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Lipids. Author manuscript; available in PMC 2017 January 06.

Published in final edited form as:

Lipids. 2016 August ; 51(8): 905–912. doi:10.1007/s11745-016-4168-3.

Caloric Restriction Normalizes Obesity-Induced Alterations on Regulators of Skeletal Muscle Growth Signaling

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Abstract

The objective of this study was to establish the impact of caloric restriction on high fat diet-induced alterations on regulators of skeletal muscle growth. We hypothesized that caloric restriction would reverse the negative effects of high fat diet-induced obesity on REDD1 and mTOR-related signaling. Following an initial 8 week period of HF diet-induced obesity, caloric restriction (CR ~30 %) was employed while mice continued to consume either a low (LF) or high fat (HF) diet for 8 weeks. Western analysis of skeletal muscle showed that CR reduced ($p < 0.05$) the obesity-related effects on the lipogenic protein, SREBP1. Likewise, CR reduced ($p < 0.05$) the obesity-related effects on the hyperactivation of mTORC1 and ERK1/2 signaling to levels comparable to the LF mice. CR also reduced ($p < 0.05$) obesity-induced expression of negative regulators of growth, REDD1 and cleaved caspase 3. These findings have implications for on the reversibility of dysregulated growth signaling in obese skeletal muscle, using short-term caloric restriction.

Keywords

mTORC1; SREBP1; Apoptosis

Introduction

Recent statistics from the Centers for Disease Control and Prevention estimate that in the United States, 16.9 % of children ages 2–19 years and 34.9 % of adults are obese [1]. In addition to the host of comorbidities that frequently accompany obesity (i.e. hypertension, insulin resistance, cardiovascular disease), skeletal muscle growth signaling is often

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Compliance with Ethical Standards

Conflict of interest

The authors have no conflicts of interest to declare.

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defective [2, 3]. Hyperactivity of the mechanistic target of rapamycin (mTOR) pathway can contribute to impaired growth signaling and insulin resistance in obese humans [3] and animals [2]. Consistent with this rationale, we previously reported that short-term treatment with the AMPK agonist and mTOR repressor, AICAR, normalized mTOR and downstream regulatory processes that control growth (e.g. mRNA translation initiation) to lean levels [4]. These and other [5] findings support the contention that normalizing mTOR during or following obesity, through the benefits of exercise, diet, or medication, restores growth signaling, insulin sensitivity, and even limit muscle mass loss.

The mTOR signaling, specifically mTOR complex 1 (mTORC1) [6, 7], pathway regulates anabolic processes, including cell proliferation, protein synthesis, and lipo-genesis. mTORC1 is a large protein complex comprised of mTOR protein and multiple subunits, specifically the rapamycin sensitive accessory protein, Raptor [8]. Active mTORC1 phosphorylates downstream target proteins p70 S6 kinase (S6K1) and subsequently ribosomal protein S6 (rpS6), promoting translation initiation through eIF3 and the 40S ribosomal subunit [9]. mTORC1 is directly activated by GTP-bound Ras-homologue enriched in brain (Rheb) protein when the tuberous sclerosis complex (TSC) is directly inactivated by Akt and/or ERK1/2 [10, 11]. Conversely, the protein regulated in development and DNA damage responses 1 [REDD1; aka DNA-damage-inducible transcript 4 (DDIT4), Dexamethasone-induced gene 2 (Dig2), and RTP801] represses mTOR. REDD1 is upregulated by various stressors, such as glucocorticoids [12], DNA damage [13], endoplasmic reticulum (ER) stress [14], and hypoxia [15, 16], among others. REDD1's mechanism of action has been reported to work through TSC2 by sequestering the modulatory protein, 14-3-3 [17], or by recruiting serine-threonine protein phosphatase 2A (PP2A) to dephosphorylate T308 on Akt [18].

Recent reports from our laboratory [3, 19, 20] and others [21, 22] have shown a metabolic and nutrient role for REDD1. As mentioned above, skeletal muscle from obese animals and humans exhibit hyperactive mTOR signaling in a fasted state that coincides with elevated REDD1 expression. In this scenario, skeletal muscle from obese mice [20] and humans [3] exhibit a blunted Akt and mTOR signaling response to growth stimuli. The physiological reason for the simultaneous increase in REDD1 in skeletal muscle during obesity is unknown, though it may be due to hyperactive mTOR signaling. Thus, when mTOR is highly activated (i.e. in an obese state), REDD1 protein is stabilized and protected from proteasomal degradation [23]. Upon inhibition of mTOR, REDD1 protein stability is reduced, leading to its degradation [23, 24], collectively suggesting reciprocal regulation of REDD1–mTOR that is dependent upon the energy status or nutrient state of the cell.

Short and long term caloric restriction promotes body mass loss [25, 26], improved insulin action [27, 28], and longevity [26]. Yet, there have been equivocal findings that caloric restriction alters the glucocorticoid cortisol [29, 30], yet no studies have examined caloric restriction effects on the glucocorticoid responsive protein, REDD1. This becomes important in the understanding of how mTOR is regulated during caloric restriction. Given that glucocorticoids or obesity/high fat diet consumption are potent stimulators of REDD1 expression, the objective of this study was to establish the impact of caloric restriction on high fat diet-induced alterations on regulators of skeletal muscle growth. We hypothesized

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that caloric restriction would reverse the negative effects of high fat diet-induce obesity on regulators of growth signaling, and normalize their expression to that of the lean controls.

Experimental Methods

Materials

A Coomassie protein assay was performed using Coomassie Plus Reagent (Thermo Scientific; Rockford, IL, USA). Western blotting was performed using a Bio-Rad mini-PROTEAN Tetra Cell system. Polyvinylidine difluoride (PVDF) membrane was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Primary antibody for SREBP-1c (NB600-582) from NOVUS biologicals (Littleton, CO, USA), antibodies for phospho-p70 S6 Kinase Thr389 (9234), phospho-S6 Ribosomal Protein Ser240/244 (5364), phospho-MEK1/2 Ser217/221 (9154), phospho-ERK1/2 Thr202/Tyr204 (4370), cleaved caspase 3 (9664), LC3A/B (4108), and GAPDH (2118), were purchased from Cell Signaling Technology (Beverly, MA, USA), and REDD1 (10638-1-AP) was purchased from Proteintech. Enhanced chemiluminescence (ECL) reagent was purchased from Bio-Rad Laboratories (Clarity western ECL). Chemiluminescence imaging was performed on a Bio-Rad ChemiDoc MP Imager.

Animals

The Institutional Animal Care and Use Committee at the University at Buffalo approved the protocols and procedures. Six-week-old, male C57Bl/6 mice (Jackson Laboratories) were housed at 22 °C in 50 % humidity with 12-h day/night cycles. This study design and characteristics of the mice were reported previously [31]. For the first 8 weeks of the study, the mice were split into four groups, one group receiving a low fat (LF) diet (5 % fat; Research Diets) and the other three groups receiving a high fat diet (60 % fat; Research Diets). Following this initial lead-in period, the LF fed mice were maintained on their diet for an additional 8 weeks, and the HF fed mice were either maintained on the same high fat (HF) diet, a low fat, caloric restricted diet (LF+CR), or a high fat, caloric restricted (HF +CR) diet for an additional 8 weeks. The caloric intake of the calorie restricted groups was ~70 % of that of the low fat, control group, an amount that has previously shown to be effective at protecting against high fat diet induced obesity in mice [32]. Per the previously published findings from this study [31], the HF fed groups all weighed significantly more than the LF fed group. Caloric restriction limited weight gain in both the LF and the HF fed groups versus the HF maintained group, with the LF+CR body weight similar to the LF fed group. Following the treatment period and a 12-h *versus* fast, blood and the plantar flexor complex (containing the medial and lateral gastrocnemius, soleus, and plantaris muscles) were collected under 3 % isoflurane. The tissue was frozen in liquid nitrogen for subsequent analysis, then the mice were euthanized while under 3 % isoflurane anesthesia.

Blood Fatty Acids

Nonesterified free fatty acids were analyzed with an enzymatic kit in accordance with the manufacturer's instructions (Wako Chemical; Richmond, VA, USA).

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Tissue Processing and Western Analysis

Per our previously published methods [20], the plantar flexor complex samples were homogenized in ten 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, USA)], where the total and cytosolic fractions were isolated and protein concentrations were determined. Equal protein (30 μg of protein) was resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto PVDF membrane (Bio-Rad Protean). After blocking in 5 % milk in tris-buffered saline (TBS) plus 0.1 % Tween-20 (TBS-T) for 1 h at room temperature, membranes were incubated with the respective primary antibody overnight 4 °C in TBS-T. Membranes were incubated with a horse-radish peroxidase (HRP)-containing secondary antibody corresponding to the primary antibody host for 1 h in a 5 % milk/TBS-T solution at room temperature. Then the protein immunoblot images were visualized following addition of ECL reagent and captured (Bio-Rad ChemiDoc MP Imager). Density measurements for the images were quantified using Bio-Rad ImageLab software, and were normalized to the appropriate control. Each sample was then normalized to the LF group, for the respective blot, and then expressed as a mean percentage of the LF group between blots.

Statistical Analysis

Statistics analyses were performed using IBM SPSS version 22.0 software. A one-way analysis of variance with a least significant difference *post hoc* test or Pearson correlation was used to determine significance between groups. The significance level was set a priori at $p < 0.05$. The results are expressed as the mean ± standard error.

Results

As previously reported by Cui *et al.* [31] and consistent with our previous reports in obese mice [4, 20], the skeletal muscle mass per body weight from the HF fed mice was lower than the LF fed mice, and there was a partial (HF + CR) or near complete (LF + CR) reduction in the high fat diet-induced body weight gain with caloric restriction *versus* the LF control mice. Thus, regulators of skeletal muscle metabolism and mass were assessed. Protein expression of the lipogenic regulator, SREBP1c, was comparable between the LF and the CR groups (Fig. 1) *versus* a higher trend in the HF group ($p = 0.08$ *versus* LF). Consistent with this, blood non-esterified fatty acids were significantly higher (Fig. 2; $p < 0.05$) in the HF fed group *versus* all other groups, which was consistent with other markers of lipid metabolism reported by Cui *et al.* [31]. A regulatory pathway of SREBP1c and subsequently lipogenesis is the mTOR pathway [6, 7]. Accordingly, mTOR phosphorylation was significantly higher ($p < 0.05$) in the HF group when compared to the LF controls (Fig. 3a), and was significantly lower ($p < 0.05$) after caloric restriction. S6K1 phosphorylation in the HF group trended higher when compared to the LF group (Fig. 3b; $p = 0.15$), and trended lower (Fig. 3b; $p = 0.07$) in the HF + CR mouse muscle. Similar to mTOR, phosphorylation of the downstream substrate of S6K1, ribosomal protein S6 (rpS6) was significantly higher ($p < 0.05$) in the HF group (Fig. 3c) when compared to the LF group, and dramatically lower

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($p < 0.05$) in the CR groups regardless of the dietary fat when compared to both the LF and HF group (Fig. 3c). Activation of a pathway that also regulates mTOR, the MEK1/2-ERK1/2 pathway, was significantly higher (Fig. 4a, b; $p < 0.05$) in the HF group when compared to the LF group. Caloric restriction was effective in reducing (Fig. 4a, b) the phosphorylation of MEK1/2 ($p = 0.08$ *versus* HF) and ERK1/2 ($p < 0.05$ *versus* HF), regardless of the dietary fat. Similar to our previous findings [20], HF diet-induced obesity promoted a significant elevation (Fig. 5; $p < 0.05$) in skeletal muscle REDD1 expression. Caloric restriction (regardless of dietary fat) normalized (Fig. 5; $p < 0.05$) REDD1 expression to levels comparable to the LF fed group. Interestingly, significant positive correlations between REDD1-MEK1/2 ($r = 0.482$; $p < 0.05$) and REDD1-ERK1/2 ($r = 0.762$; $p < 0.001$) were observed. Extending upon these findings of negative regulators of muscle growth, caloric restriction (regardless of dietary fat) significantly reduced ($p < 0.05$) HF diet-induced skeletal muscle expression of the apoptotic protein, cleaved caspase 3 (Fig. 6a) to levels comparable to the LF group. The LC3-II/I ratio, an indicator of autophagy, was significantly increased (Fig. 6a; $p < 0.05$) after HF diet-induced obesity, and was significantly reduced ($p < 0.05$) following caloric restriction.

Discussion

The goal of the study was to determine the impact of caloric restriction on high fat diet-induced alterations on regulators of skeletal muscle growth. Accordingly, following an initial 8-week period of HF diet-induced obesity, CR (~30 %) was employed while mice continued to consume either a LF or HF diet for 8 weeks. CR mitigated the obesity-related effects on two negative regulators of skeletal muscle growth, REDD1 and cleaved caspase 3. CR also attenuated obesity-related elevation in skeletal muscle mTORC1 and ERK1/2 pathway activation. Consistent with our previous findings showing that skeletal muscle REDD1 is responsive to overnutrition, such as high fat diet-induced obesity, the change in REDD1 following a nutrient stimulus that limits caloric intake is proportional in response.

Models of obesity show high circulating concentration of glucose, insulin, branched chain amino acids, glucocorticoids, and cytokines among others, which bathe skeletal muscle in catabolic and anabolic stimuli [33–35]. The current data coupled with our previous findings show that elevated REDD1 expression in fasted muscle from high-fat fed, obese mice is associated with reduced TSC2 complex formation [4], and can promote Rheb GTP loading and mTORC1 signaling (low raptor-mTOR association, elevated S6K1 and rpS6 phosphorylation). Thus, hyperactive mTORC1 can coincide with elevated REDD1 expression, as reported by us [20] and others [23].

Branched chain amino acids (BCAA) are a well-established mTOR agonist in muscle [36, 37], and elevated concentrations of BCAA appear to contribute to insulin resistance in obese tissues [38, 39]. Though not measured in the current study, high circulating concentrations of amino acids in the obese [38, 39] may constitutively activate the Rag pathway (and mTOR), partially explaining the inability of REDD1 to inhibit mTORC1, since REDD1 and growth factors signal to mTORC1 through TSC [40, 41]. Conversely, the elevation of glucocorticoids, a REDD1-agonist, can contribute to the irregular and blunted mTORC1 responses in the obese under both fasted and fed conditions [22, 33–35]. In addition to

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upregulating REDD1 protein expression while consuming a HF diet, glucocorticoid-induced REDD1 expression can promote apoptosis [42] and autophagy [43], which is consistent with the observed increase in cleaved caspase 3 and the LC3-II/I ratio, markers of apoptosis and autophagy, respectively. An increase in protein catabolism has been shown to restore the available free amino acid pool [44, 45], promoting mTOR activation and cell survival. Alterations in plasma glucocorticoids during obesity or caloric restriction would help explain the concomitant increase of both REDD1 and mTOR during obesity, and their reduction after caloric restriction.

The inhibitory role of REDD1 on mTOR activity in skeletal muscle was first reported in dexamethasone treated rodents [12], as well as in models of skeletal muscle atrophy [22, 46–48]. Recent data from our laboratory and others report that REDD1 expression is elevated in skeletal muscle, cardiac, and liver from obese and/or high fat fed mouse models. Moreover, skeletal muscle REDD1 expression is downregulated or upregulated during a short-term fasting-to-fed state transition or short-term fasting, respectively [22]. REDD1 expression appears to be closely linked to the nutrient and the hormonal state of the cell, and these current and previous findings suggest differential regulation of REDD1 during acute (i.e. feeding or fasting) and chronic states (i.e. obesity or caloric restriction). This study supports these findings, and extends upon our previous work to show that chronic nutrient alterations (i.e. high diet or caloric restriction) can regulate REDD1 expression.

Another mechanism that may control REDD1 expression is the very protein that it inhibits, mTOR. When mTOR is highly active, 26S proteasome-dependent degradation of REDD1 is inhibited. Under conditions that inhibit mTOR, REDD1 protein stability is reduced via the HUWE1 ubiquitin ligase, which leads to degradation [23]. By upregulating REDD1 expression, mTOR may act in a manner to self-regulate, which could be advantageous during obesity. TXNIP (also known as VDUP1 or TBP2) is induced by various types of cellular stress, including oxidative stress, UV irradiation, heat shock and apoptotic signaling [49, 50], is also a binding partner of REDD1 that promotes its stabilization and mTOR inhibition.

The half-life of the REDD1 protein is estimated to be 5–90 min [51], depending upon the cell type, then undergoes degradation through a CUL4-DDB1-regulated ubiquitin ligation [24, 52]. The mechanism controlling REDD1 degradation remains to be completely elucidated, though recent findings suggest that MEK–ERK signaling may play a role in REDD1 expression. Constitutively active MEK prevents REDD1 degradation even in the presence of cyclohexamide or CUL4 overexpression. Consistent with our findings in human skeletal muscle from type 2 diabetic, obese individuals [3], the current data show that skeletal muscle from obese mice exhibit hyperactivation of MEK–ERK in a fasted state through an undefined mechanism or regulator. Similar to the reports of other laboratories [53], we report that caloric restriction reduces basal MEK–ERK activation state. Sustained, long-term activation of ERK in either the cytosol or the nucleus can promote apoptosis [54]. Consistent with previous findings in obese animal models [55], these data show that caloric restriction can reverse high fat diet-induced increases in skeletal muscle elevated cleaved caspase 3, indicating that apoptosis was reduced. When obese mice are treated with the MEK inhibitor, U0126, blood glucose and insulin action are improved [56]. Likewise, when

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ERK1 is ablated in ob/ob mice, glucose metabolism and insulin sensitivity were improved and inflammatory cytokines were reduced [57]. Thus, elevated basal ERK1/2 activation and subsequent maintenance of REDD1 expression during obesity may be an attempt to limit inappropriate mTOR activation under conditions that promote apoptosis.

In conclusion, these data show that caloric restriction can reverse the negative effects of a high fat diet-induced obesity on regulators of skeletal muscle growth. Specifically, caloric restriction of high fat fed obese mice reduced the basal hyperactivation of mTORC1 and ERK1/2 signaling, as well as reduced REDD1 expression, that was associated with changes in markers for apoptosis and autophagy. Thus, caloric restriction may be used as a non-pharmacologic approach to mitigating aberrant growth signaling in obese skeletal muscle.

Acknowledgments

The authors would like to thank Mingxia Cui for excellent support throughout the project.

Abbreviations

CR	Caloric restriction
ERK	Extracellular signal-regulated kinase
HF	High fat
LF	Low fat
MEK	MAPK/ERK kinase
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
REDD1	Regulated in development and DNA damage responses 1
rpS6	Ribosomal protein S6, S6K1 p70 ribosomal protein S6 kinase-1
SREBP1c	Sterol regulatory element-binding protein 1c

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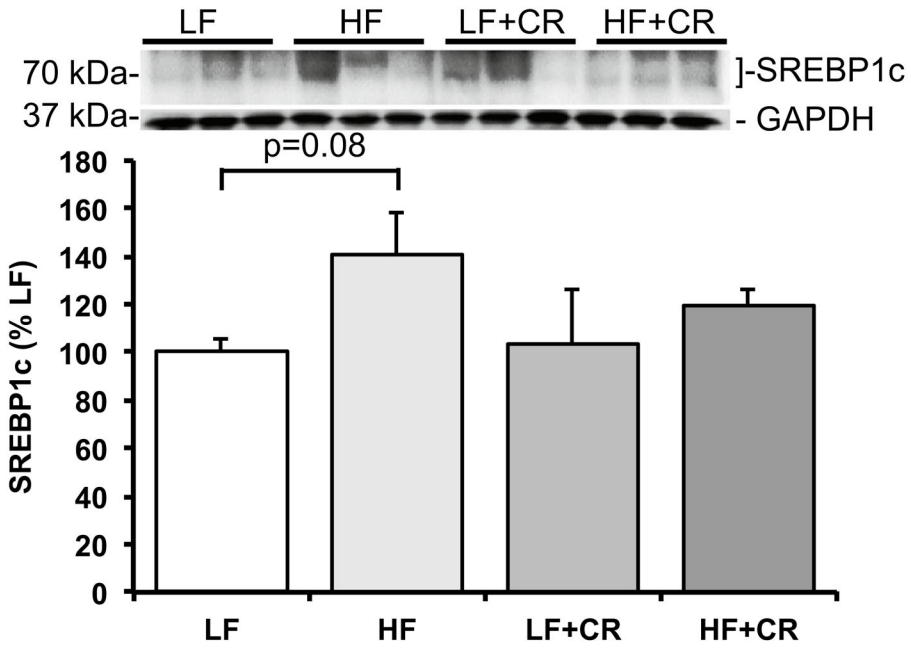

Fig. 1.

Skeletal muscle SREBP1c expression in low fat and high fat fed, calorically restricted mice. Equal protein from low (LF) and high fat (HF) fed, calorically restricted (CR) mouse muscle homogenates were analyzed by Western blot analysis for SREBP1c and GAPDH, then normalized to GAPDH. Representative Western blots are shown. Means marked with an *asterisk* are significantly different $p < 0.05$ *versus* LF ($n = 6$/group)

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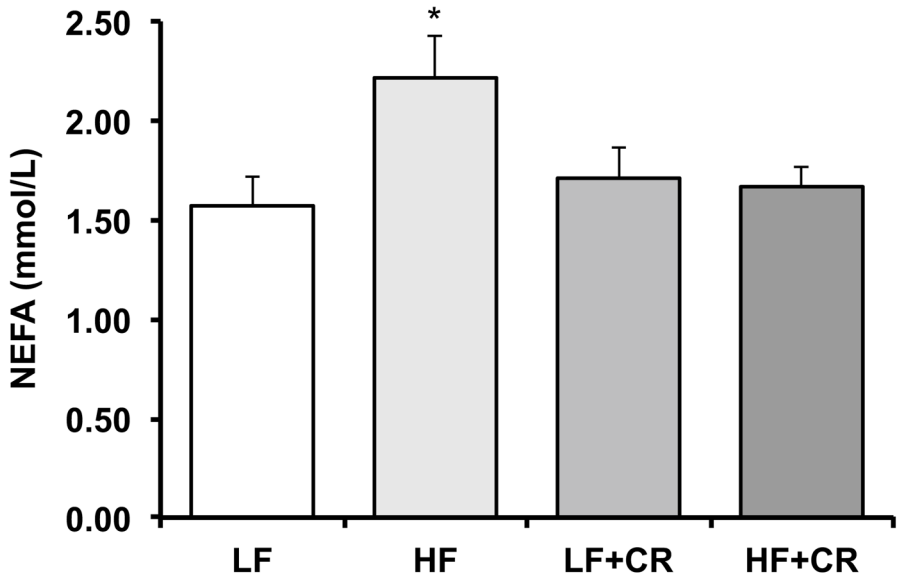

Fig. 2.

Serum nonesterified fatty acids (NEFA) in low fat and high fat fed, calorically restricted mice. Means marked with an *asterisk* are significantly different $p < 0.05$ *versus* LF ($n = 6$/group)

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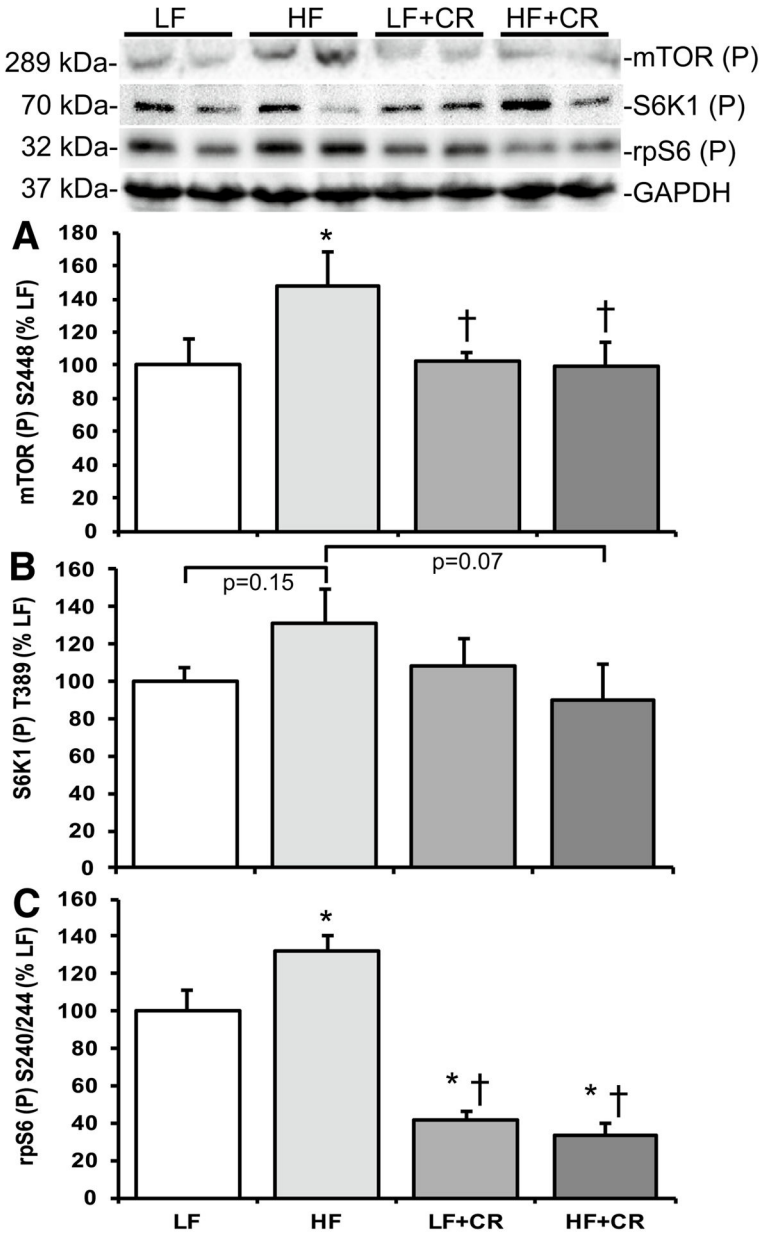

Fig. 3.

Skeletal muscle mTORC1 signaling activation in low fat and high fat fed, calorically restricted mice. Equal protein from low (LF) and high fat (HF) fed, calorically restricted (CR) mouse muscle homogenates were analyzed by Western blot analysis for **a** mTOR S2448 phosphorylation, **b** S6K1 T389 phosphorylation, **c** rpS6 S240/244 phosphorylation, and GAPDH, then normalized to GAPDH. Representative Western blots are shown. Means marked with an *asterisk* are significantly different $p < 0.05$ *versus* LF ($n = 6$/group)

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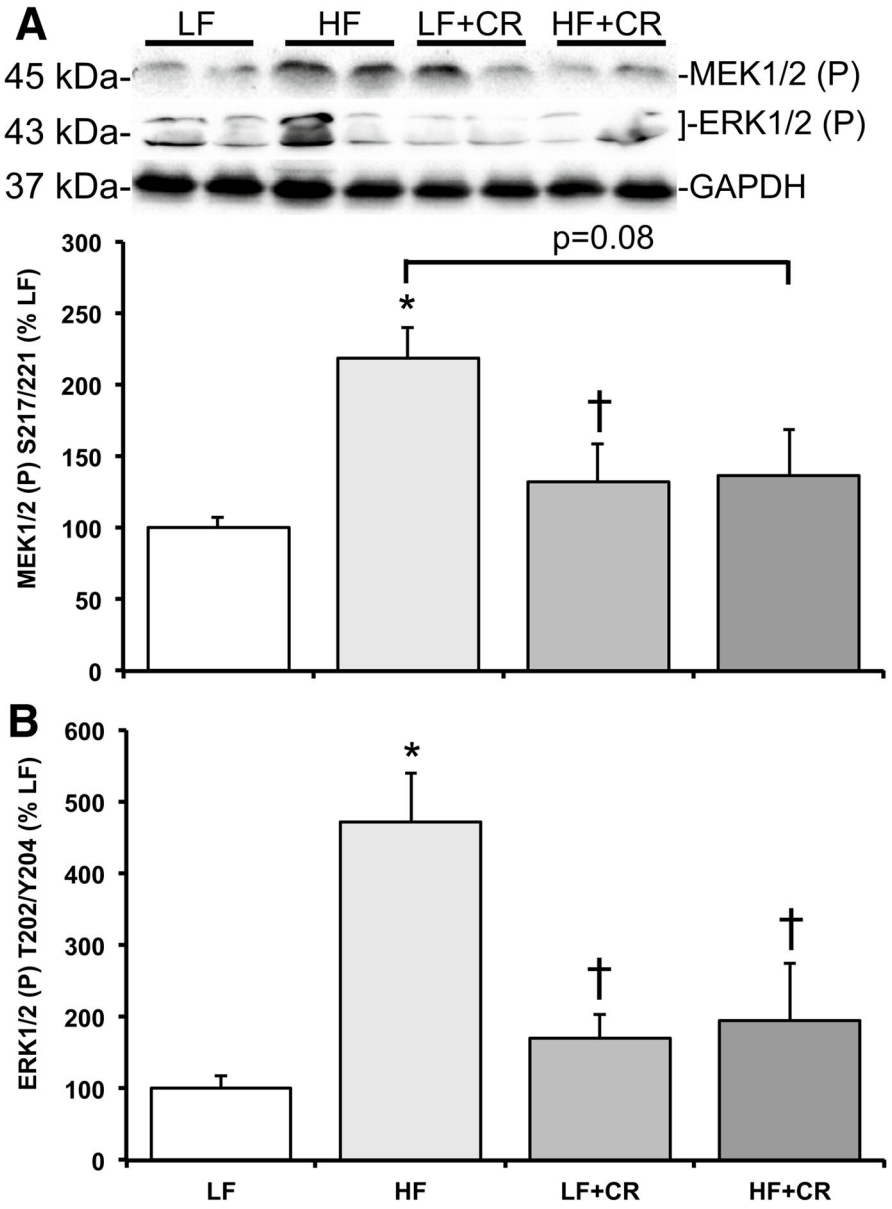

Fig. 4.

Skeletal muscle MEK–ERK signaling activation in low fat and high fat fed, calorically restricted mice. Equal protein from low (LF) and high fat (HF) fed, calorically restricted (CR) mouse muscle homogenates were analyzed by Western blot analysis for **a** MEK1/2 S217/221 phosphorylation, **b** ERK1/2 T202/Y204 phosphorylation, and GAPDH, then normalized to GAPDH. Representative Western blots are shown. Means marked with an *asterisk* are significantly different $p < 0.05$ *versus* LF and *dagger* are significantly $p < 0.05$ *versus* HF ($n = 6$/group)

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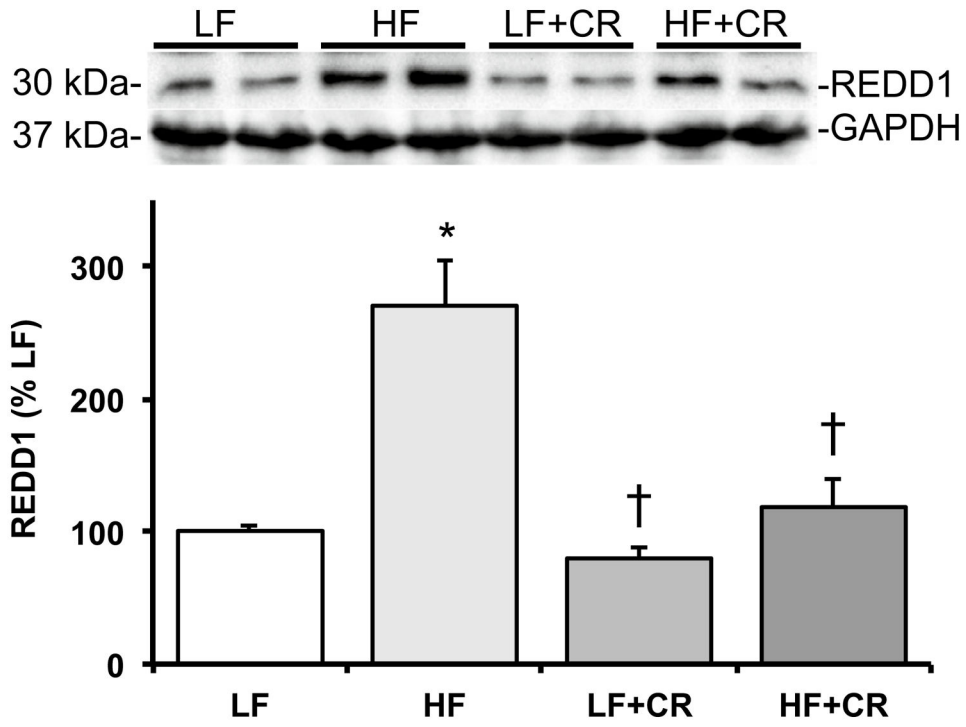

Fig. 5.

Skeletal muscle REDD1 expression in low fat and high fat fed, calorically restricted mice. Equal protein from low (LF) and high fat (HF) fed, calorically restricted (CR) mouse muscle homogenates were analyzed by Western blot analysis for REDD1 and GAPDH, then normalized to GAPDH. Representative Western blots are shown. Means marked with an *asterisk* are significantly different $p < 0.05$ *versus* LF and *dagger* are significantly $p < 0.05$ *versus* HF ($n = 6$/group)

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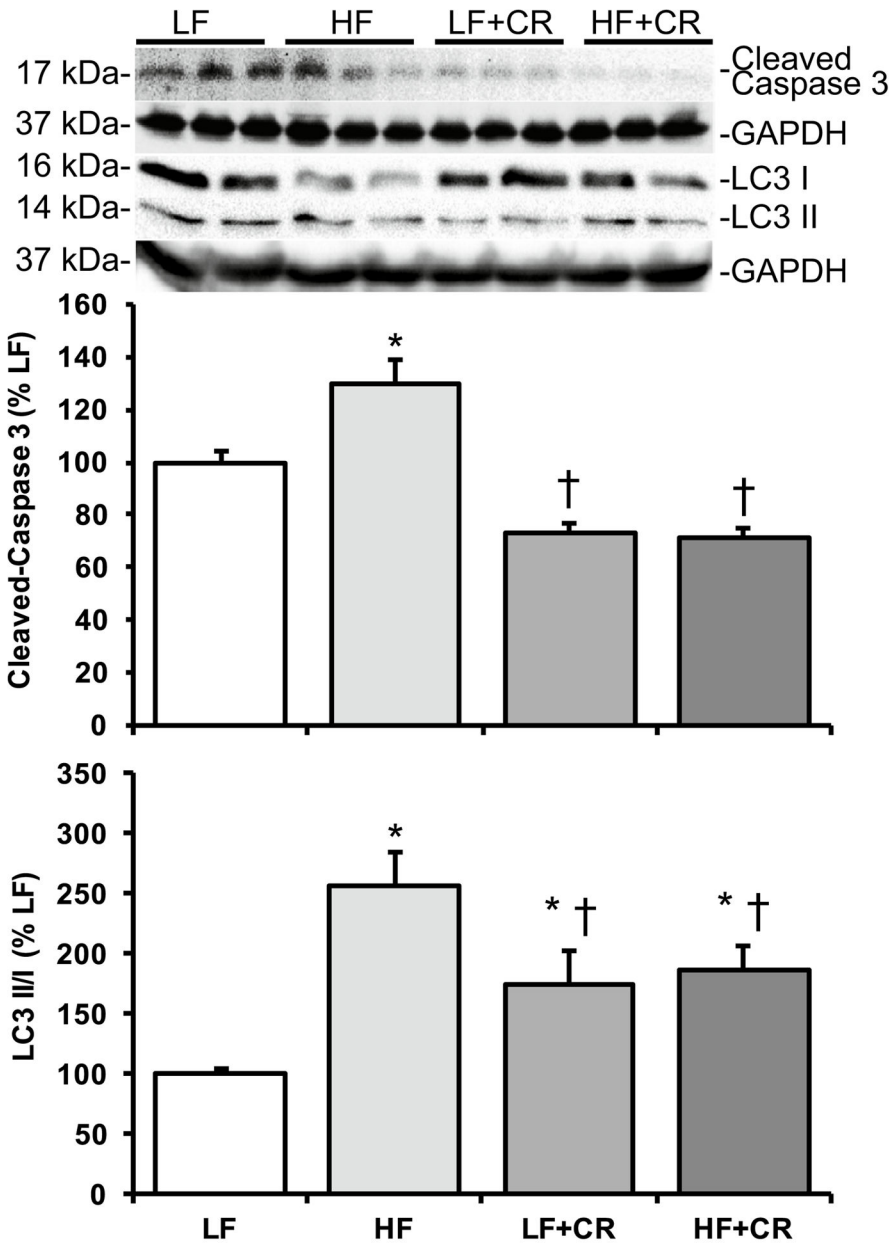

Fig. 6.

Skeletal muscle expression of cleaved caspase 3 and LC3 in low fat and high fat fed, calorically restricted mice. Equal protein from low (LF) and high fat (HF) fed, calorically restricted (CR) mouse muscle homogenates were analyzed by Western blot analysis for **a** cleaved caspase 3, **b** LC3-II/I ratio, and GAPDH, then normalized to GAPDH. Representative Western blots are shown. Means marked with an *asterisk* are significantly different $p < 0.05$ *versus* LF and *dagger* are significantly $p < 0.05$ *versus* HF ($n = 6$/group)