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Lack of REDD1 reduces whole body glucose and insulin tolerance, and impairs skeletal muscle insulin signaling

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ABSTRACT

A lack of the REDD1 promotes dysregulated growth signaling, though little has been established with respect to the metabolic role of REDD1. Therefore, the goal of this study was to determine the role of REDD1 on glucose and insulin tolerance, as well as insulin stimulated growth signaling pathway activation in skeletal muscle. First, intraperitoneal (IP) injection of glucose or insulin were administered to REDD1 wildtype (WT) versus knockout (KO) mice to examine changes in blood glucose over time. Next, alterations in skeletal muscle insulin (IRS-1, Akt, ERK 1/2) and growth (4E-BP1, S6K1, REDD1) signaling intermediates were determined before and after IP insulin treatment (10 min). REDD1 KO mice were both glucose and insulin intolerant when compared to WT mice, evident by higher circulating blood glucose concentrations and a greater area under the curve following IP injections of glucose or insulin. While the REDD1 KO exhibited significant though blunted insulin-stimulated increases ($p < 0.05$) in Akt S473 and T308 phosphorylation versus the WT mice, acute insulin treatment has no effect ($p < 0.05$) on REDD1 KO skeletal muscle 4E-BP1 T37/46, S6K1 T389, IRS-1 Y1222, and ERK 1/2 T202/Y204 phosphorylation versus the WT mice. Collectively, these novel data suggest that REDD1 has a more distinct role in whole body and skeletal muscle metabolism and insulin action than previously thought.

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1. Introduction

Skeletal muscle is a site for insulin action and glucose disposal and comprises a large portion of fat free mass. Skeletal muscle is positively associated with metabolic homeostasis [1], and the stability of muscle mass is central to insulin sensitivity and glucose metabolism [2]. Insulin regulates glucose uptake into skeletal muscle via the phosphatidylinositol-3 kinase (PI3K)/Akt pathway, resulting in the recruitment of glucose transporters to the plasma membrane [3]. Insulin also activates mammalian target of rapamycin (mTOR) through the PI3K–Akt pathway, which phosphorylates and inhibits the tuberous sclerosis complex 2 (TSC2), thus promoting GTPase activity of the mTOR kinase, Rheb [4]. mTOR is controlled by hormonal and nutrient cues, such as those altered during a fasted or a fed state [5], and regulates protein synthesis, autophagy, metabolism and cell survival [4]. mTOR exists in two interdependent multi-protein complexes, raptor containing mTOR complex 1 (mTORC1) and rictor containing mTOR complex 2 (mTORC2) [6,7]. mTORC1 phosphorylates two downstream substrates, the eukaryotic initiation factor (eIF) 4E binding protein-1 (4E-BP1) and p70 ribosomal protein S6 Kinase-1 (S6K1) [8]. Skeletal muscle from obese or insulin resistant rodents has an attenuated response to nutrient and growth stimuli [9–11], associated with dysregulated mTORC1 signaling. mTORC1 signaling activation should be reduced during a fasted state, though is hyperactive in obese and insulin resistant models [9–12]. Hyperactive mTORC1 negatively feeds back to inhibit the insulin receptor substrate-1 (IRS-1) by reducing tyrosine phosphorylation and promoting serine phosphorylation [9,12], downregulating insulin signaling.

A repressor of mTORC1, the protein regulated in development and DNA damage responses 1 (REDD1; also known as DDIT4 and RTP801) was initially reported as a stress-regulated protein [13] (e.g. hypoxia [14], glucocorticoids [15], DNA damage [16], endoplasmic reticulum (ER) stress [17]). However, recent findings have suggested a nutrient role for REDD1 [11,18,19] that is central to the insulin-mediated activation of Akt and mTOR. Our recent work [11] suggests that a loss of REDD1 limits skeletal muscle's ability to respond appropriately to nutrient cues. REDD1's exact mechanism of action on mTORC1 remains unclear, though recent findings [19] suggest that following a PP2A-dependent Akt T308 dephosphoryla-

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tion, reduced TSC2 phosphorylation promotes Rheb GTPase loading. Still, much remains to be determined about REDD1 and its role in metabolism. Thus, the goal of this study was to determine the role of REDD1 in the control of whole body glucose tolerance and skeletal muscle insulin action. We hypothesized that a loss of REDD1 leads to reductions in whole body glucose and insulin tolerance, and impairments in skeletal muscle insulin signaling.

2. Methods

2.1. Animals

The Institutional Animal Care and Use Committee at the University at Buffalo approved the protocols and procedures. All mice were housed at 21 °C in 50% humidity with 12/12 h light/dark cycle on a standard chow diet (Harlan; Cat# 2018). 3–4 month old wild-type and RTP801 (REDD1) knockout C57Bl/6x129SvEv mice (generated by Lexicon Inc.; Woodland, TX for Quark Pharmaceuticals Inc.; Fremont, CA) [20].

2.2. Intraperitoneal glucose tolerance (ipGTT) and insulin tolerance (ipITT) tests

Following a 12 h fast, blood glucose concentrations were determined through the tail vein. A small nick was made at the distal quarter of the tail with a sterile scalpel blade, and then a drop of blood was placed onto the test strip and measured by a handheld glucometer (OneTouch Ultra, LifeScan Inc.). At this time, blood loss was stopped by application of a styptic pencil to the nick area. Then 2 g/kg BW sterile glucose (Sigma) was administered via intraperitoneal (ip) injection with a sterile 0.3 mL syringe/26 gauge needle. The mouse was then returned to its cage until the next collection timepoint and every timepoint thereafter. Blood glucose was assessed by the same method described above at 15, 30, 60, 90, and 120 min.

Following a 6 h fast (to limit possible terminal reductions in blood glucose that may occur with a 12 h fast), blood glucose concentration was determined through the tail vein. Again, a small nick at the distal quarter of the tail was made on the tail vein with a sterile scalpel blade, then a drop of blood was placed onto the test strip and measured by a handheld glucometer (OneTouch Ultra, LifeScan Inc.). At this time, blood loss was stopped by application of a styptic pencil to the nick area. Next, 0.5 IU/kg BW insulin (Humulin, Eli Lilly) was administered by intraperitoneal injection with a sterile 0.3 mL syringe/26 gauge needle, and blood glucose was determined at 7.5, 15, 30, 45, and 90 min through the tail vein. The mouse was then returned to its cage until the next collection timepoint and every timepoint thereafter. Changes in glucose were plotted over time for both the ipGTT and ipITT, then the area under the curve (AUC) was also calculated [21].

2.3. Insulin injection

Following a 12 h fast, the animals were placed under 3.5% isoflurane anesthesia for the duration of the procedure. Then the right plantar flexor complex (containing the medial and lateral gastrocnemius, soleus, and plantaris muscles) was removed and immediately placed it in liquid nitrogen for subsequent analysis. Insulin (Humulin, Eli Lilly) was then injected into the intraperitoneal space at a concentration of 0.5 IU/kg BW. After 10 min, the remaining (left) plantar flexor complex was removed and placed immediately into liquid nitrogen for subsequent analysis.

2.4. Tissue homogenization

The plantar flexor complex muscle samples collected during the insulin injection protocol were homogenized in 10 volumes of CHAPS-containing buffer [40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM-glycerophosphate, 40 mM NaF, 1.5 mM sodium vanadate, 0.3% CHAPS, 0.1 mM PMSF, 1 mM benzamidine, 1 mM DTT, and protease inhibitors (#04693116001, Roche, Indianapolis, IN)]. An initial aliquot of the homogenate, serving as a total muscle lysate, was collected for Western analysis. The homogenate was further clarified by a 1000*rcf* centrifugation for 5 min (at 4 °C), and the supernatant was retained (i.e. cytoplasmic enriched fraction). A small aliquot of each sample was taken for the determination of protein concentration for each sample. An equal volume of 2X sodium dodecyl sulfate loading buffer was added to each fraction, boiled for 5 min, then Western analysis was performed.

2.5. Western blotting

First, equal quantities of protein (Coomassie; Thermo) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred onto a PVDF membrane. After blocking in 5% non-fat milk in tris-buffered saline (TBS) plus 0.1% Tween-20 (TBS-T) for 1 h, membranes were incubated with anti-phospho primary antibodies (Cell Signaling) for Akt S473, Akt T308, IRS-1 Y1222, ERK 1/2 T202/Y204, S6K1 T389, or 4E-BP1 T37/46 and total antibodies for REDD1 (Proteintech), S6K1 (Cell Signaling), and GAPDH (Cell Signaling) in TBS-T overnight at 4 °C. Membranes were then washed and incubated with the respective secondary antibody for 1 h in a 5% non-fat milk/TBS-T solution at room temperature. The membranes were washed in TBS-T, then visualized via ECL and then quantified by measuring the luminescent signal using a Bio-Rad ChemiDoc MP Imager. The blots were analyzed using Bio-Rad Image Lab software and the data was expressed as a percentage of the respective control group for each individual blot.

2.6. Statistical analysis

Statistics were performed using IBM SPSS v22.0.0 software. The results are expressed as the mean ± standard error. Comparisons were made for each variable using independent or paired (where appropriate) two-tailed *t*-test or a one-way analysis of variance, Tukey HSD *post hoc* test, to establish significant differences between groups (only after the F statistic indicated an overall significance). The significance levels was set *a priori* at $p < 0.05$.

3. Results

3.1. Glucose and insulin tolerance

Though not significantly different from one another, the REDD1 KO mice had higher blood glucose concentrations after a 12 h fast (Fig. 1A), which is consistent with our previously reported findings [11]. However, 15 and 30 min after the glucose injection, blood glucose concentrations were significantly higher (Fig. 1A; $p < 0.05$) in the REDD1 KO mice when compared to the WT mice at those same timepoints. Thereafter, both the REDD1 WT and KO mice displayed similar blood glucose concentrations for the remainder of the test. Consistent with this, the area under the curve (AUC) was correspondingly higher (Fig. 1A Inset; $p < 0.05$) for the REDD1 KO versus the WT mice.

For the insulin tolerance test, the mice were fasted for only 6 h to limit detrimentally low glucose concentrations following insulin

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