Insulin facilitation of muscle protein synthesis following resistance exercise in hindlimb-suspended rats is independent of a rapamycin-sensitive pathway

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RODENT HINDLIMB SUSPENSION has been used as a ground-based model for studying musculoskeletal changes that occur during space flight (3, 6–8, 19–21, 26, 27, 34) and disuse because of comparable decreases in the size of selected hindlimb muscles (1, 6, 7, 25, 27). The soleus muscle, predominately composed of slow-twitch fibers, atrophies to the greatest extent with hindlimb unloading, which is due, in part, to a reduction of muscle protein synthesis (12, 30). Mechanisms associated with this reduction of muscle protein synthesis are not completely understood but may involve alterations of signaling controlling the mRNA translational process.

Recent studies have implicated the Akt-mammalian target of rapamycin (mTOR) pathway as a key component in the signal transduction for the control of protein synthesis in skeletal muscle (4, 5, 12, 29). It has been suggested that higher mTOR activity leads to elevated muscle mass, whereas reduced activity is associated with muscle atrophy. Rapamycin, a potent inhibitor of mTOR activity, almost completely blocked compensatory hypertrophy of soleus muscle during synergistic ablation (4). However, in that same study, recovery from atrophy induced by normal ambulation after hindlimb suspension was not completely blocked by rapamycin in soleus muscle (4). Thus it appears that restoration of muscle after atrophy can occur, in part, because of mechanisms that are independent of the AKT-mTOR pathway.

Our group explored the effect of resistance exercise on maintenance of muscle mass during hindlimb suspension. Using flywheel technology as a form of resistance exercise, we (12) have shown that this type of exercise was useful in attenuating the losses of muscle and bone mass and maintaining rates of muscle protein synthesis during a 28-day hindlimb suspension study. To date, little is known about the effect of intermittent resistance exercise on signaling for muscle protein synthesis during muscle unloading, particularly at the onset of training. The purpose of this study was to examine the effect of resistance exercise, a known stimulant of muscle hypertrophy, on rates of protein synthesis with and without insulin, since this hormone is a potent agonist of the mTOR pathway (23, 24) and is a necessary component for postresistance exercise elevations of muscle protein synthesis (15).

METHODS

Animals and operant conditioning. Male Sprague-Dawley rats (7 mo old) were used for this study, and methods were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences. Before experiments, all animals were

operate conditioned to engage in rat resistance exercise training as described previously (12, 15, 33). Briefly, all rats were operantly conditioned to press an illuminated lever in a specially designed cage to avoid a brief foot shock stimulus (<1 mA, 60 Hz, 1–5 V). The movement facilitated by the entrainment process required full extension and flexion of the hindlimbs. All animals were entrained to conduct this movement in an upright or standing position using the hindlimb-suspended training apparatus positioned in a vertical manner, so that once the animal was hindlimb suspended, the animal was familiar with the exercise equipment (12). The movement facilitated by the entrainment process is similar to the “squat,” as seen in traditional weight room settings, and the movement is the same in both the upright (vertical) and hindlimb-suspended (horizontal) environments. Once appropriately entrained, the animals engaged in the flywheel training protocol (described below) with very little or no shock. Entrainment to reach this point required approximately four distinct sessions lasting 30–45 min, with each session separated by 48–72 h. All familiarization and resistance exercise sessions were conducted at the onset of the dark cycle.

Hindlimb suspension: After operant conditioning, rats were randomly assigned to either control (n = 8), hindlimb-suspended (HS; n = 10), or hindlimb-suspended with resistance exercise (HSRE; n = 10) groups. Animals in the HS and HSRE groups were hindlimb suspended for a 4-day period, similar to previously described methods (8, 26). Briefly, for HS and HSRE groups, animals were anesthetized by Nembutal injection (ip), and a harness with a hook was attached to their tails. The tail device was connected via a thin steel cable to a pulley that slid on a vertically adjustable stainless steel bar that ran longitudinally above a high-sided cage with standard floor dimensions. The system is designed so that the rats cannot rest their hindlimbs against any side of the cage.

Resistance exercise: We have developed a modified version of the human flywheel resistance exercise apparatus (2, 12). Briefly, resistance-exercised rats were tethered via a tail cast apparatus that was spooled around an inertia wheel located on the outside of the resistance exercise apparatus. The rat was allowed to place its feet on a shock grid at one end of the cage, and a bar capable of illumination was located in the apparatus opposite the shock grid. After the entrainment period described above, the animals engaged in resistance exercise. For resistance exercise, upon illumination of the bar, the animal fully extended its hindlimbs in an effort to depress the bar. As a result of this extension, the rat pulled against the tether, using enough force to overcome the mass of the wheel (similar to the unwinding of a yo-yo). Once the tether was fully unspooled at the rat’s full extension, the momentum or inertia of the spinning wheel forced the tether to spool again. This spooling action facilitated movement of the animal back to the original starting state. Once the animal was back in the starting position, the bar was illuminated again, facilitating another repetition by the animal. The movement is similar to a squat, as performed by humans, and involved movement at the hip, knee, and ankle joints. When needed, shock was applied briefly (<1 s) to facilitate movement of the animal.

The resistance exercise protocol consisted of two exercise sessions over a 4-day period. Each session was separated by 48 h and consisted of two sets of a maximum of 25 repetitions (or point of failure; i.e., the animal would not respond to the illuminated bar even with a brief foot shock). A resistance exercise session required ~15 min to complete. Force developed by the animal was recorded using a load cell (Entran Devices, Fairfield, NJ) attached to the flywheel apparatus, which was integrated to a personal computer. Data acquisition was obtained by software programmed by the investigative team and collected data at a rate of 40×/s throughout the 25-repetition period of each set.

In vitro assessment of muscle protein synthesis. Approximately 16 h after the last exercise session (on day 4), animals were anesthetized, and soleus muscles were dissected from origin to insertion and weighed. Soleus muscle was selected because it is very susceptible to disuse atrophy using these methods (4, 30). Furthermore, this muscle is very active during this type of exercise activity, responds with elevated rates of protein synthesis (14, 15), and is a suitable muscle for the study of muscle protein synthesis in vitro (12). Sixteen hours postexercise was chosen to study rates of synthesis, since previous studies have demonstrated elevated rates of synthesis during this period (15, 18). HS and HSRE animals were suspended for the same period of time. Animals from control, HS, and HSRE groups were used at the same time of day for the assessment of muscle protein synthesis so that comparisons among groups could be made.

Rates of soleus muscle protein synthesis were assessed in vitro, as previously described (12). Briefly, soleus muscles were sectioned longitudinally into strips weighing 47.1 ± 1.8 mg and placed into specially designed clamps to maintain muscle length. Muscle strips were then placed into a Krebs-Henseleit buffer (K-HB), maintained at 37°C, and gassed with humidified O2 (95%)-CO2 (5%). The medium contained all amino acids at physiological levels, 5 mM D-glucose, and 4.5% bovine serum albumin, dialyzed against 40 volumes of K-HB for 48 h before inclusion in the buffer. After a 15-min incubation period, the muscle strips were transferred to wells containing 5 ml of medium that included a 2 mM concentration of cold (nonradioactive) phenylalanine plus 1 μCi/ml radiolabeled [1-2,3,4,5,6-3H]phenylalanine. After a 35-min incubation period in the presence of the radioisotope, strips were placed into liquid nitrogen, frozen at −80°C, and later assessed for incorporation of tritiated phenylalanine into TCA-precipitable extracts (32), corrected for the specific radioactivity of the incubation medium. Time was recorded for each muscle strip from the start of the radiolabeling period to the point of freezing so that rates of muscle protein synthesis could be expressed per unit time.

Muscle protein determinations for all strips were conducted using the bicinchoninic acid assay (Sigma, St. Louis, MO). Identical medium was used for all soleus muscle experiments. Rates of muscle protein synthesis were expressed as nanomoles phenylalanine (F) incorporated per gram of muscle per hour.

To validate our incubation methodology, we examined in a separate group of rats the effect of a 45-min incubation using identical medium on muscle ATP concentration compared with muscle from age-matched control rats that was quick-frozen to the temperature of liquid nitrogen in situ. The purpose of this measure was to ensure that the overall mass of the incubated soleus strips used for the study of muscle protein synthesis did not affect the permeability of oxygenated medium, which would ultimately lead to diminished ATP concentration and limited physiological function. For this subset of studies, ATP concentration was examined in perchloric acid muscle extracts using an enzyme methodology and fluorometry as described by Passonneau and Lowry (28). The ATP concentrations from incubated soleus muscle strips with masses ranging from 40 to 49 mg were similar to control values frozen in situ (P < 0.05), suggesting that strips of this size, as used for the determination of muscle protein synthesis, may be used with present incubation methodologies (see Fig. 1).

To further validate our in vitro incubation model, we assessed rates of soleus muscle protein synthesis in vitro in a separate group of rats from control animals (n = 5) and compared them with values obtained using in situ hindlimb preparation methodologies (control; n = 5), as described previously (12, 14, 15). Because no differences were observed between rates of synthesis as measured in vitro and in situ (253.3 ± 26.4 vs. 249.5 ± 20.8 nmol F incorporated/g muscle −1·h −1, respectively; P = 0.573), it was determined that the in vitro method was suitable for this study.

Activation/inhibition signal transduction studies. To assess signal transduction pathways after resistance exercise, soleus muscles were incubated for ~35 min with or without insulin (20,000 μU/ml) and with or without rapamycin (1 μM). When appropriate, insulin was provided at supraphysiological concentrations to ensure that insulin signaling pathways were maximized during incubations for the assessment of muscle protein synthesis (22). Furthermore, insulin has been demonstrated to be a necessary component of muscle protein
synthesis after resistance exercise (9, 14, 15). Rapamycin was provided when necessary as an upstream inhibitor of mTOR, and we have been successful in inhibiting mTOR with rapamycin at this concentration (13). Rapamycin was used alone or together with insulin to determine the effect of mTOR signaling on the postexercise protein synthesis response.

Statistics. Analysis of variance was used to compare means of muscle protein synthesis rates among treatments (∆insulin and/or ∆rapamycin) and between groups. Force production between sets or body mass (g), soleus mass (mg), or soleus mass (mg)-to-body mass (g) ratio between groups was also compared with analysis of variance. Differences among means were considered significant when P < 0.05. When F ratios were significant, a Student-Newman-Keuls test was used to compare relevant means when multiple comparisons were tested. All data are expressed as means ± SE.

RESULTS

Resistance exercise performance during hindlimb suspension. Rats that engaged in resistance exercise were able to complete 19.75 ± 1.46 of the desired 25 repetitions per set over the exercise paradigm. It has been our experience that animals do not typically complete 100% of the repetitions at the onset of training after hindlimb suspension because of addition of resistance from the operant conditioning paradigm and/or fatigue during the experimental protocol. Data related to maximum force per set and the force profile curve through all repetitions are shown in Fig. 2. Force production has been demonstrated to elicit a training effect in adult rats (12) and is similar to forces applied during earlier studies (9, 10, 14, 15).

Resistance exercise maintains muscle mass during short-term hindlimb suspension. Four days of hindlimb suspension, with or without resistance exercise, resulted in a significant loss of body mass (P < 0.05) compared with age-matched control animals (Table 1). Soleus mass was also reduced with HS compared with control animals (P < 0.05), but soleus muscle of HSRE rats was not different from that of controls (P > 0.05; Table 1). When expressed as soleus mass-to-body mass ratios, control and HSRE ratios were similar, whereas the ratio of the HS group was significantly lower (P < 0.05). Together, these data suggest that, although 4 days of hindlimb suspension result in an acute reduction of body mass, the addition of resistance exercise during this time period is adequate to attenuate losses of soleus muscle mass in this model.

Insulin-mediated increase in soleus muscle protein synthesis studies after resistance exercise. Rates of soleus muscle protein synthesis were measured at the end of the 4-day period, in vitro, with and without insulin. For HSRE animals, the measurement was 16 h after the last bout of exercise. Acute administration of insulin had no effect on rates of soleus muscle protein synthesis in control animals, as demonstrated previously in studies using similar techniques (11, 14, 15). Hindlimb suspension resulted in diminished rates of soleus muscle protein synthesis (P < 0.05), compared with controls, that were unaffected by an acute administration of insulin (Fig. 3). With only two sessions of resistance exercise on days 1 and 7-mo-old male Sprague-Dawley rats. Measures were taken at the end of the 4-day period, which was 16 h after the last bout of exercise for the HSRE rats. *P < 0.05, different from control within comparison.

Table 1. Body and soleus mass of control, 4-day HS, and 4-day HSRE rats

<table>
<thead>
<tr>
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<th>Control (n = 8)</th>
<th>HS (n = 10)</th>
<th>HSRE (n = 10)</th>
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<tr>
<td>Body mass, g</td>
<td>468.3±12.6</td>
<td>432.9±8.3*</td>
<td>434.0±5.3*</td>
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<tr>
<td>Soleus mass, mg</td>
<td>196.6±8.0</td>
<td>166.8±5.6*</td>
<td>181.4±5.7*</td>
</tr>
<tr>
<td>Soleus mass (mg)-to-body mass (g) ratio</td>
<td>0.42±0.01</td>
<td>0.38±0.07*</td>
<td>0.42±0.01</td>
</tr>
</tbody>
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Data are expressed as means ± SE; n = no. of rats. Body mass and soleus mass were measured at the time of death. Hindlimb-suspended (HS) rats were hindlimb suspended for 4 days, and hindlimb-suspended with resistance exercise (HSRE) rats engaged in 2 resistance exercise bouts during the 4-day hindlimb suspension period (days 1 and 3). Control rats were age-matched 7-mo-old male Sprague-Dawley rats. Measures were taken at the end of the 4-day period, which was 16 h after the last bout of exercise for the HSRE rats.
The purpose of this study was to examine the effect of acute resistance exercise on signaling pathways involved in protein synthesis in soleus muscles from HS rats. Hindlimb suspension is associated with a loss of muscle mass, which, in part, is the result of significant reductions of muscle protein synthesis (12, 30, 31). The most important finding from this study is that hindlimb suspension facilitates an adaptive insulin-signaling response whereby protein synthesis is mediated through a yet-undefined signaling pathway that is insensitive to rapamycin (independent of mTOR). We base this observation on our findings that rapamycin did not inhibit insulin-facilitated muscle protein synthesis after hindlimb suspension. We speculate that the muscle has acquired an adaptive response as a compensatory mechanism in an attempt to maintain mass during muscle unloading, albeit somewhat unsuccessfully.

In the present investigation, the addition of resistance exercise attenuated the decrement in soleus muscle mass compared with HS animals without exercise, suggesting that our resistance exercise paradigm was adequate for maintenance of mass within the first 4 days of hindlimb suspension. Furthermore, insulin facilitated rates of synthesis in exercised muscle, and the augmented rates were higher than in controls, findings that are consistent with other studies (14, 15). We expanded on previous work (12, 15) by demonstrating that the anabolic response to resistance exercise with insulin is maintained even in the presence of rapamycin. This novel finding suggests that the signaling adaptations that occur with hindlimb suspension are sufficient to permit the transient elevation of rates of protein synthesis by resistance exercise. Thus resistance exer-

DISCUSSION

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3 of hindlimb suspension, rates of synthesis without insulin were similar to those in the HS group (P > 0.05), but the addition of insulin significantly increased rates of synthesis to levels greater than controls (P < 0.05; Fig. 3). Together, these results suggest that resistance exercise is sufficient to stimulate rates of muscle protein synthesis in HS animals when the muscle is incubated in insulin-sufficient medium.

**Hindlimb suspension facilitates involvement of rapamycin-insensitive pathways for soleus muscle protein synthesis.** To determine the involvement of the mTOR pathway in muscle protein synthesis in control, HS, and HSRE rats, soleus muscles were incubated in the presence of 1 µM rapamycin, with or without insulin. In control animals, rapamycin significantly decreased (P < 0.05) rates of synthesis, and insulin did not overcome (rescue) this inhibition (Fig. 4, top). That rates of synthesis in the presence of rapamycin with insulin were similar to rates of synthesis with rapamycin only suggests that signaling for protein synthesis in soleus muscle from control animals operates through a rapamycin-sensitive pathway.

In HS animals, where rates of protein synthesis were already diminished with hindlimb unloading, rates of synthesis were further diminished (P < 0.05) with rapamycin (Fig. 4, middle). However, unlike in control animals, rapamycin had no effect on rates of protein synthesis in HS animals when insulin was added to the medium, suggesting that insulin signaling for protein synthesis is independent of the rapamycin-sensitive pathway under these conditions.

In HSRE animals, rapamycin inhibited soleus muscle protein synthesis to the same extent as in control and HS groups (Fig. 4, bottom). The addition of insulin resulted in augmented rates of protein synthesis in both rapamycin-treated and untreated muscle of HSRE rats, suggesting that the anabolic response of resistance exercise with hindlimb suspension is not dependent on a rapamycin-sensitive pathway under these conditions.
cise may be an important countermeasure to the losses of muscle mass that occur during muscle unloading.

The resistance exercise protocol in the present study was adequate for the preservation of muscle mass at the 4th day of hindlimb suspension, likely due to the postexercise elevations of muscle protein synthesis. It has been reported that losses of muscle mass with hindlimb suspension are associated with both a reduction of muscle protein synthesis and an elevation of muscle proteolysis (30), with the latter being more responsive to insulin (31). It was shown that acute administration of insulin in streptozotocin-induced diabetic rats during hindlimb suspension attenuated losses of muscle mass, and the reduction of atrophy occurred without an apparent increase in muscle protein synthesis (31). This suggests that the role of insulin in muscle protein metabolism without prior exercise may primarily affect muscle proteolysis rather than synthesis. The present study supports this concept, since the diminished rates of synthesis in soleus muscle of HS rats were not normalized with the acute administration of insulin in vitro. Thus resistance exercise may serve to normalize muscle mass by facilitating rates of muscle protein synthesis in the face of the accelerated proteolysis brought on by hindlimb suspension. It must be noted that we used supraphysiological levels of insulin to facilitate signaling for muscle protein synthesis in the present study, and, therefore, it is unknown whether physiological levels of insulin are sufficient to augment postexercise rates of protein synthesis with 4 days of hindlimb suspension. Future work will systematically assess the role of physiological levels of insulin on muscle protein synthesis during hindlimb suspension with and without resistance exercise in vivo.

Our studies were conducted in vitro, with and without rapamycin or insulin, thereby blocking signaling through the mTOR pathway to determine its role in muscle protein synthesis. Therefore, the present study cannot discount the involvement of the rapamycin-sensitive pathway for overall rates of muscle protein synthesis, in vivo, with hindlimb suspension. Insulin stimulation of soleus muscle protein synthesis is dependent on the mTOR pathway in control animals, as the reduction of protein synthesis in the presence of rapamycin was not overcome by the addition of insulin. By contrast, in HS animals, insulin restored rates of protein synthesis in the presence of the inhibitor, suggesting that alternative pathways influence rates of protein synthesis with hindlimb suspension independently of exercise.

In summary, hindlimb suspension results in a reduction of muscle protein synthesis, and this reduction is associated with a concomitant decrease in dependence of signaling through a rapamycin-sensitive pathway. Although this adaptation in insulin signaling is not understood at this time, we suggest that it serves a compensatory role to maintain muscle mass, albeit unsuccessfully, in an atrophying environment. The addition of resistance exercise allowed for maintenance of muscle mass, partly as a result of elevated rates of muscle protein synthesis. Furthermore, the augmented rates of synthesis after exercise in the presence of insulin were not dependent on a rapamycin-sensitive pathway. These findings may have important implications for our understanding of the control of muscle protein homeostasis under atrophying conditions, particularly as it relates to the use of countermeasures to prevent muscle atrophy.

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GRANTS

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