REDD2 expression in rat skeletal muscle correlates with nutrient-induced activation of mTORC1: responses to aging, immobilization, and remobilization

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Kelleher AR, Pereira SL, Jefferson LS, Kimball SR. REDD2 expression in rat skeletal muscle correlates with nutrient-induced activation of mTORC1: Responses to aging, immobilization, and remobilization. Am J Physiol Endocrinol Metab 308: E122–E129, 2015. First published November 18, 2014; doi:10.1152/ajpendo.00341.2014.—In a previous study (Kelleher AR, Kimball SR, Dennis MD, Schilder RJ, and Jefferson LS. Am J Physiol Endocrinol Metab 304: E229–236, 2013.), we observed a rapid (i.e., 1–3 days) immobilization-induced repression of mechanistic target of rapamycin complex 1 (mTORC1) signaling in hindlimb skeletal muscle of young (2-mo-old) rats that was associated with elevated expression of regulated in development and DNA-damage response (REDD) 1 and REDD2. The present study extends that observation to include an assessment of those parameters in soleus muscle of the immobilized hindlimb of various-aged rats as well as in response to remobilization. Male Sprague-Dawley rats aged 2, 9, and 18 mo were subjected to unilateral hindlimb immobilization for 7 days, whereas one group of the 9-mo-old animals underwent 7 days of remobilization. Soleus muscle mass-to-body mass ratio declined with age, with the loss of muscle mass following hindlimb immobilization being inversely proportional to age. Compared with 2-mo-old rats, the older rats exhibited reduced mTORC1 signaling in the nonimmobilized limb in association with elevated REDD2, but not REDD1, mRNA expression. In the 2-mo-old rats, 7 days of hindlimb immobilization attenuated mTORC1 signaling and induced REDD2, but not REDD1, mRNA expression. In contrast, hindlimb immobilization did not further attenuate the age-related reduction in mTORC1 signaling nor further enhance the age-related induction of REDD2 mRNA expression in 9- and 18-mo-old rats. Across ages, REDD1 mRNA was not impacted by immobilization. Finally, remobilization elevated mTORC1 signaling and lowered REDD2 mRNA expression, with no impact on REDD1 gene expression. In conclusion, changes in mTORC1 signaling associated with aging, immobilization, and remobilization were inversely proportional to alterations in REDD2 mRNA expression.

LOSS OF SKELETAL MUSCLE MASS and function in the elderly leads to increased risk of falls and fractures, physical frailty, and increased morbidity and mortality (11, 39, 53). A period of inactivity (e.g., limb immobilization, bed rest, etc.) worsens the problem, since it leads to acute skeletal muscle atrophy (52). Both age- and inactivity-induced muscle atrophy are thought to be in large part a consequence of the development of resistance to nutrient (particularly amino acids)-induced stimulation of muscle protein synthesis (42, 49). In young adult humans (e.g., ~25 yr old), muscle protein synthesis is stimulated in response to consumption of relatively small quantities (e.g., 7–10 g) of essential amino acids, whereas in older adults (e.g., ~65 yr old) a comparable amount of amino acids is ineffective (31, 32). However, ingesting larger quantities of amino acids (e.g., 25–30 g) effectively stimulates muscle protein synthesis in both young and older individuals (55, 56). Notably, the responsiveness of muscle protein synthesis in the elderly to small quantities of essential amino acids can be overcome by increasing the leucine content of the mixture (21, 32). Similar results have been reported for studies in rats in which the age-related resistance of skeletal muscle protein synthesis to stimulation by a protein diet can be overcome by supplementation with leucine (14). Combined, the results from both human and rat studies suggest that the age-related loss of muscle responsiveness to amino acids is largely due to impaired leucine sensitivity.

The anabolic effects of amino acids in muscle are due in large part to mechanistic target of rapamycin complex 1 (mTORC1)-mediated stimulation of protein synthesis (for review, see Ref. 24). Upon activation by amino acids, hormones, or exercise, mTORC1 phosphorylates protein substrates at specific residues, including p70 ribosomal protein S6 kinase 1 (p70S6K1) on Thr389 and uncoordinated-51 like autophagy activating kinase 1 (ULK1) on Ser757, which promotes mRNA translation and represses autophagy, respectively (23, 34, 44). The key role played by mTORC1 in determining muscle size is evident in studies in which conditional deletion of mTORC1 components (e.g., raptor or mTOR) leads to development of a muscle dystrophy phenotype with reduced muscle weight and fiber cross-sectional area (7, 50). In a previous study, we observed an immobilization-induced attenuation of mTORC1 signaling that was resistant to a nutrient-induced stimulation (33). We also found that immobilization for 3 days led to induction in the expression of mRNAs for two mTORC1 repressors, regulated in development and DNA-damage response (REDD) 1 and REDD2, encoded by the DDIT4 and DDIT4L genes, respectively. Consequently, the question arises as to the role of REDD1 and REDD2 in contributing to the loss of skeletal muscle that occurs in response to aging and remobilization.

In the present study, we tested the hypothesis that REDD1 and/or REDD2 mRNA expression is elevated in muscle from older compared with young rats in association with attenuated mTORC1 signaling and that the immobilization-induced stimulation of REDD1 and/or REDD2 expression is exacerbated in older compared with young rats. We also tested the hypothesis that remobilization reverses the immobilization-induced induc-
tion of REDD1 and/or REDD2 expression in conjunction with restoration of mTORC1 signaling.

METHODS

Animals. Male Sprague-Dawley rats of age 2, 9, and 18 mo (Charles River Laboratories, Wilmington, MD; and Harlan Laboratories, Indianapolis, IN) were housed in wire cages in a temperature (25°C)- and light-controlled environment. Rats were provided rodent chow (AIN-93M; Research Diets, New Brunswick, NJ) and water ad libitum. Before hindlimb immobilization, rats were adapted to a reversed 12:12-h light-dark cycle (lights off at 0700) for 1 wk. Animal facilities and experimental protocols were approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine.

Experimental design. Rats were anesthetized by isoflurane inhalation (2.5%) and subjected to unilateral hindlimb immobilization by casting as described previously (33). Hindlimbs were immobilized for 7 days before removal of the soleus muscle for subsequent analysis. An additional group of 9-mo-old rats was subjected to unilateral hindlimb immobilization for 7 days, and, following removal of the cast, they were allowed to remobilize for 7 days. Before the day of tissue harvest, all rats were fasted overnight (18 h) but allowed free access to water. On tissue harvest days, rats were individually caged and provided with similar amounts of rodent chow (AIN-93M) for 10 min. One rat did not eat and was excluded from the study. The remaining rats on average consumed 3.2 ± 0.2 g of food, and there was no significant difference in food consumption among the groups as assessed by ANOVA with Holm-Sidak correction for multiple comparisons. Thus, all rats were in a fed state at the time of the analysis. The animals were subsequently anesthetized using isoflurane and remained anesthetized for the remainder of the experiment. The soleus muscle was removed from the immobilized and the contralateral nonimmobilized (control) hindlimb 45 min after the start of chow feeding. This time point was selected based on previous studies showing that the peak response of mTORC1 signaling in skeletal muscle to a nutrient-induced stimulus occurs between 45 and 60 min (4). Muscle from the nonimmobilized hindlimb was used as a control based on the observation in our previous study (33) that nutrient-induced mTORC1 signaling responded identically in the soleus of the nonimmobilized hindlimb of an immobilized rat compared with the soleus of a rat not subjected to immobilization.

Sample preparation and immunoblot procedure. The soleus muscle was chosen for analysis in the present study so that the results could be compared with those in our previous study investigating the effect of unilateral hindlimb immobilization on mTORC1 signaling in young rats (33). Soleus muscle was dissected from each hindlimb, weighed, and processed for analysis of mRNA expression and phosphorylation state of key proteins in the mTORC1 signaling pathway as described previously (33). For immunoblot analysis, polyvinylidene difluoride membranes were incubated with primary antibodies recognizing proteins phosphorylated on specific residues, including p70S6K1 Thr389, ULK1 or Akt (Cell Signaling Technology) or p70S6K1. Blots were developed using a FluorChem M Multifluor System (ProteinSimple, San Jose, CA) and analyzed using AlphaView (ProteinSimple, San Jose, CA) and analyzed using AlphaView (ProteinSimple) and Genetools (Syngene, Cambridge, MA) software.

Measurement of mRNA expression. RNA was isolated from soleus muscle harvested 45 min after the start of chow feeding using the TRIzol method (Life Technologies, Grand Island, NY) and prepared for real-time polymerase chain reaction as previously described (33). Primers were purchased from Applied Biosystems, including Ddit4 (REDD1) (assay ID: Rn01433735_g1), Ddit4l (REDD2) (Rn00589659_g1), Trim63 (MuRF1) (Rn00590197_m1), Fbxo32 (MAFbx) (Rn00591730_m1), and Tbp (TATA-binding protein) (Rn01455646_m1). Tbp mRNA expression was compared against the expression of other common housekeeping genes, including β-actin, Hprt1, Polr2a, and Rpl32. Tbp mRNA expression was used as an internal control, since its expression did not change substantially with aging or in response to 7 days of immobilization or remobilization.

Statistical analysis. Results from individual groups are presented as means ± SE. Outliers were determined using Grubb’s test (α-level 0.05) and excluded from further analysis. Data for the immobilized rats was analyzed by two-way ANOVA with Newman-Keuls correction for multiple comparisons with age and immobilization status as variables. The effects of remobilization were assessed by comparing data from the 9-mo-old immobilized rats with that obtained from rats in the remobilization group by ANOVA with Newman-Keuls correction for multiple comparisons. Nonlinear regression (curve fit) analysis was used to assess the relative relationship between p70S6K1 phosphorylation on Thr389 and REDD2 mRNA expression for all samples. All comparisons were performed using GraphPad Prism 6 software. Differences between groups were considered significant at P ≤ 0.05.

RESULTS

Effect of age and remobilization on immobilization-induced loss of muscle mass. As shown in Table 1, the soleus muscle mass-to-body mass ratio declined with age, being significantly lower (P < 0.05) in 18-mo-old rats compared with both 2- and 9-mo-old rats. In response to 7 days of hindlimb immobilization, muscle mass-to-body mass ratio was reduced in rats from all age groups. Notably, the magnitude of the loss of muscle mass following hindlimb immobilization was inversely proportional to age, where the muscle mass-to-body mass ratio was reduced 40, 15, and 8% in the soleus muscle from the immobilized hindlimb of 2-, 9-, and 18-mo-old rats, respectively, when compared with the soleus muscle from the contralateral nonimmobilized hindlimb (referred to hereafter as the control hindlimb). No difference in muscle mass-to-body mass ratio was observed between the soleus muscle from the control hindlimb of 9-mo-old immobilized and remobilized rats. Following 7 days of remobilization, muscle mass-to-body mass ratio was further reduced (P < 0.05) in the soleus muscle from the remobilized hindlimb compared with either the immobilized or the control hindlimb of 9-mo-old rats. Similar results were obtained for the gastrocnemius and plantaris muscles (Table 1).

Effect of age, immobilization, and remobilization on mTORC1 signaling. To gain an understanding of the potential molecular events responsible for age- and immobilization-induced skeletal muscle atrophy, rats were provided with a nutrient stimulus to produce an anabolic response that was assessed by analysis of the phosphorylation state of residues on two proteins known to be direct targets of mTORC1, i.e., p70S6K1 Thr389 and ULK1 Ser757. The analysis demonstrated that aging was associated with a 53 and 78% attenuation of the nutrient-induced stimulation of p70S6K1 phosphorylation on Thr389 in the soleus muscle from the control hindlimb of 9- and 18-mo-old rats, respectively, relative to the soleus muscle from the control hindlimb of 2-mo-old rats (Fig. 1). In response to 7 days of hindlimb immobilization, the nutrient-induced stimulation of p70S6K1 on Thr389 was reduced 68 and 48% in the soleus muscle from the immobilized hindlimb relative to the control hindlimb of 2- and 9-mo-old rats, respectively, whereas it was unchanged from the control value in 18-mo-old rats. Nutrient-induced stimulation of ULK1...
phosphorylation on Ser\(^{757}\) also declined with age, and, in response to immobilization, it was reduced 48% relative to the control hindlimb of 2-mo-old rats. No difference was observed in ULK1 phosphorylation state between hindlimbs of 9- or 18-mo-old rats. Following 7 days of remobilization, mTORC1 signaling was augmented in the soleus muscle from the remobilized limb relative to the control hindlimb, i.e., phosphorylation of p70S6K1 on Thr\(^{389}\) and ULK1 on Ser\(^{757}\) was elevated. 

Effect of age, immobilization, and remobilization on Akt activation. As a biomarker of an upstream signaling input to mTORC1, phosphorylation of Akt on Ser\(^{473}\) was assessed as an index of the activation state of the kinase. The assessment demonstrated that aging was associated with attenuation of the nutrient-induced activation of Akt in soleus muscle from the control hindlimb of 9- and 18-mo-old rats relative to the soleus muscle from the control hindlimb of 2-mo-old rats (Fig. 2). In response to 7 days of hindlimb immobilization, the nutrient-induced phosphorylation of Akt on Ser\(^{473}\) was reduced 30% in the soleus muscle from the immobilized hindlimb relative to the soleus from the control hindlimb of 2-mo-old rats. No difference in nutrient-induced phosphorylation of Akt on Ser\(^{473}\) was observed between the soleus muscle from the immobilized and the control hindlimb of 9- and 18-mo-old rats. Following 7 days of remobilization, phosphorylation of Akt on Ser\(^{473}\) was augmented 90% in the soleus muscle from the remobilized limb relative to the soleus muscle from the control hindlimb.

Effect of age, immobilization, and remobilization on REDD1 and REDD2 mRNA expression. To gain an understanding of the mechanism(s) responsible for the observed responses of mTORC1 signaling to aging, hindlimb immobilization, and remobilization, the mRNA expression for two repressors of the pathway, i.e., REDD1 and REDD2, was assessed. In contrast to our earlier study showing an increase in REDD1 mRNA expression following 1, 2, or 3 days of immobilization (33), its expression was unchanged from the control value following 7 days of immobilization (Fig. 3A). Moreover, no change in REDD1 mRNA expression was observed in response to aging or remobilization. In contrast, aging was associated with increased expression of REDD2 mRNA, i.e., its expression was increased ~250 and 300% in the soleus muscle from the control hindlimb of 9- and 18-mo-old rats, respectively, relative to the soleus muscle from the control hindlimb of 2-mo-old rats (Fig. 3B). In response to 7 days of hindlimb immobilization, REDD2 mRNA expression was increased >400% in the soleus muscle from the immobilized hindlimb relative to the soleus muscle from the control hindlimb of 2-mo-old rats. In 9-mo-old rats, REDD2 mRNA expression in the soleus muscle from the immobilized hindlimb relative to the soleus muscle from the control hindlimb was further increased by 77% above the aging-induced response; however, this trend did not reach statistical significance \((P = 0.16)\). Moreover, in 18-mo-old rats, immobilization did not enhance REDD2 mRNA expression above the aging-induced response. Following 7 days of remobilization, REDD2 mRNA expression was repressed 38% in the soleus muscle from the remobilized limb relative to the soleus muscle from the control hindlimb and was significantly lower than the REDD2 mRNA expression in the soleus muscle from the immobilized hindlimb of 9-mo-old rats.

Relationship between p70S6K1 Thr\(^{389}\) phosphorylation and REDD2 mRNA expression. Across age groups and conditions, there appeared to be an association between mTORC1 signaling and REDD2 mRNA expression in the soleus muscle from rat hindlimbs, i.e., the phosphorylation of p70S6K1 on Thr\(^{389}\) and ULK1 on Ser\(^{757}\) was inversely proportional to REDD2 mRNA expression. A scatter plot with the natural logarithm (ln) of REDD2 mRNA expression and the phosphorylation of p70S6K1 on Thr\(^{389}\) plotted on the x- and y-axes, respectively, revealed a linear relationship between these two variables. As shown in Fig. 4, low REDD2 mRNA expression was associated with high phosphorylation of p70S6K1 on Thr\(^{389}\), and increases in REDD2 mRNA expression were associated with reductions in phosphorylation of p70S6K1 on Thr\(^{389}\). Fitting a linear regression to these points revealed a negative correlation \((r^2 = 0.53)\) between REDD2 mRNA expression and phosphorylation of p70S6K1 on Thr\(^{389}\) \((P < 0.0001)\). Similarly,

### Table 1. Young rats are more susceptible to hindlimb immobilization-induced loss of muscle mass than older rats

<table>
<thead>
<tr>
<th>Muscle and Age</th>
<th>Muscles</th>
<th>Muscle Mass, g</th>
<th>Muscle Mass/Body Mass (×10,000)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immob or recov limb</td>
<td>Control limb</td>
<td>Immob or recov limb</td>
</tr>
<tr>
<td>Soleus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mo</td>
<td>0.098 ± 0.007*#+</td>
<td>0.163 ± 0.007#</td>
<td>3.13 ± 0.19*#</td>
</tr>
<tr>
<td>9 mo</td>
<td>0.174 ± 0.002</td>
<td>0.204 ± 0.008</td>
<td>4.18 ± 0.19*#</td>
</tr>
<tr>
<td>9 mo (recov)</td>
<td>0.143 ± 0.010*#</td>
<td>0.186 ± 0.009</td>
<td>3.45 ± 0.18*</td>
</tr>
<tr>
<td>18 mo</td>
<td>0.188 ± 0.004</td>
<td>0.202 ± 0.010</td>
<td>3.29 ± 0.20*#</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2 mo</td>
<td>1.196 ± 0.044*#+</td>
<td>1.809 ± 0.027*#+</td>
<td>37.3 ± 1.32*#</td>
</tr>
<tr>
<td>9 mo</td>
<td>2.213 ± 0.069*</td>
<td>2.603 ± 0.097</td>
<td>49.4 ± 1.42*+</td>
</tr>
<tr>
<td>9 mo (recov)</td>
<td>1.466 ± 0.073*#</td>
<td>2.287 ± 0.075</td>
<td>35.4 ± 1.13*#</td>
</tr>
<tr>
<td>18 mo</td>
<td>2.106 ± 0.091*</td>
<td>2.543 ± 0.093</td>
<td>39.3 ± 1.33*#</td>
</tr>
<tr>
<td>Plantaris</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mo</td>
<td>0.237 ± 0.006*#+</td>
<td>0.344 ± 0.010#+</td>
<td>7.42 ± 2.78*#</td>
</tr>
<tr>
<td>9 mo</td>
<td>0.421 ± 0.016*</td>
<td>0.501 ± 0.024+</td>
<td>9.23 ± 0.33*</td>
</tr>
<tr>
<td>9 mo (recov)</td>
<td>0.310 ± 0.023*#</td>
<td>0.475 ± 0.008</td>
<td>7.49 ± 0.45*#</td>
</tr>
<tr>
<td>18 mo</td>
<td>0.459 ± 0.017*</td>
<td>0.557 ± 0.020</td>
<td>8.56 ± 0.28*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Rat 2, 9, and 18 mo of age had one hindlimb immobilized (Immob) for 7 days, and an additional group of 9-mo-old animals had their casts removed after 7 days and were allowed to remodel for an additional 7 days (recov). Body weights were 320.5 ± 5.3, 456.5 ± 4.4, and 537.5 ± 21.4 g for 2-, 9-, and 19-mo-old rats, respectively, and 414 ± 16.6 g for 9-mo-old rats after recovery for 7 days. *P < 0.05 compared with control limb; #P < 0.05 compared with equivalent limb in 9-mo-old rats; and +P < 0.05 compared with equivalent limb in 18-mo-old rats.
Effect of age, immobilization, and remobilization on atrogenic mRNA expression. To determine whether other biomarkers of muscle atrophy change coordinately with REDD2, the mRNA expression of the E3 ubiquitin ligases, MAFbx and MuRF1 (also known as “atrogenes”; see Ref. 8), was assessed. The mRNA expression of MAFbx (Fig. 5A) was elevated 63 and 47% in the soleus muscle of the control hindlimb of 9- and 18-mo-old rats, respectively, compared with the soleus muscle of the control hindlimb of 2-mo-old rats, although the difference was not statistically significant by ANOVA. Moreover, after 7 days of immobilization, MAFbx mRNA expression was higher in soleus muscle of the immobilized compared with the control hindlimb in each age group, although the difference at 18 mo was only significant by t-test. Aging had no significant effect on the mRNA expression of MuRF1 (Fig. 5B). Interestingly, although MuRF1 was elevated in the soleus muscle of the immobilized hindlimb of 2-mo-old rats as assessed by t-test, it was not elevated in response to immobilization in 9- and 18-mo-old rats. In response to 7 days of remobilization, MAFbx expression was lower in soleus muscle of the remobilized hindlimb compared with the soleus muscle of the immobilized hindlimb of 9-mo-old rats. However, MAFbx mRNA expression was not statistically different between the soleus muscle of the remobilized and the nonimmobilized hindlimb of the same rat. In contrast, MuRF1 mRNA expression was lower in the soleus muscle of the remobilized hindlimb compared with the control hindlimb of 9-mo-old rats.

DISCUSSION

In both animals (3) and humans (15), nutrients induce stimulation of protein synthesis through activation of the mTORC1 signaling pathway. In agreement with the impaired responsiveness of muscle protein synthesis to stimulation by nutrients, the sensitivity of mTORC1 signaling to activation by amino acids is also attenuated in muscle from older individuals compared with young adults (26). In the present study, a similar phenomenon was observed in rats, i.e., aging was associated with a proportional reduction in nutrient-induced activation of mTORC1 signaling. Notably, the decline in sensitivity of mTORC1 to nutrient-induced activation was inversely correlated with changes in expression of REDD2, which, in addition to REDD1, is a dominant repressor of the pathway. Many studies have focused on the role of REDD1 and/or REDD2 in repressing mTORC1 signaling under conditions of cell stress (13, 19, 41), with many fewer assessing their role under physiological conditions. However, recent reports have demonstrated an inverse correlation between changes in mTORC1 signaling and REDD1 and/or REDD2 expression in skeletal muscle in response to fasting and refeeding (43) as well as after a bout of resistance exercise (16, 17, 25). The present study extends these observations to show that elevated REDD2 mRNA expression in 9- and 18-mo-old rats is associated with an attenuation of nutrient-induced activation of mTORC1 signaling. The mechanism through which aging acts to induce REDD2 expression is unknown. However, it is notable that many of the stressors that have been shown to enhance REDD1 and/or REDD2 gene transcription, e.g., en-

![Graphs and tables related to the discussion on REDD2 expression with aging and immobilization.](http://ajpendo.physiology.org/)
RED2 expression with aging and immobilization

Fig. 2. Feeding-induced activation of Akt is blunted with age and hindlimb immobilization in skeletal muscle from young vs. older rats and is augmented following remobilization. Rats 2, 9, and 18 mo of age had one hindlimb immobilized for 7 days, and an additional group of 9-mo-old animals had their casts removed after 7 days and were allowed to remobilize for an additional 7 days. Relative phosphorylation of Akt on Ser^473 in the soleus muscle was assessed by protein immunoblot analysis. Bars represent the mean phospho-to-total protein ratio in the soleus muscle from control (dark gray), immobilized (black), and remobilized (light gray) hindlimbs. No differences were observed in total Akt protein expression. Samples were run on the same blot, but not in contiguous lanes. Noncontiguous lanes are denoted by a white line. For each condition, samples from two animals are shown. Data are means ± SE, n = 5–6 rats/group. *P < 0.05 compared with control limb. #P < 0.05 compared with equivalent limb in 18-mo-old rat.

Fig. 3. Regulated in development and DNA-damage response (REDD) 2, but not REDD1, mRNA expression increases with age and becomes unresponsive to immobilization, but is repressed following remobilization. REDD1 and REDD2 mRNA expression in the soleus muscle was assessed by Taqman gene expression assay. Rats 2, 9, and 18 mo of age had one hindlimb immobilized for 7 days, and an additional group of 9-mo-old animals had their casts removed after 7 days and were allowed to remobilize for an additional 7 days. Bars represent the mean REDD1 mRNA (A) or REDD2 mRNA (B)-to-TATA-binding protein (Tbp) mRNA ratio in the soleus muscle from control (dark gray), immobilized (black), and remobilized (light gray) hindlimbs expressed as a percentage of the same ratio in the soleus muscle from nonimmobilized hindlimbs of 9-mo-old rats. Tbp gene expression was verified against three common loading controls as a reliable housekeeping gene. Data are means ± SE, n = 4–6 rats/group. *P < 0.05 compared with control limb. #P < 0.05 compared with equivalent limb in 9-mo-old rat. †P < 0.05 compared with control limb by paired t-test.
may have resulted from the stress of the immobilization procedure. In contrast, based on the results presented here, REDD2 induction would seem to be the more relevant response to immobilization. Such a possibility is consistent with the finding that REDD2 mRNA expression is enriched in skeletal muscle compared with other tissues (45). It should be noted that the data presented herein are for mRNA and not protein expression for REDD2. Presently, assessing changes in REDD2 protein expression is not feasible due to the lack of anti-REDD2 antibodies that recognize the rat protein.

Another key result of the present study is the observation that the enhanced nutrient-induced activation of mTORC1 signaling that occurs in response to remobilization is associated with reduction in REDD2 mRNA expression. This result agrees with earlier ones that have also observed an enhancement of mTORC1 signaling in skeletal muscle following 1–7 days of remobilization (10, 35, 36, 46). Repression of REDD2 mRNA expression during remobilization would provide a mechanism for relieving the resistance to nutrient-induced activation of mTORC1 signaling following immobilization. Notably, despite the activation of mTORC1 signaling, muscle mass continued to fall during the period of remobilization. A similar phenomenon has been previously reported (35, 40). The molecular basis for the delayed recovery of muscle mass with remobilization is unknown. One potential mechanism could be linked to myonuclear apoptosis that occurs during immobilization and is still prevalent during reloading (27, 51). It is also tempting to speculate that it may be related to a reduction in RNA content, and thus ribosome abundance. In young growing animals, ribosome number is elevated to allow for higher rates of protein synthesis after an anabolic stimulus, whereas fewer ribosomes would be needed to maintain the relatively static muscle mass in older animals. Thus, an increase in ribosome number in the older animals may be required for muscle hypertrophy following remobilization. Notably, ribosome bio-

genesis, which is under the control of mTORC1 signaling (30), is reduced during hindlimb suspension and immobilization (5) and enhanced during reloading following hindlimb suspension (28). Due to the relatively slow turnover of ribosomes, it is not surprising to observe a delay in the response of skeletal muscle protein synthesis and growth (i.e., mass) to immobilization and/or remobilization in muscle from older animals.

![Graph showing the relationship between REDD2 mRNA expression and p70S6K1 Thr389 phosphorylation](image-url)

**Fig. 4.** REDD2 mRNA expression is inversely proportional to phosphorylation of p70S6K1 on Thr389 in rat soleus muscle. All data points represent an individual rat soleus muscle from 2-, 9-, and 18-mo-old rats that had one hindlimb immobilized for 7 days and an additional group of 9-mo-old animals that had their casts removed after 7 days and were allowed to remobilize for an additional 7 days. Data for REDD2 mRNA expression and relative p70S6K1 phosphorylation on Thr389 were taken from Figs. 3B and 1A, respectively. A negative correlation ($r^2 = 0.5372$) between REDD2 mRNA expression and phosphorylation of p70S6K1 on Thr389 ($P < 0.0001$) was established by fitting a linear regression to these points.

![Graph showing MAFbx and MuRF1 mRNA expression](image-url)

**Fig. 5.** MAFbx mRNA expression increases with age and 7 days of immobilization, whereas MuRF1 mRNA expression increases with immobilization only in 2-mo-old animals and decreases in response to remobilization. MAFbx and MuRF1 mRNA expression in the soleus muscle was assessed by Taqman gene expression assay. Rats 2, 9, and 18 mo of age had one hindlimb immobilized for 7 days, and an additional group of 9-mo-old animals had their casts removed after 7 days and were allowed to remobilize for an additional 7 days. Bars represent the mean MAFbx mRNA ($A$)- or MuRF1 mRNA ($B$)-to-Tbp mRNA ratio in the soleus muscle from control (dark gray), immobilized (black), and remobilized (light gray) hindlimbs expressed as a percentage of the same ratio in the soleus muscle from control hindlimbs of 9-mo-old rats. Tbp gene expression was verified against three common loading controls as a reliable housekeeping gene. Data are means ± SE, $n = 4–6$ rats/group. *$P < 0.05$ compared with control limb. $#P < 0.05$ compared with equivalent limb in 9-mo-old rat. ++$P < 0.05$ compared with control limb by unpaired $t$-test. $S$P $< 0.05$ vs. equivalent limb in 9- or 18-mo-old rat.
Models of both aging- and inactivity-induced muscle atrophy are associated with insulin resistance in skeletal muscle (9, 11, 48, 57, 59, 60). The alterations in the nutrient-induced stimulation of phosphorylation of Akt on Ser\(^{473}\) observed in the present study in response to aging and hindlimb immobilization and remobilization are in agreement with other reports (10, 29, 46, 60). It remains to be determined whether these changes in phosphorylation of Akt on Ser\(^{473}\) are linked with changes in REDD2 mRNA expression. Thus, future studies are needed to determine the contributions of induced REDD2 mRNA expression vs. attenuated Akt phosphorylation on mTORC1 signaling during aging, hindlimb immobilization, and remobilization.

Increased expression of the atrogenes, MAFbx and MuRF1, is considered to be an early marker of disuse atrophy (1, 35, 54) because expression of the mRNAs encoding the proteins is rapidly induced after limb immobilization in association with loss of muscle mass (8, 22). The results of the present study showing that, in 2-mo-old rats, expression of both MAFbx and MuRF1 mRNA is upregulated after 7 days of hindlimb immobilization is in agreement with these previous reports. However, unlike MAFbx mRNA that exhibited increased abundance in older (i.e., 2- compared with 9-mo-old) rats, MuRF1 mRNA expression in the present study was unaffected by age. This finding corroborates a previous report that MuRF1 mRNA expression was increased in muscle from older compared with younger rats (12) but disagrees with other studies reporting that MuRF1 mRNA expression is either unchanged (2) or decreased (18) during aging. Conflicting findings regarding the effect of age on MAFbx mRNA expression have also been reported, with different studies showing increased (12), decreased (18) or no change (2) in older compared with younger animals. The reason for the discrepant findings among studies is unclear but is likely due to differences in the muscle being examined, the age of the animals, or species.

In conclusion, nutrient-induced activation of mTORC1 signaling was enhanced and REDD2 mRNA expression was reduced in skeletal muscle from young compared with older rats. Moreover, mTORC1 signaling was enhanced by 7 days of remobilization in association with reduced REDD2 mRNA expression. No differences in REDD1 mRNA expression were observed in skeletal muscle due to aging, hindlimb immobilization, or remobilization. Therefore, REDD2 expression appears to play a prominent role in the regulation of mTORC1 signaling in skeletal muscle during aging, and hindlimb immobilization and remobilization, and consequently in modulating skeletal muscle mass.

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DISCLOSURES

SLP is an employee of Abbott Nutrition R&D. Otherwise, the authors declare no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS


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