Nutrient-Induced Stimulation of Protein Synthesis in Mouse Skeletal Muscle Is Limited by the mTORC1 Repressor REDD1^{1–3}

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Abstract

Background: In skeletal muscle, the nutrient-induced stimulation of protein synthesis requires signaling through the mechanistic target of rapamycin complex 1 (mTORC1). Expression of the repressor of mTORC1 signaling, regulated in development and DNA damage 1 (REDD1), is elevated in muscle during various atrophic conditions and diminished under hypertrophic conditions. The question arises as to what extent REDD1 limits the nutrient-induced stimulation of protein synthesis.

Objective: The objective was to examine the role of REDD1 in limiting the response of muscle protein synthesis and mTORC1 signaling to a nutrient stimulus.

Methods: Wild type REDD1 gene (REDD1^{+/+}) and disruption in the REDD1 gene (REDD1^{-/-}) mice were feed deprived for 16 h and randomized to remain feed deprived or refed for 15 or 60 min. The tibialis anterior was then removed for analysis of protein synthesis and mTORC1 signaling.

Results: In feed-deprived mice, protein synthesis and mTORC1 signaling were significantly lower in REDD1^{+/+} than in REDD1^{-/-} mice. Thirty minutes after the start of refeeding, protein synthesis in REDD1^{+/+} mice was stimulated by 28%, reaching a value similar to that observed in feed-deprived REDD1^{-/-} mice, and was accompanied by increased phosphorylation of mTOR (Ser2448), p70S6K1 (Thr389), and 4E-BP1 (Ser65) by 81%, 167%, and 207%, respectively. In refed REDD1^{-/-} mice, phosphorylation of mTOR (Ser2448), p70S6K1 (Thr389), and 4E-BP1 (Ser65) were significantly augmented above the values observed in refed REDD1^{+/+} mice by 258%, 405%, and 401%, respectively, although protein synthesis was not coordinately increased. Seventy-five minutes after refeeding, REDD1 expression in REDD1^{+/+} mice was reduced (~15% of feed-deprived REDD1^{+/+} values), and protein synthesis and mTORC1 signaling were not different between refed REDD1^{+/+} mice and REDD1^{-/-} mice.

Conclusions: The results show that REDD1 expression limits protein synthesis in mouse skeletal muscle by inhibiting mTORC1 signaling during periods of feed deprivation and that a reduction in its expression is necessary for maximal stimulation of protein synthesis in response to refeeding. *J Nutr* 2015;145:708–13.

Keywords: mammalian target of rapamycin, refeeding, 70-kDa ribosomal protein S6 kinase, eukaryotic initiation factor 4E binding protein 1, *DDIT4*, atrophy, hypertrophy

Introduction

Skeletal muscle mass is determined by coordination between rates of protein synthesis and protein degradation (1). In healthy

muscle in which mass is maintained, rates of synthesis and degradation wax and wane throughout the diurnal cycle in response to periods of feed deprivation and refeeding but remain in an overall net balance. A shift in favor of either net synthesis or net degradation results in muscle hypertrophy or atrophy, respectively (1). Although it is known that nutrients induce a shift in favor of net synthesis (1–8), various conditions such as limb immobilization, aging, sepsis, alcohol abuse, and cancer-induced cachexia show resistance to the stimulation of protein synthesis (a phenomenon termed anabolic resistance) (9–13).

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³ Supplemental Figures 1 and 2 and Supplemental Table 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

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Protein synthesis is regulated in part by signaling through the mechanistic target of rapamycin in complex 1 (mTORC1)⁶ (14). This protein kinase integrates a variety of cellular stimuli including nutrient availability and hormones to regulate mRNA translation through phosphorylation of the 70-kDa ribosomal protein S6 kinase 1 (p70S6K1) and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (15). In human skeletal muscle, signaling through mTORC1 is required for the nutrient-induced stimulation of protein synthesis because administration of rapamycin completely blocks the response (3). Thus, understanding mechanisms that limit nutrient-induced activation of mTORC1 will aid in development of therapies that enhance signaling through this complex to preserve muscle mass during anabolic resistant conditions.

Expression of the repressor of mTORC1 signaling, regulated in development and DNA damage 1 (REDD1; encoded by the *DDIT4* gene), is elevated in skeletal muscle during various atrophic conditions (e.g., feed deprivation) and diminished under hypertrophic conditions (e.g., refeeding) (11, 12, 16, 17). For example, the nutrient-induced stimulation of mTORC1 in diabetic rats is blunted in concert with elevated REDD1 expression (17). Likewise, REDD1 expression is elevated in immobilized skeletal muscle in which nutrient-induced activation of mTORC1 signaling is repressed (11). Moreover, the expression of REDD1 is elevated during other atrophic conditions (12, 18–20). Thus, the purpose of the present study was to examine the contribution of increased REDD1 expression to the decrease in muscle protein synthesis and mTORC1 signaling in feed-deprived mice and to determine whether increased REDD1 expression limits the feeding-induced stimulation of protein synthesis in skeletal muscle.

Methods

Animals. All experiments were performed on male mice 9–12 wk of age (~25 g in weight). Mice with a chromosomal disruption in the gene coding REDD1 (REDD1^{-/-}) were generated by Lexicon Genetics, Inc. (The Woodlands, Texas), and permission to use them was generously granted by Dr. Elena Feinstein (Quark Pharmaceuticals). Male, wild-type, age-matched B6/129F1 control mice (REDD1^{+/+}) were obtained from Taconic (Hudson, New York) or from those bred in The Pennsylvania State University College of Medicine central animal quarters. All mice were housed in a temperature (25°C)- and light (12:12 h light:dark cycle)-controlled environment. Mice were provided rodent unpurified diet (Harlan-Teklad 2018) and water ad libitum. The macronutrient composition of this diet is outlined in **Supplemental Table 1**. The Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine approved the animal facilities and all of the experimental protocols, which also comply with NIH guidelines.

Experimental methods. Three days before each study, all mice were individually housed and food pellets were placed on the bedding of the cage floor to acclimate them to the experimental conditions. The night before each study, all mice were feed deprived beginning at 1600. The next morning (~0900), mice from each genotype were randomized to remain feed deprived or have access to food pellets on the cage floor for

either 15 or 60 min. After the refeeding period, food was removed from the refeed group and all mice (both feed deprived and refeed) were injected with radioactive tracer and muscle protein synthesis was measured as previously described (16). Fifteen minutes postinjection (i.e., at 30 or 75 min from the start of refeeding, respectively), the tibialis anterior muscles were extracted, frozen in liquid nitrogen, and stored at -80°C. Each study was replicated 2–4 times on separate days.

Western blot analysis. Western blot analysis was conducted as previously described (16, 21). Antibodies against p70S6K1 (Thr389), 4E-BP1 (Ser65), mTOR (Ser2448), and total mTOR, were obtained from Cell Signaling Technology. Antibodies against total p70S6K1 and 4E-BP1 were obtained from Bethyl Laboratories. Antibodies against REDD1 were obtained from ProteinTech. Appropriate secondary antibodies were obtained from Bethyl Laboratories.

Statistical analysis. All data are presented as mean ± SEM. Two-factor ANOVA was used to determine the effect of refeeding or genotype on the dependent variables in the 15-min refeeding experiment. One-factor ANOVA was used to determine the effect of refeeding on the dependent variables in the 60-min refeeding experiment. Unpaired Student's *t* test was used post hoc to determine specific differences if a significant *F* value was observed by either 2-factor ANOVA or 1-factor ANOVA. Pearson product-moment correlation analysis was used to determine relations between REDD1 and rates of protein synthesis or mTORC1 signaling in the 15-min refeeding experiment. All analyses were performed using Graphpad Prism software and Microsoft Excel software. Statistical significance for all analyses was set at *P* ≤ 0.05.

Results

Refeeding for 15 min does not affect REDD1 protein expression. REDD1^{+/+} and REDD1^{-/-} mice consumed the same amount of food during the 15-min refeeding period (**Supplemental Figure 1**). Furthermore, REDD1 expression in muscle of refeed REDD1^{+/+} mice did not differ from feed-deprived REDD1^{+/+} mice at this time point after refeeding.

REDD1 expression limits the nutrient-induced stimulation of protein synthesis and activation of mTORC1 signaling. After the 15-min refeeding, the rate of skeletal muscle protein synthesis was stimulated by 28% ± 12% (*P* < 0.05) in muscle of refeed REDD1^{+/+} mice relative to feed-deprived REDD1^{+/+} control mice (**Figure 1A**). Protein synthesis in feed-deprived REDD1^{-/-} mice was significantly greater (*P* < 0.05) relative to feed-deprived REDD1^{+/+} values (**Figure 1A**), and the protein synthetic rate in refeed REDD1^{-/-} mice did not differ from feed-deprived REDD1^{-/-} values. However, the protein synthetic rate in refeed REDD1^{-/-} mice was significantly greater (*P* < 0.05) than the values observed in refeed REDD1^{+/+} mice.

Fifteen minutes of refeeding REDD1^{+/+} mice activated mTORC1 signaling, as assessed by phosphorylation of mTOR at Ser2448, p70S6K1 at Thr389, and 4E-BP1 at Ser65, and was significantly increased (*P* < 0.05) relative to feed-deprived REDD1^{+/+} values (**Figure 1B–D**). Similar to a previous report (22), mTORC1 signaling was elevated in muscle from feed-deprived REDD1^{-/-} compared with feed-deprived REDD1^{+/+} mice. Moreover, 15 min of refeeding REDD1^{-/-} mice further increased phosphorylation of mTOR (Ser2448), p70S6K1 (Thr389), and 4E-BP1 (Ser65) above the values observed in both feed-deprived REDD1^{+/+} and feed-deprived REDD1^{-/-} mice (**Figure 1B–D**). Representative Western blots are shown in **Figure 1E**. Notably, a significant linear negative correlation was observed after 15 min of refeeding in REDD1^{+/+} mice between REDD1 protein expression and rates of protein synthesis (*P* < 0.05), phosphorylation of mTOR (Ser2448; *P* < 0.05),

⁶ Abbreviations used: mTORC1, mechanistic target of rapamycin in complex 1; p70S6K1, 70-kDa ribosomal protein S6 kinase 1; Rag, ras-related GTP binding; REDD1, regulated in development and DNA damage 1; REDD1^{-/-}, disruption in the REDD1 gene; REDD1^{+/+}, wild type REDD1 gene; Rheb, ras homolog enriched in brain; TSC2, tuberous sclerosis complex 2; ULK1, uncoordinated movement-51-like kinase 1; 4E-BP1, eukaryotic initiation factor 4E binding protein 1.

FIGURE 1 Rates of protein synthesis and mTORC1 signaling after 15 min of refeeding in REDD1^{+/+} and REDD1^{-/-} mice. (A) Rate of protein synthesis in the tibialis anterior expressed as a percent of the REDD1^{+/+} feed-deprived value. The ratio of phosphorylated to total protein for (B) mTOR (Ser2448), (C) p70S6K1 (Thr389), and (D) 4E-BP1 (Ser65) as determined by Western blot analysis. (E) Representative Western blots. Values are mean $\pm$ SEM ($n = 5$ –8 per group). *Significant effect of refeeding within genotype at $P < 0.05$. #Significant effect of genotype within feed-deprived or re-fed condition at $P < 0.05$. F, feed deprived; F $\times$ G, feeding $\times$ genotype; M.E., main effect; mTOR, mechanistic target of rapamycin; mTORC1, mechanistic target of rapamycin in complex 1; p-mTOR, phosphorylated mechanistic target of rapamycin; p-p70S6K1, p70S6K1 phosphorylated at Thr389; p-4E-BP1, 4E-BP1 phosphorylated at Ser65; p70S6K1, 70-kDa ribosomal protein S6 kinase 1; R, re-fed; REDD1, regulated in development and DNA damage 1; REDD1^{-/-}, disruption in the REDD1 gene; REDD1^{+/+}, wild type REDD1 gene; 4E-BP1, eukaryotic initiation factor 4E binding protein 1.

p70S6K1 (Thr389; $P < 0.05$), and phosphorylation of 4E-BP1 (Ser65; $P < 0.05$; Table 1 and Supplemental Figure 2).

After 60 min of refeeding, loss of REDD1 results in values for rates of protein synthesis and mTORC1 signaling similar to those obtained in re-fed REDD1^{-/-} animals. After 60 min of refeeding, there was no significant difference in the amount of feed consumed by either genotype (Figure 2A). REDD1 expression in re-fed REDD1^{+/+} mice was significantly reduced ($P < 0.05$) to nearly undetectable values (Figure 2B). The rate of protein synthesis was significantly increased ($P < 0.05$) in re-fed compared with feed-deprived REDD1^{+/+} mice (Figure 2C). However, in contrast to the shorter feeding period where protein synthesis in re-fed REDD1^{+/+} mice was lower compared with re-fed REDD1^{-/-} mice (Figure 1A), 75 min after initiating refeeding protein synthesis was not significantly different in re-fed REDD1^{+/+} mice compared with REDD1^{-/-} mice (Figure

2C). Furthermore, refeeding REDD1^{+/+} mice increased phosphorylation of both p70S6K1 (Thr389) (Figure 2D) and 4E-BP1 (Ser65) (Figure 2E) ($P < 0.05$) relative to feed-deprived REDD1^{+/+} values with no significant difference observed in phosphorylation of either protein in re-fed REDD1^{+/+} mice compared with REDD1^{-/-} mice. Representative Western blots are shown in Figure 2F. These data provide further support for the conclusion that REDD1 expression acts to limit the magnitude of the nutrient-induced stimulation of protein synthesis and activation of mTORC1 signaling in mouse skeletal muscle.

Discussion

In the present study we found that in REDD1^{+/+} mice re-fed for a short (i.e., 15 min) period of time, the feeding-induced stimulation of protein synthesis in skeletal muscle occurred before a detectable reduction in REDD1 expression. Notably, feed-deprived REDD1^{-/-} mice exhibited higher rates of protein synthesis than feed-deprived REDD1^{+/+} mice, and protein synthesis was greater in re-fed REDD1^{-/-} than in re-fed REDD1^{+/+} mice. After a more prolonged feeding period (i.e., 60 min), REDD1 expression in REDD1^{+/+} mice was dramatically reduced compared with feed-deprived REDD1^{+/+} mice and the nutrient-induced stimulation of protein synthesis was not different in re-fed REDD1^{-/-} than REDD1^{+/+} mice at this time point. Combined, these findings support the conclusion that REDD1 functions to limit rates of protein synthesis in mouse skeletal muscle during feed-deprived states and that a reduction in REDD1 abundance is necessary for achieving maximal stimulation of protein synthesis after refeeding. These conclusions are

TABLE 1 Pearson product-moment correlation matrix for 15-min re-fed REDD1^{+/+} mice ($n = 5$)¹

	REDD1 protein content (r)	P
Protein synthesis	-0.94	<0.02
mTOR (Ser2448)	-0.96	<0.01
p70S6K1 (Thr389)	-0.88	<0.05
4E-BP1 (Ser65)	-0.90	<0.05

¹ mTOR, mechanistic target of rapamycin; p70S6K1, 70-kDa ribosomal protein S6 kinase 1; REDD1, regulated in development and DNA damage 1; REDD1^{+/+}, wild type REDD1 gene; 4E-BP1, eukaryotic initiation factor 4E binding protein 1.

FIGURE 2 REDD1 protein expression, rates of protein synthesis, and mTORC1 signaling after 60 min of refeeding in REDD1^{+/+} and REDD1^{-/-} mice. (A) Food consumption after 60-min refeeding. (B) REDD1 protein expression was determined by Western blot analysis and was normalized to ponceau stain. (C) Rate of protein synthesis in the tibialis anterior expressed as a percent of the REDD1^{+/+} feed-deprived value. The ratio of phosphorylated to total protein was determined for (D) p70S6K1 (Thr389) and (E) 4E-BP1 (Ser65) by Western blot analysis. (F) Representative Western blots. Values are mean $\pm$ SEM ($n = 3-5$ per group). Bars with differing letters are significantly different, $P < 0.05$. F, feed deprived; mTORC1, mechanistic target of rapamycin in complex 1; N.D., none detected; N.S., nonspecific band; p-p70S6K1, p70S6K1 phosphorylated at Thr389; p-4E-BP1, 4E-BP1 phosphorylated at Ser65; p70S6K1, 70-kDa ribosomal protein S6 kinase 1; R, refed; REDD1, regulated in development and DNA damage 1; REDD1^{-/-}, disruption in the REDD1 gene; REDD1^{+/+}, wild type REDD1 gene; 4E-BP1, eukaryotic initiation factor 4E binding protein 1.

supported by results of previous studies. For example, REDD1 mRNA and protein expression are reduced in parallel with increased rates of protein synthesis both in human skeletal muscle after resistance exercise and in mice after muscle contractions (16, 23). Conversely, aerobic exercise in rodents represses protein synthesis in skeletal muscle in concert with increased REDD1 expression (24, 25). Nonetheless, REDD1-independent mechanism(s) for limiting nutrient-induced stimulation of protein synthesis must exist, because in the present study protein synthesis was not further enhanced by refeeding feed-deprived REDD1^{-/-} mice. This point is discussed further in the following paragraphs.

The feeding-induced stimulation of protein synthesis observed in the present study was associated with activation of the mTORC1 signaling pathway. Previous studies showed the stimulation of protein synthesis that occurs in response to refeeding or oral administration of amino acids (e.g., Leu) to a feed-deprived animal is blocked by prior administration of the mTORC1 inhibitor, rapamycin (3, 7, 26), demonstrating the important role played by mTORC1 in the response. The results of the present study provide further support for this conclusion by showing that mTORC1 signaling is inversely proportional to the abundance of the mTORC1 repressor, REDD1. Thus, when REDD1 expression was highest, e.g., in feed-deprived REDD1^{+/+} mice, mTORC1 signaling was lowest, and when REDD1 expression was low, e.g., in REDD1^{-/-} mice or in REDD1^{+/+} mice refed for 60 min, mTORC1 signaling was high. Indeed, in feed-deprived REDD1^{+/+} mice, phosphorylation of both

p70S6K1 and 4E-BP1 was almost too low to measure. Although the increase in REDD1 abundance that occurs in response to feed deprivation almost certainly contributes to the reduction in mTORC1 signaling and protein synthesis, by itself the increase is probably insufficient to account completely for the reduction in either because short-term refeeding increased both protein synthesis and mTORC1 signaling without significantly reducing REDD1 abundance. This result suggests that one or more feeding-induced signaling pathways to mTORC1 can still be activated in the presence of REDD1, although the response of the kinase is blunted because of the presence of the repressor.

At least 2 pathways are involved in the feeding-induced stimulation of protein synthesis and mTORC1 signaling, the insulin-sensitive tuberous sclerosis complex 2/ras homolog enriched in brain (TSC2/Rheb) pathway and the amino acid-sensitive ras-related GTP binding (Rag) pathway. In this regard, clamping insulin at fasting values prevents the stimulation of protein synthesis and activation of mTORC1 signaling that occurs in response to oral administration of Leu to feed-deprived rats (27). Moreover, feeding a protein-deficient diet to feed-deprived rats increases plasma insulin concentrations to the same extent as feeding an isocaloric protein-containing meal; however, neither protein synthesis nor mTORC1 signaling are changed relative to that observed in feed-deprived rats (28). Overall, the results support the conclusion that signaling through both the insulin and the amino acid pathways is required to maximally activate mTORC1 and thereby stimulate muscle protein synthesis (29-31). Further support for a combined

effect of insulin and amino acids on mTORC1 signaling is provided by studies using cells in culture. For example, in serum- and Leu-deprived Rat2 fibroblasts, treatment with either Leu or insulin activates mTORC1 signaling, but in combination they generate an additive effect (15). Cells expressing a constitutively active Rheb variant are resistant to serum, but not Leu, deprivation-induced repression of mTORC1 signaling (15). In contrast, cells expressing constitutively active Rag variants are resistant to Leu, but not serum, deprivation-induced repression of mTORC1 signaling (15). Cells expressing constitutively active variants of both Rheb and Rags are resistant to both serum and Leu deprivation-induced repressed signaling (15). Thus, signaling through both pathways is required for maximal activation of mTORC1.

REDD1 is thought to repress mTORC1 signaling by activating the GTPase activity of TSC2 toward Rheb, leading to increased GDP loading and inactivation of Rheb (32–34). Thus, the increased expression of REDD1 in muscle from feed-deprived mice would be expected to lead to activation of TSC2 and thus to inactivation of Rheb; signaling through the amino acid/Rag pathway would be expected to remain intact. However, as discussed previously, because Rheb · GTP is required for optimal amino acid-induced activation of mTORC1, it might be expected that REDD1-mediated inactivation of Rheb would also blunt the stimulatory effect of amino acids on mTORC1 signaling. Support for this hypothesis is provided by a study (35) showing that ectopic expression of the REDD1-related protein, REDD2, in muscle cells inhibited basal mTORC1 signaling and also blunted the stimulatory effect of Leu. Thus, the increase in REDD1 expression that occurs in response to feed deprivation likely attenuates mTORC1 signaling through a mechanism involving activation of TSC2 and accumulation of Rheb in the inactive GDP-bound form. Reduced GTP loading of Rheb would not only directly interfere with mTORC1 activation but would also indirectly perturb signaling through the Rag pathway.

An apparent contradictory finding in the present study was that refeeding feed-deprived REDD1^{-/-} mice further augmented the activation of mTORC1 signaling, but had no effect on protein synthesis. A similar effect was observed in previous studies. For example, electrically induced muscle contractions stimulate protein synthesis and mTORC1 signaling in REDD1^{+/+} mice (16). However, in REDD1^{-/-} mice, contractions further augment mTORC1 signaling but have no effect on protein synthesis. Moreover, mTORC1 signaling in skeletal muscle is activated in direct proportion to the amount of Leu administered by oral gavage, as demonstrated by a linear increase in p70S6K1 phosphorylation up to a dose of 1.35 g of Leu per kilogram of body weight (2). In contrast, protein synthesis is maximally stimulated at 10–15% of the maximum dose of Leu. The molecular basis for the disconnect between activation of mTORC1 signaling and protein synthesis is unknown. Although speculative, the additional activation of mTORC1 signaling beyond what is necessary to maximally stimulate protein synthesis may be necessary to bring other downstream responses into play. One example might be to increase the capacity of the tissue to synthesize protein by promoting ribosome biogenesis, because both ribosomal DNA transcription (36) and translation of mRNAs encoding ribosomal proteins (14) require signaling through mTORC1. Another example might be to promote a more prolonged inhibition of autophagy through phosphorylation and inhibition of uncoordinated movement-51-like kinase 1 (ULK1), a protein necessary for the initial stages of autophagic puncta formation (37). Confirmation of such speculation requires further investigation.

Overall, the results of the present study provide compelling support for the conclusion that REDD1 acts as a nutrient-sensitive repressor of mTORC1 signaling and protein synthesis. Notably, it does not prevent completely the feeding-induced stimulation of protein synthesis. Instead, the results are consistent with the conclusion that REDD1 acts as a governor that initially limits the magnitude of the feeding-induced activation of mTORC1 and stimulation of protein synthesis until its expression is reduced. The results are likely applicable to other conditions in which REDD1 expression is elevated in skeletal muscle. For example, REDD1 expression is elevated during various atrophic conditions such as cancer cachexia (19, 38), limb immobilization (11), sepsis (12), and alcohol abuse (18) and likely contributes to the repressed nutrient-induced stimulation of protein synthesis and activation of mTORC1 signaling in those conditions. Therefore, pharmacologic strategies that target REDD1 in skeletal muscle during these and other atrophic conditions may help to restore protein synthesis and offset or reverse the loss of muscle mass.

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