mTORC1 signaling (7, 18). In the present study, EE also increased phosphorylation of Akt, an upstream stimulator of mTORC1. Although animal data regarding post-EE Akt activation especially under fasting conditions are limited, an increase in Akt phosphorylation has been reported previously in humans after fasting (7, 13) and after RE under fasting conditions (22). Further studies are warranted to investigate the effect of specific EE protocol or fasting state on RE-induced anabolic responses.

As is generally known and has been discussed above, RE increases muscle protein synthesis. However, it is also known to activate signaling pathways that lead to inhibition of muscle protein synthesis, especially during and immediately after exercise. The immediate response is known to reflect energy balance and metabolic stress during exercise, and anabolic responses of RE or high-intensity muscle contraction are inhibited mainly by AMPK and Ca$^{2+}$ signaling (16, 55). We observed a significant but temporal increase in AMPK and Raptor phosphorylation after a bout of high-intensity isometric muscle contraction. Previous studies also reported that AMPK and Raptor are activated by high-intensity isometric contraction of animal skeletal muscle (39, 44, 55), whereas eccentric contraction failed to activate AMPK (57). Thus, an acute bout of RE activates both inhibitory and stimulatory signaling pathways in skeletal muscle, and the balance between these pathways ultimately determines the muscle protein response.

In summary, we observed that p70S6K phosphorylation was decreased after RE when EE was performed after it but not when EE was performed before RE. This may be due to the inactivation of mTORC1 activity via simultaneous activation of AMPK signaling, suggesting that when RE and EE are performed concurrently, the mTORC1 signaling induced by any prior bout of RE may be downregulated by the subsequent bout of EE.

**GRANTS**

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**DISCLOSURES**

The authors declare no conflicts of interest, financial or otherwise.

**AUTHOR CONTRIBUTIONS**

R.O., K.S., K.M., K.N., and S.F. conception and design of research; R.O., K.S., and K.M. performed experiments; R.O. analyzed data; R.O. and S.F. interpreted results of experiments; R.O. prepared figures; R.O. and S.F. drafted manuscript; R.O., K.S., K.M., K.N., and S.F. approved final version of manuscript; K.S., K.M., K.N., and S.F. edited and revised manuscript.

**Fig. 6.** Phospho-Akt Thr$^{308}$ and Ser$^{473}$ (A), mTOR Ser$^{2448}$ (B), and p70S6K Thr$^{389}$ (C) after concurrent exercise. Values are means ± SE. *$P < 0.05$ vs. CON; +$P < 0.05$ vs. EE before RE.

**Fig. 7.** Muscle protein synthesis after concurrent exercise. Values are means ± SE. *$P < 0.05$ vs. CON; +$P < 0.05$ vs. EE before RE. MW, molecular weight.
REFERENCES


