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Elevated Corticosterone Associated with Food Deprivation Upregulates Expression in Rat Skeletal Muscle of the mTORC1 Repressor, REDD1[1–3]

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Abstract

Food deprivation induces a repression of protein synthesis in skeletal muscle in part due to reduced signaling through the mammalian target of rapamycin complex 1 (mTORC1). Previous studies have identified upregulated expression of the protein Regulated in DNA Damage and Development (REDD1) as an important mechanism in the regulation of mTORC1 activity in response to a variety of stresses. Our goal in this investigation was to determine whether modulation of REDD1 expression occurs in response to food deprivation and refeeding, and, if it does, to ascertain if changes in REDD1 expression correlate with altered mTORC1 signaling. As expected, mTORC1 signaling was repressed after 18 h of food deprivation compared with freely-fed control rats and quickly recovered after refeeding for 45 min. Food deprivation caused a dramatic rise in REDD1 mRNA and protein expression; refeeding resulted in a reduction to baseline. Food deprivation is characterized by low-serum insulin and elevated glucocorticoid concentrations. Therefore, initially, alloxan-induced type I diabetes was used to minimize the food deprivation- and refeeding-induced changes in insulin. Although diabetic rats exhibited upregulated REDD1 expression compared with nondiabetic controls, there was no direct correlation between REDD1 mRNA expression and serum insulin levels, and insulin treatment of diabetic rats did not affect REDD1 expression. In contrast, serum corticosterone levels correlated directly with REDD1 mRNA expression ($r = 0.68$; $P = 0.01$). Moreover, inhibiting corticosterone-mediated signaling via administration of the glucocorticoid receptor antagonist RU486 blocked both the food deprivation- and diabetes-induced increase in REDD1 mRNA expression. Overall, the results demonstrate that changes in REDD1 expression likely contribute to the regulation of mTORC1 signaling during food deprivation and refeeding. J. Nutr. 139: 828–834, 2009.

Introduction

Food deprivation induces a repression of global rates of protein synthesis in various tissues, including skeletal muscle (1). The repression of protein synthesis is partially mediated by reduced signaling through the mammalian target of rapamycin complex 1 (mTORC1)[4] to mechanisms involved in mRNA translation (2). The mTORC1 complex includes the protein kinase mTOR, regulatory associated protein of mTOR, Ras homolog enriched in brain, and LST8 (or GβL for G-protein β-like protein) (3). The best-characterized substrates phosphorylated by mTORC1 are eukaryotic initiation factor (eIF) 4E binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1) (4). Phosphorylation of 4E-BP1 results in dissociation of the protein from eIF4E (5,6), allowing eIF4E to bind to eIF4G to form the mRNA cap-binding complex, eIF4F (7,8). Upon assembly of eIF4F at the m^7GTP cap, the 40S ribosomal subunit is recruited to form the 48S preinitiation complex. However, when 4E-BP1 is hypophosphorylated, as occurs in muscle during food deprivation, it tightly binds eIF4E, preventing assembly of the eIF4F complex and therefore the 48S preinitiation complex. Phosphorylation of S6K1 by mTORC1 enables it to bind phosphoinositide-dependent protein kinase 1 (9,10), which subsequently phosphorylates S6K1, activating it and allowing it to phosphorylate ribosomal protein S6 (11), a component of the 40S ribosomal subunit, eIF4B (12,13), and eukaryotic elongation factor 2 kinase (14). Thus, food deprivation-induced repression of mTORC1 signaling downregulates both the initiation and elongation phases of mRNA translation, resulting in reduced protein synthesis.

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[3] Supplemental Figure 1 is available with the online posting of this paper at jn.nutrition.org.
[4] Abbreviations used: 4E-BP1, eukaryotic initiation factor 4E binding protein 1; eIF, eukaryotic initiation factor; mTORC1, mammalian target of rapamycin complex 1; REDD1, Regulated in DNA Damage and Development 1; S6K1, ribosomal protein S6 kinase 1; TSC2, tuberous sclerosis complex 2.
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The protein Regulated in DNA Damage and Development 1 (REDD1; also referred to as Rtp801 and DDIT4) has been shown in several studies to act as an inhibitor of mTORC1 signaling. Though its mechanism of action is relatively uncharacterized, repression of mTORC1 signaling requires the upstream mTORC1 repressor tuberous sclerosis complex 2 (TSC2) (15–17) and may require the release of TSC2 from a 14–3-3 protein (18). Adverse conditions, including ATP depletion (17), DNA damage (19), endoplasmic reticulum stress (20,21), and hypoxia (15,16,22,23), transcriptionally induce REDD1 expression through various mechanisms, including hypoxia inducible factor-1α during hypoxia (16,23) and p53 in the case of DNA damage (24). Recent investigations by our laboratory utilizing the synthetic glucocorticoid dexamethasone have shown exogenous glucocorticoids induce REDD1 mRNA and protein expression in skeletal muscle as well as in L6 myoblasts (25).

Elevation of circulating glucocorticoids, namely corticosterone in the rodent and cortisol in humans, occurs with food deprivation. Diminished food intake also reduces the release of gut hormones that signal pancreatic β-cells to secrete insulin. Elevated serum corticosterone and low insulin are, therefore, hallmarks of the food-deprived state [reviewed in (26)]. We sought in this study to determine, first, whether REDD1 expression increases in concert with the established decrease in mTORC1 activity during food deprivation and, second, possible roles for insulin and corticosterone in regulating REDD1 expression in food-deprived rats.

Materials and Methods

Rat care. The animal facilities and the experimental protocol used in the studies reported herein were reviewed and approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University College of Medicine. Male Sprague-Dawley rats (~150 g; Charles River) were maintained on a 12-h-light:-dark cycle and consumed a standard diet [Harlan-Teklad Rodent Chow 8604 (27)] and water ad libitum. All experiments were performed at the beginning of the light cycle.

Experimental design. Rats were food deprived or allowed to eat ad libitum for 18 h before the experiment. The morning of the experiment, some food-deprived rats were allowed to refeed for 45 min prior to killing. Diabetes was induced in postabsorptive rats by intravenous injection of alloxan (48 mg/kg body weight in 0.155 mol/L saline) (Sigma). A urine sample was taken the day prior to the experiment to confirm the presence of hyperglycemia ($\geq$0.025 mol/L glucose); any rats that failed to exceed this threshold were excluded from the experiment. Approximately 54 h following alloxan injection, overnight food deprivation was begun and at 72 h, rats were either killed or refed for 45 min prior to killing. Mean weight of food consumed during the refeeding period was comparable between groups. Some of the rats received an intraperitoneal injection of 1 U of insulin 55 min prior to killing (10 min before start of refeeding). Alternatively, rats received an intraperitoneal injection of RU486 (25 mg/kg body weight in 25 g/L ethanol) (Sigma) at the beginning of the 18-h period of food deprivation and again 2 h prior to killing. Control rats were injected with an equal volume of vehicle alone.

Sample collection. Rats were killed by decapitation. Serum obtained from trunk blood was centrifuged at 1800 $\times$ *g*; 10 min at 4°C. The right hindlimb gastrocnemius was quickly excised and a small portion was homogenized and centrifuged as described previously (25). Remaining supernatant was also stored at −80°C. The left hindlimb gastrocnemius was flash-frozen between aluminum blocks precooled in liquid nitrogen and then stored at −80°C.

Serum corticosterone measurement. Serum corticosterone was analyzed using the Correlate-EIA Cortisol Enzyme Immunoassay kit (catalog no. 900–071, Assay Designs) according to the manufacturer's instructions. Results were confirmed using the Corticosterone Enzyme Immunoassay kit (catalog no. 900–097) from the same manufacturer.

Analysis of protein expression and phosphorylation status. REDD1, S6K1 total protein and phosphorylated at Thr389, 4E-BP1, and Akt total protein and phosphorylated at Ser473 were quantitated by protein Western blot analysis as described previously (25).

Analysis of REDD1 mRNA expression. Total RNA was isolated from frozen muscle via homogenization in Trizol buffer (catalog no. 15596–018, Invitrogen) and subsequently purified according to the manufacturer's protocol. RNA quality and concentration were determined using the NanoDrop Spectrophotometer with associated NanoDrop ND-1000 3.5.1 software (Thermo Fisher Scientific). One microgram of isolated RNA from each sample was used to generate complementary cDNA using the High Capacity cDNA Reverse Transcription kit (part no. 4368814, Applied Biosystems). REDD1 mRNA in each sample was determined in triplicate by quantitative real-time PCR analysis using the primers previously described (25) and the QuantiTect SYBR Green PCR kit (catalog no. 204143, Qiagen). The REDD1 mRNA value was normalized to β-actin mRNA for each sample.

Statistical analysis. Data are presented as means ± SEM. Data were analyzed by the Prism version 5.0b for Macintosh statistical software package (GraphPad Software), using either an unpaired *t* test or 1- or 2-way ANOVA, where appropriate. In *t* tests, if a difference between means were detected, a nonparametric Mann-Whitney test was performed. In the 2-way ANOVA, the variables tested were nutritional status (i.e. freely fed, food deprived, refed) and experimental diabetes. If a statistical difference was detected by ANOVA, the data were subjected to post hoc nonparametric Dunn analysis for the effect of nutritional status or unpaired *t* test for the effect of experimental diabetes. Statistical outliers were identified using the Grubb's test outlier calculator provided by GraphPad Software and excluded from analyses. In all analyses, $P < 0.05$ was considered significant.

Results

Effects of food deprivation and refeeding on biomarkers of mTORC1 activation. Overnight food deprivation has been shown to reduce signaling through mTORC1 (28). In the present study, overnight food deprivation of the rats caused the proportion of 4E-BP1 in the hyperphosphorylated γ-form to decline to ~30% of the control, freely fed value, while refeeding for 45 min following the deprivation caused a recovery in 4E-BP1 phosphorylation to the control value (**Supplemental Fig. 1***A*). Like 4E-BP1, S6K1 also resolves into multiple electrophoretic forms based on its phosphorylation states. Quantification of the hyperphosphorylated β-, γ-, and δ-forms showed a food deprivation-induced decline in overall S6K1 phosphorylation that reverted toward the control pattern upon refeeding (Supplemental Fig. 1*B*).

Effect of food deprivation and refeeding on REDD1 expression. Having confirmed the effect of food deprivation and refeeding on mTORC1 signaling, we next sought to investigate possible mechanisms mediating the effects. Overnight food deprivation increased expression of the mTORC1 repressor, REDD1, over 2.5-fold in the gastrocnemius compared with freely fed controls (**Fig. 1***A*). Upon refeeding, REDD1 protein expression returned to the control level. REDD1 mRNA expression changed in concert with the protein, being increased to ~500% of the control value with food deprivation and returning to baseline when rats were refed (Fig. 1*B*).

Effect of diabetes on food deprivation and refeeding-induced changes in REDD1 expression. To minimize the

fluctuations in insulin levels associated with food deprivation and refeeding, the studies described above were repeated using rats with experimentally induced type I diabetes. The effectiveness of this model in minimizing variations in insulin concentrations was confirmed (**Fig. 2***A*). Thus, in control rats, insulin concentrations were changed 5-fold by food deprivation and refeeding, whereas in diabetic rats, concentrations of the hormone varied <2-fold in response to feeding status. Two-way ANOVA analysis revealed an effect of experimental diabetes ($P = 0.0002$) and nutritional state ($P = 0.0033$) on serum insulin concentrations, with no significant interaction. However, the analysis revealed a trend ($P = 0.0657$) for such an interaction. To assess the effectiveness of diabetes in attenuating food deprivation- and refeeding-induced changes in mTORC1 signaling, S6K1 phosphorylation status was measured. Both the decrease in S6K1 hyperphosphorylation caused by food deprivation and the increase caused by refeeding were severely blunted in diabetic compared with control rats (Fig. 2*B*,*C*). In addition, the food deprivation- and refeeding-induced changes in phosphorylation of S6K1 on Thr389, a residue directly phosphorylated by mTORC1 (29,30), were largely absent in diabetic rats. Two-way ANOVA analysis revealed an effect of experimental diabetes ($P = 0.0043$) and nutritional state ($P = 0.0160$) on serum insulin concentrations, as well as an interaction ($P = 0.0242$). However, even though mTORC1 signaling was attenuated in diabetic compared with control rats, food deprivation and refeeding still induced changes in REDD1 expression. Indeed, REDD1 protein expression was 3-fold greater in gastrocnemius from either food-deprived or refed diabetic rats compared with controls (**Fig. 3***A*). Two-way ANOVA analysis revealed an effect of experimental diabetes ($P < 0.0001$) and nutritional state ($P < 0.0001$) on REDD1 protein expression, as well as an interaction ($P < 0.0001$). REDD1 mRNA expression was at least 2-fold greater in muscle from diabetic compared with control rats regardless of feeding status (Fig. 3*B*). Two-way ANOVA analysis revealed an effect of experimental diabetes ($P = 0.0003$) and nutritional state ($P = 0.0017$) on REDD1 mRNA expression, as well as an interaction ($P = 0.0294$).

To determine whether low insulin concentrations in the diabetic rats were responsible for the upregulated REDD1 expression, diabetic rats were injected with insulin just prior to their refeeding period. Insulin administration significantly increased serum insulin concentrations (**Fig. 4***A*) as well as upregulated insulin signaling within the gastrocnemius, as indicated by phosphorylation of Akt

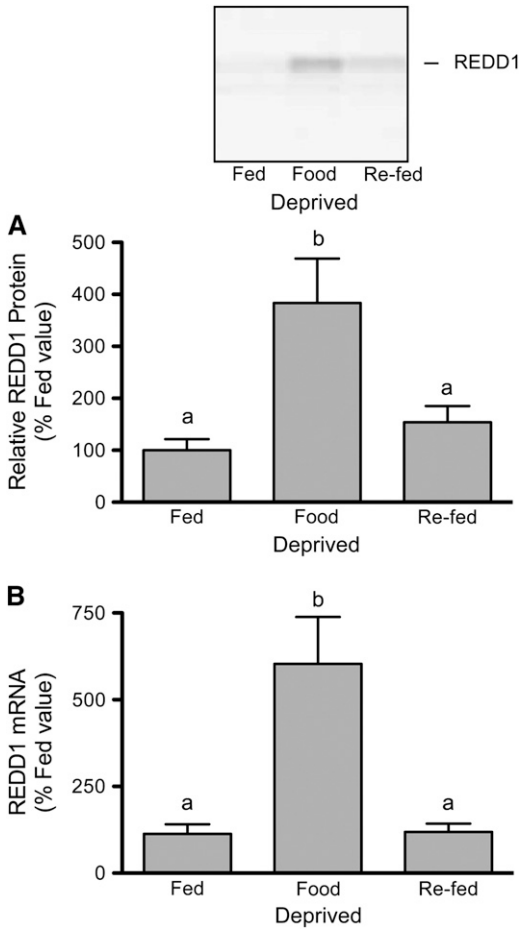

FIGURE 1 REDD1 protein (*A*) and mRNA expression (*B*) in gastrocnemius muscle of rats that consumed food ad libitum (Fed), were food deprived for 18 h, or were refed for 45 min after an 18-h deprivation (Re-fed). Values are means ± SEM, $n \pm 6$. Means without a common letter differ, $P < 0.05$.

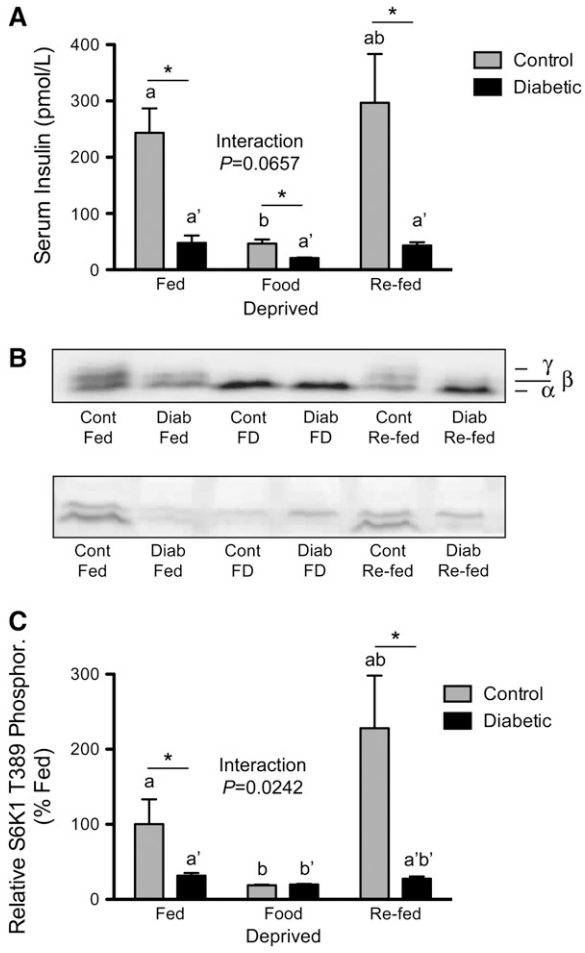

FIGURE 2 Serum insulin level (*A*), S6K1 hyperphosphorylation (*B*), and S6K1 phosphorylation on Thr389 (*C*) in gastrocnemius muscle of control (gray bars) and diabetic (black bars) rats that consumed food ad libitum (Fed), were food deprived for 18 h, or were refed for 45 min after an 18-h deprivation (Re-fed). Values are means ± SEM, $n = 5$–6. Within diabetic and control groups, means without a common letter differ, $P < 0.05$. *Different from corresponding control, $P < 0.05$.

on Ser473 (Fig. 4*B*). However, insulin administration did not affect REDD1 protein expression in refed diabetic rats (Fig. 4*C*).

Effect of glucocorticoid receptor inhibition on REDD1 expression. In addition to reduced insulin, elevated glucocorticoid concentrations are a well-recognized feature of food deprivation (26). In the present study, food deprivation led to a significant increase in the serum corticosterone concentration in control rats (**Fig. 5***A*) and the food deprivation-induced increase was exacerbated in diabetic rats, in which serum corticosterone concentrations increased 4-fold with food deprivation (Fig. 5*B*). Importantly, there was a significant positive correlation between serum corticosterone concentrations and muscle REDD1 mRNA expression in fed and food-deprived control (**Fig. 6***A*) and diabetic (Fig. 6*C*) rats. Moreover, RU486 administration to fed and food-deprived control (Fig. 6*B*) and diabetic (Fig. 6*D*) rats not only prevented the food deprivation- and diabetes-induced increase in REDD1 expression but also abolished the relationship between corticosterone concentration and REDD1 mRNA expression.

Discussion

Food deprivation causes a repression of global rates of protein synthesis in skeletal muscle (1). In part, the repression is a result of downregulated signaling through the mTORC1 complex, resulting in reduced phosphorylation of its substrates S6K1 and

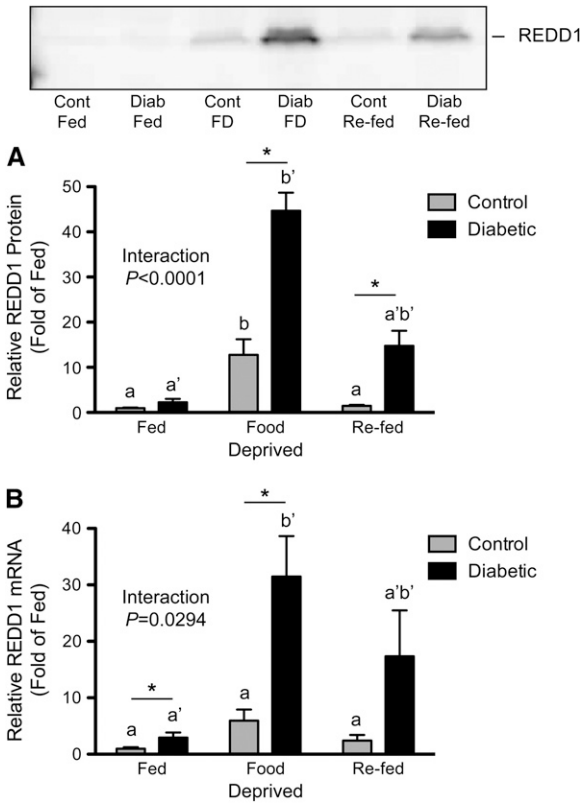

FIGURE 3 REDD1 protein (*A*) and mRNA expression (*B*) in gastrocnemius muscle of control (gray bars) and diabetic (black bars) rats that consumed food ad libitum (Fed), were food deprived for 18 h, or were refed for 45 min after an 18-h deprivation (Re-fed). Values are means ± SEM, $n = 6$. Within diabetic and control groups, means without a common letter differ, $P < 0.05$. *Different from corresponding control, $P < 0.05$.

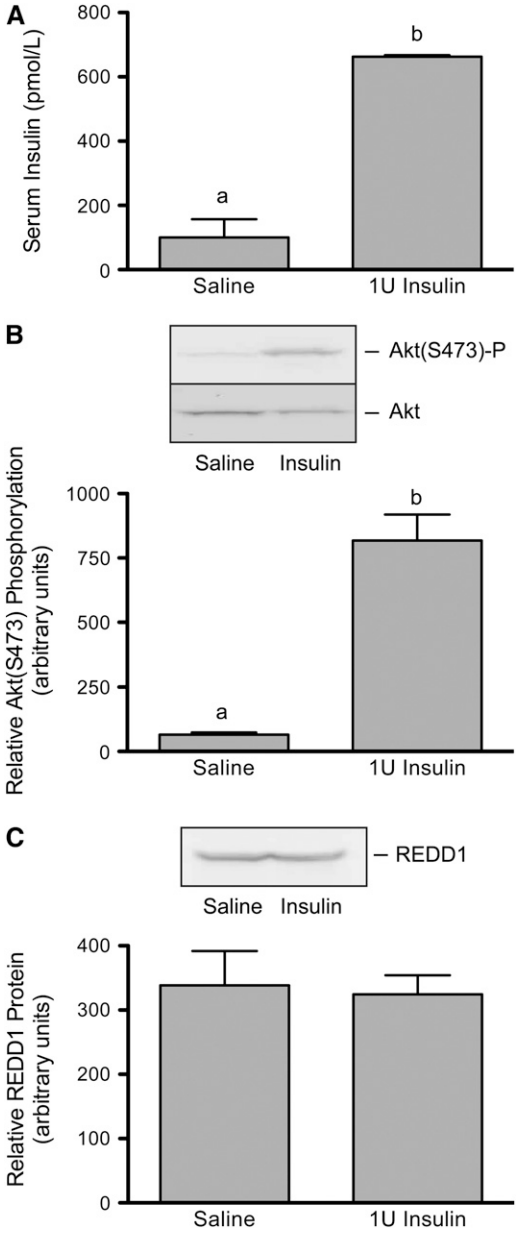

FIGURE 4 Serum insulin concentration (*A*), Akt(Ser473) phosphorylation (*B*), and REDD1 expression (*C*) in gastrocnemius muscle of control and insulin-treated diabetic rats. Values are means ± SEM, $n = 6$. Means without a common letter differ, $P < 0.05$.

4E-BP1 (28). Multiple upstream inputs to mTORC1 are influenced by food deprivation. For example, serum insulin concentrations fall during food deprivation, resulting in decreased signaling through the class I phosphatidylinositol 3-kinase and the protein kinase Akt. Akt phosphorylates multiple proteins involved in regulating mTORC1 signaling, including TSC2 (31) and PRAS40 (32–34), and phosphorylation of these inhibitory proteins causes an increase in mTORC1 activity. Amino acids also activate mTORC1 via a mechanism requiring Ca^{2+}/calmodulin (35) and the class III phosphatidylinositol 3-kinase vacuolar protein sorting 34 (36,37). Other effectors such as mitogen-activated protein 4K3 (38) and a family of small GTPases, the Rag proteins, have also been identified as necessary for amino acid-induced mTORC1 activation (39). Glucose also functions as a regulator of mTORC1. A decrease in available glucose to utilize in generating ATP results in an elevated

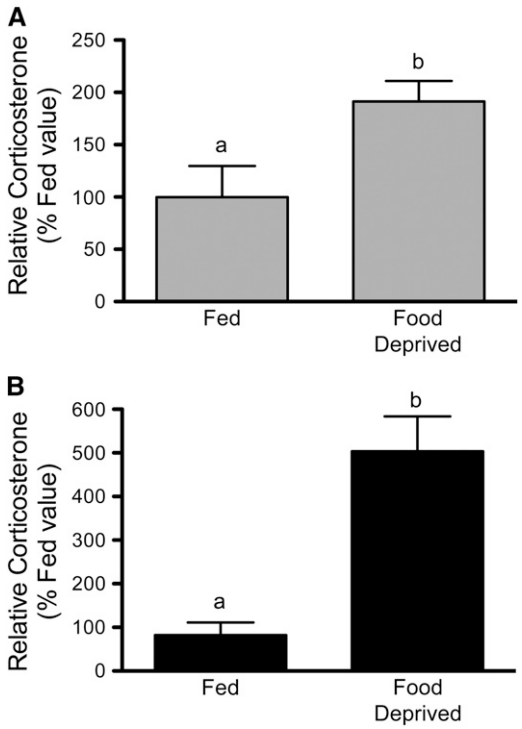

FIGURE 5 Serum corticosterone concentrations in fed and food-deprived control (*A*) or diabetic (*B*) rats. (*B*) Serum corticosterone concentrations were measured in serum from fed and food-deprived diabetic rats. Values are means ± SEM, $n = 6$. Means without a common letter differ, $P < 0.05$.

AMP:ATP ratio, activating the AMP-activated protein kinase [reviewed in (40)]. AMP-activated protein kinase phosphorylates the mTORC1 regulatory proteins TSC2 (41) and regulatory associated protein of mTOR (42), as well as mTOR itself (43); all 3 events are associated with decreased mTORC1 signaling.

REDD1 has been shown in many in vitro studies to be a negative regulator of mTORC1 activity and, thus, a regulator of mRNA translation. Hypoxia induces REDD1 expression (15,16,22,23) and was dependent on hypoxia inducible factor-1α–mediated transcription of the REDD1 gene (16,23). Energy depletion, induced via either glucose withdrawal or treatment with 2-deoxyglucose, also induces REDD1 expression (17). Other in vitro inducers of REDD1 include DNA damage (19) and endoplasmic reticulum stress (20,21).

There have been few studies documenting the in vivo role of REDD1 in regulating mTORC1 activity. Previous work by our laboratory showed exogenous dexamethasone administration to rats induced REDD1 mRNA and protein expression in gastrocnemius muscle in conjunction with decreased mTORC1 signaling (25). Recently, Lang et al. (44) showed that acute alcohol administration, known to downregulate mTORC1 activity and protein synthesis, elevated REDD1 mRNA and protein in gastrocnemius muscle. Also, Drummond et al. (45) showed that REDD1 mRNA in vastus lateralis muscle (like gastrocnemius, a predominantly fast-twitch muscle) decreased 3 h following low-intensity resistance exercise with blood flow restriction in human volunteers, consistent with the increased mTORC1 signaling and protein synthesis observed (46).

In the present study, we sought to determine whether or not REDD1 might be a mediator of reduced mTORC1 signaling in skeletal muscle during food deprivation. As anticipated, phosphorylation of the best-characterized substrates of mTORC1, 4E-BP1 and S6K1, decreased significantly in muscle of food-deprived rats compared with freely fed rats. Refeeding for 45 min caused a recovery in mTORC1 signaling. Consistent with a possible role for REDD1 in repressing mTORC1 signaling, REDD1 mRNA and protein increased dramatically in muscle during food deprivation and returned to baseline in refed rats. The feeding-induced decrease in REDD1 mRNA and protein was rapid, occurring within 45 min. This result is consistent with other studies by our laboratory using cells in culture that suggest the half-life of the REDD1 protein to be <10 min (47).

Insulin regulates the expression of genes encoding proteins that play important roles in various metabolic pathways. Therefore, in the present study, a possible role for changes in insulin concentrations in mediating the food deprivation- and refeeding-induced changes in muscle REDD1 expression was assessed using a short-term model of type I diabetes to minimize the food deprivation- and refeeding-induced changes in insulin levels. Indeed, insulin concentrations in freely fed diabetic rats were similar to those in food-deprived controls and food deprivation reduced the serum insulin concentration to only approximately one-half the value for control diabetic rats. The effectiveness of the model in minimizing food deprivation- and refeeding-induced changes in mTORC1 signaling was demonstrated by the significantly repressed phosphorylation of S6K1 on Thr389 in skeletal muscle from both freely fed and refed diabetic rats compared with controls. Importantly, REDD1 expression was significantly greater in muscle from freely fed diabetic compared with control rats. Moreover, the food deprivation-induced increase in REDD1 expression was magnified in diabetic rats. These findings are consistent with the idea that insulin normally acts as a repressor of REDD1 expression in muscle in the fed or refed state. However, insulin administration to diabetic rats did not affect REDD1 expression. Additionally, insulin concentration and REDD1 mRNA were not correlated (data not shown). The lack of effect of insulin in repressing REDD1 expression was not due to a failure to activate signaling

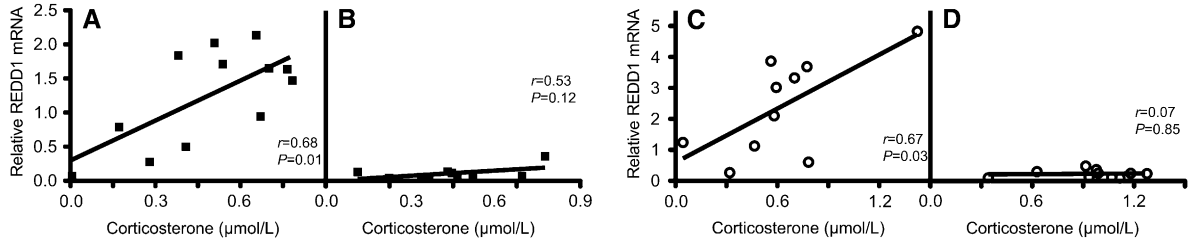

FIGURE 6 Serum corticosterone concentration is directly proportional to REDD1 mRNA expression in gastrocnemius of control (*A*) and diabetic (*C*) rats administered vehicle but not in control (*B*) or diabetic (*D*) rats administered RU486.

pathways in muscle, because Akt phosphorylation was significantly elevated in insulin-treated compared with control rats.

Because dexamethasone potently upregulates REDD1 expression (25), another possible mechanism through which diabetes and food deprivation might act to upregulate expression of the protein is through increased corticosterone levels. As expected, serum corticosterone levels increased in both control and diabetic food-deprived compared with fed rats. Moreover, corticosterone concentrations and REDD1 mRNA expression were positively correlated in both control and diabetic rats. Treatment with the glucocorticoid receptor antagonist RU486 greatly decreased the food deprivation- and diabetes-induced upregulation of REDD1 mRNA expression and also abolished the relationship between corticosterone concentrations and REDD1 mRNA expression. Combined, the results of the present study show that increased corticosterone concentrations that occur during food deprivation and type 1 diabetes are required for increased REDD1 mRNA and protein expression in muscle. Previous studies (21,25) showing that dexamethasone by itself upregulates REDD1 mRNA and protein expression in cells in culture suggest that the food deprivation-induced increase in glucocorticoid concentration is likely sufficient for upregulated REDD1 expression.

In summary, this study has shown that both food deprivation- and experimentally induced type I diabetes induce expression of REDD1 mRNA and protein. Consistent with a role for REDD1 in repressing the activity of the mTORC1 signaling pathway, REDD1 protein expression was inversely proportional to the activation state of the pathway as assessed by S6K1 phosphorylation on T389 as well as hyperphosphorylation of both S6K1 and 4E-BP1. Moreover, the food deprivation- and diabetes-induced upregulation of REDD1 expression was unlikely to be a result of altered insulin signaling, because insulin administration to food-deprived, diabetic rats did not lower the elevated REDD1 expression. Instead, glucocorticoid signaling appears to play a critical role in upregulating REDD1 expression in muscle in response to either an overnight food deprivation or an experimental model of type 1 diabetes. Overall, the results of the study strongly suggest that physiological alterations in glucocorticoid concentrations are sufficient to modulate REDD1 expression in skeletal muscle.

Acknowledgments

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