

OPEN ACCESS

Leucine alleviates dexamethasone-induced suppression of muscle protein synthesis via synergy involvement of mTOR and AMPK pathways

Xiao J. Wang*¹, Xin Yang*¹, Ru X. Wang*, Hong C. Jiao*, Jing P. Zhao*, Zhi G. Song* and Hai Lin*²

*Department of Animal Science, Shandong Agricultural University, Taian, Shandong 271018, P.R. China

Synopsis

Glucocorticoids (GCs) are negative muscle protein regulators that contribute to the whole-body catabolic state during stress. Mammalian target of rapamycin (mTOR)-signalling pathway, which acts as a central regulator of protein metabolism, can be activated by branched-chain amino acids (BCAA). In the present study, the effect of leucine on the suppression of protein synthesis induced by GCs and the pathway involved were investigated. *In vitro* experiments were conducted using cultured C2C12 myoblasts to study the effect of GCs on protein synthesis, and the involvement of mTOR pathway was investigated as well. After exposure to dexamethasone (DEX, 100 μ mol/l) for 24 h, protein synthesis in muscle cells was significantly suppressed ($P < 0.05$), the phosphorylations of mTOR, ribosomal protein S6 protein kinase 1 (p70s6k1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1) were significantly reduced ($P < 0.05$). Leucine supplementation (5 mmol/l, 10 mmol/l and 15 mmol/l) for 1 h alleviated the suppression of protein synthesis induced by DEX ($P < 0.05$) and was accompanied with the increased phosphorylation of mTOR and decreased phosphorylation of AMPK ($P < 0.05$). Branched-chain amino transferase 2 (BCAT2) mRNA level was not influenced by DEX ($P > 0.05$) but was increased by leucine supplementation at a dose of 5 mmol/l ($P < 0.05$).

Key words: AMPK, glucocorticoid, leucine, mTOR, muscle cell, protein synthesis.

Cite this article as: Bioscience Reports (2016) 36, e00346, doi:10.1042/BSR20160096

INTRODUCTION

The maintenance of skeletal muscle mass is of paramount importance for motility and systemic energy homeostasis [1]. The control of muscle mass is determined by a dynamic balance between the anabolic and catabolic processes involving proteins [2]. Adrenal glucocorticoids (GCs) are well known to regulate an array of physiological processes, including protein metabolism, thus contributing to whole-body homeostasis. It has been demonstrated that GCs are negative muscle protein regulators, and many pathological conditions, including muscle atrophy, are associated with an increase in circulating GCs levels [3]. In rat, muscle protein synthesis is inhibited as early as 4 h after the administration of GCs [4,5]. Shah et al. [6] showed that the injection of dexamethasone (DEX), a synthetic GC, acutely diminished protein synthesis rates to 59% of control values in skeletal muscle from young rats. The inhibition of mRNA translation initiation appears

to be a major mechanism by which GCs result in the inhibition of protein synthesis [7].

Mammalian target of rapamycin (mTOR) is a crucial component of the anabolic machinery for protein synthesis, which senses and integrates signals from growth factors, environmental stress factors, nutrient availability, and energy status. mTOR has been shown to exist in two complexes (mTORC1 and mTORC2) [8]. mTORC1 is essential for the maintenance of muscle mass and function [9,10]. mTORC1 signals to ribosomal protein S6 protein kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1), which are currently the two best-known downstream effectors of mTOR signalling, and control the protein synthetic pathway [11]. Wang et al. [12] demonstrated that the mTOR pathway is negatively regulated in the presence of excessive GCs.

AMP-activated protein kinase (AMPK), a highly sensor of cellular energy status, is activated under conditions of low intracellular ATP. AMPK acts as a major catabolic regulator in response

Abbreviations: AMPK, AMP-activated protein kinase; BCAA, branched-chain amino acids; BCAT, branched-chain amino transferase; DEX, dexamethasone; 4EBP1, eukaryotic initiation factor 4E binding protein 1; GCs, glucocorticoids; GR, glucocorticoid receptor; MAP4K3, mitogen-activated protein kinase kinase kinase 3; mTOR, mammalian target of rapamycin; p70s6k, ribosomal protein S6 protein kinase; Rheb, RAS homologue enriched in brain.

¹ These authors contributed equally to the study.

² To whom correspondence should be addressed (email hailin@sdau.edu.cn).

to energy stress, in part through its inhibition of the mTORC1 pathway. The activation of AMPK directly phosphorylates both TSC2 and Raptor to inhibit mTORC1 activity by a dual-pronged mechanism [13,14]. Kimura et al. [15] also revealed that AMPK appears to provide an overriding switch linking p70s6k regulation to cellular energy metabolism. It is generally known GCs act as mediators in whole-body energy redistribution. However, whether GCs-driven protein synthesis regulation requires the co-operation of AMPK and mTOR is yet unknown.

Branched-chain amino acids (BCAA) are one of the major signals that activate mTORC1. Leucine can provide energy by conversion to ketoisocaproate, which is oxidized via the TCA cycle; leucine could decrease AMPK α phosphorylation and AMPK activity in rats [16] and C2C12 cells [17]. We thus hypothesized that BCAA supplementation could alleviate the negative effect of GCs on protein synthesis by evoking mTOR pathway with the synergy of AMPK pathway.

In the present study, we investigated whether GCs inhibit protein synthesis via the mTOR and AMPK signalling pathways, and the involvement of BCAA was examined. Herein, cultured C2C12 myoblasts were used as a model for muscle growth. DEX, a synthetic GC that is specific for the GCs receptor and delayed plasma clearance [18], was employed to induce a hyperglucocorticoid milieu. Our results indicate that GCs repress protein synthesis, likely through the involvement of both mTOR and AMPK pathways. In addition, the effects of GCs on protein synthesis and mTOR and AMPK pathways could be attenuated by leucine supplementation. Our study originally demonstrates the synergy involvement of mTOR and AMPK in the interaction between GCs and BCAA on muscular protein synthesis. This finding provides a novel insight into the metabolic perturbations associated with long-term GCs use and dietetical therapy in clinical setting.

MATERIALS AND METHODS

Myoblasts culture and *in vitro* treatments

C2C12 myoblasts (CCTCC) were cultured in DMEM (HyClone) supplemented with 10% fetal bovine serum (HyClone), 100 U/ml penicillin and 100 μ g/ml streptomycin (Solarbio) at 37°C in a humidified atmosphere containing 5% CO₂. When cells were 70% confluent, the proliferation medium was replaced with a differentiation medium, DMEM containing 2% horse serum. After 84 h, cells were incubated for 12 h with serum-free DMEM.

After a 12-h incubation in serum-free medium, the myoblasts were exposed to DMEM-LM (Thermo) with or without DEX (100 μ mol/l) for 24 h. At 23 h of the DEX exposure, leucine (Sigma) was added to DEX treated cells for the following 1 h, with a concentration of 5, 10 or 15 mmol/l. After this, all cells were immediately subjected to an additional 30-min puromycin exposure (1 μ mol/l, Sigma) and then the detection of protein synthesis using an anti-puromycin antibody (Figure 1A), or were directly collected for mRNA and protein analysis (Figure 1B).

Protein synthesis rate analysis

To measure the muscle protein synthesis rate, we used a technique involving the labelling of newly synthesized polypeptides with low concentrations of puromycin, then the detection of these proteins using an anti-puromycin antibody [19]. After DEX and leucine administration, 1 μ mol/l puromycin was added to all wells, and the cells were incubated for an additional 30 min. Cells were then collected and subjected to Western blotting analysis using an anti-puromycin antibody as described below. The accumulation of puromycin-conjugated peptides into nascent peptide chains reflects the rate of protein synthesis in many different *in vitro* and *in vivo* conditions [19–21].

Protein preparation and western blot

Protein concentration was determined using the BCA assay kit (Beyotime). The samples were boiled at 100°C for 5 min in 5 \times sample buffer. The protein extracts were electrophoresed in 7.5–10% SDS polyacrylamide gels (Bio-Rad Laboratories) according to the Laemmli method [22]. The separated proteins were then transferred on to a nitrocellulose membrane in Tris–glycine buffer containing 20% methanol. The membranes were blocked and immunoblotted with a 1:1000 dilution of a primary antibody including anti-puromycin (KeraFAST), anti-P-mTOR (Ser²⁴⁴⁸), anti-mTOR, anti-P-p70S6K (Thr³⁸⁹), anti-p70S6K, anti-P-4EBP1 (Thr^{37/46}), anti-4EBP1, anti-P-AMPK (Thr¹⁷²) and anti-AMPK (Beverly, MA, USA).

The proteins were detected using either goat anti-rabbit IgG (H + L)-HRP conjugated secondary antibody (1:2000, Bio-Rad Laboratories) or HRP-labelled goat anti-mouse IgG (H + L) secondary antibody (1:1000, Beyotime) with enhanced chemiluminescence (ECL) plus western blot detection reagents (Beyotime). β -Actin was used as an internal control (Beyotime). Western blots were developed and quantified using BioSpectrum 810 with VisionWorksLS 7.1 software (UVP LLC). The protein level was quantified by normalizing total proteins with β -actin, and by normalizing phosphorylated proteins with their total pairs.

RNA preparation and analysis

Gene expression was measured using real-time RT-PCR. Briefly, total RNA from cells was extracted using TRIzol (Invitrogen). The quantity and quality of the isolated RNA were determined using a biophotometer (Eppendorf) and agarose gel electrophoresis. Next, reverse transcription was performed using an RT reaction (10 μ l) that consisted of 500 ng total RNA, 5 mmol/l MgCl₂, 1 μ l RT buffer, 1 mmol/l dNTP, 2.5 U AMV, 0.7 nmol/l oligo d(T) and 10 units ribonuclease inhibitor (TaKaRa). The cDNA was amplified in a 20 μ l PCR reaction containing 0.2 μ mol/l of each specific primer (Sangon) and SYBR green master mix (TaKaRa). Real-time PCR was performed at 95°C for 10 s of predenaturation, followed by 40 cycles, and each cycle consisted of denaturation at 95°C for 5 s and annealing and extension at 60°C for 40 s. Primers against β -actin was used as internal controls to normalize the differences between individual samples. The primer sequences for mouse are listed in Table 1. Standard

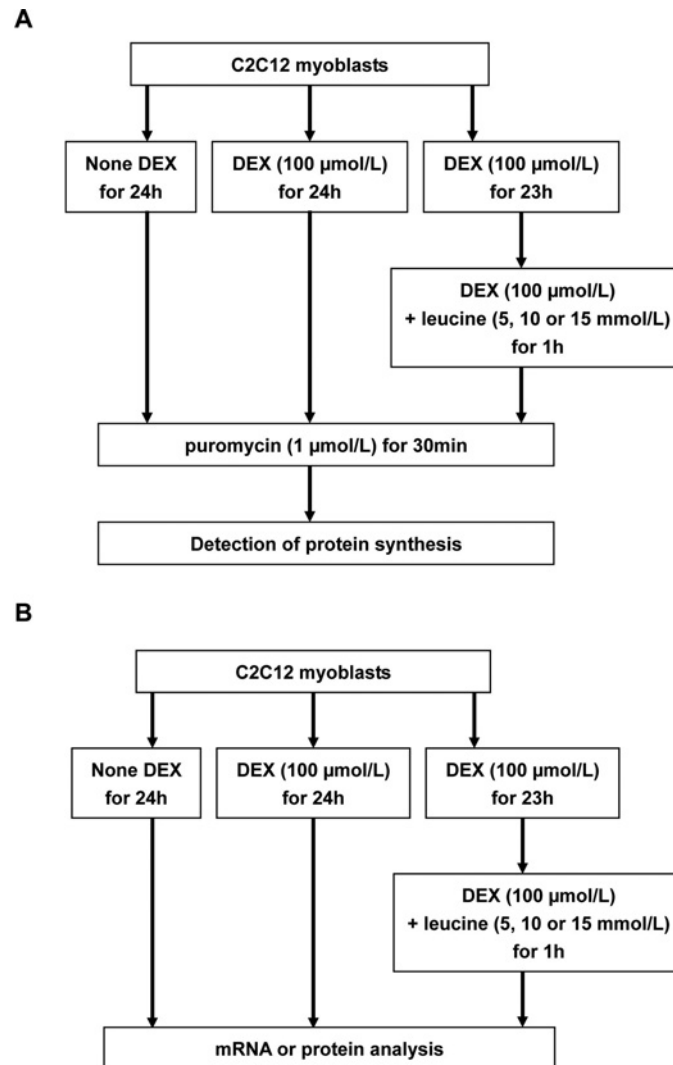


Figure 1 The flowchart of study design

The myoblasts were incubated with or without DEX (100 $\mu\text{mol/l}$) for 24 h. At 23 h of the DEX exposure, leucine (5, 10 or 15 mmol/l) was added to DEX treated cells for the following 1 h. After this, all cells were immediately subjected to an additional 30-min puromycin exposure (1 $\mu\text{mol/l}$) and then the detection of protein synthesis (**A**), or were directly collected for mRNA and protein analysis (**B**).

curves were generated using pooled cDNA from the samples that were assayed, and the comparative CT method ($2^{-\Delta\Delta\text{CT}}$) was used to quantify mRNA expression, as described by Livak and Schmittgen [23]. All of the samples were run in duplicate, and the primers were designed to span an intron to avoid genomic DNA contamination. All samples were included in the same assay for one gene to avoid inter-assay variability.

Statistical methods

All the data were subjected to one-way ANOVA analysis with the Statistical Analysis Systems statistical software package (Version 8e, SAS Institute). The homogeneity of variances among groups was confirmed using Bartlett's test (SAS Institute). When the

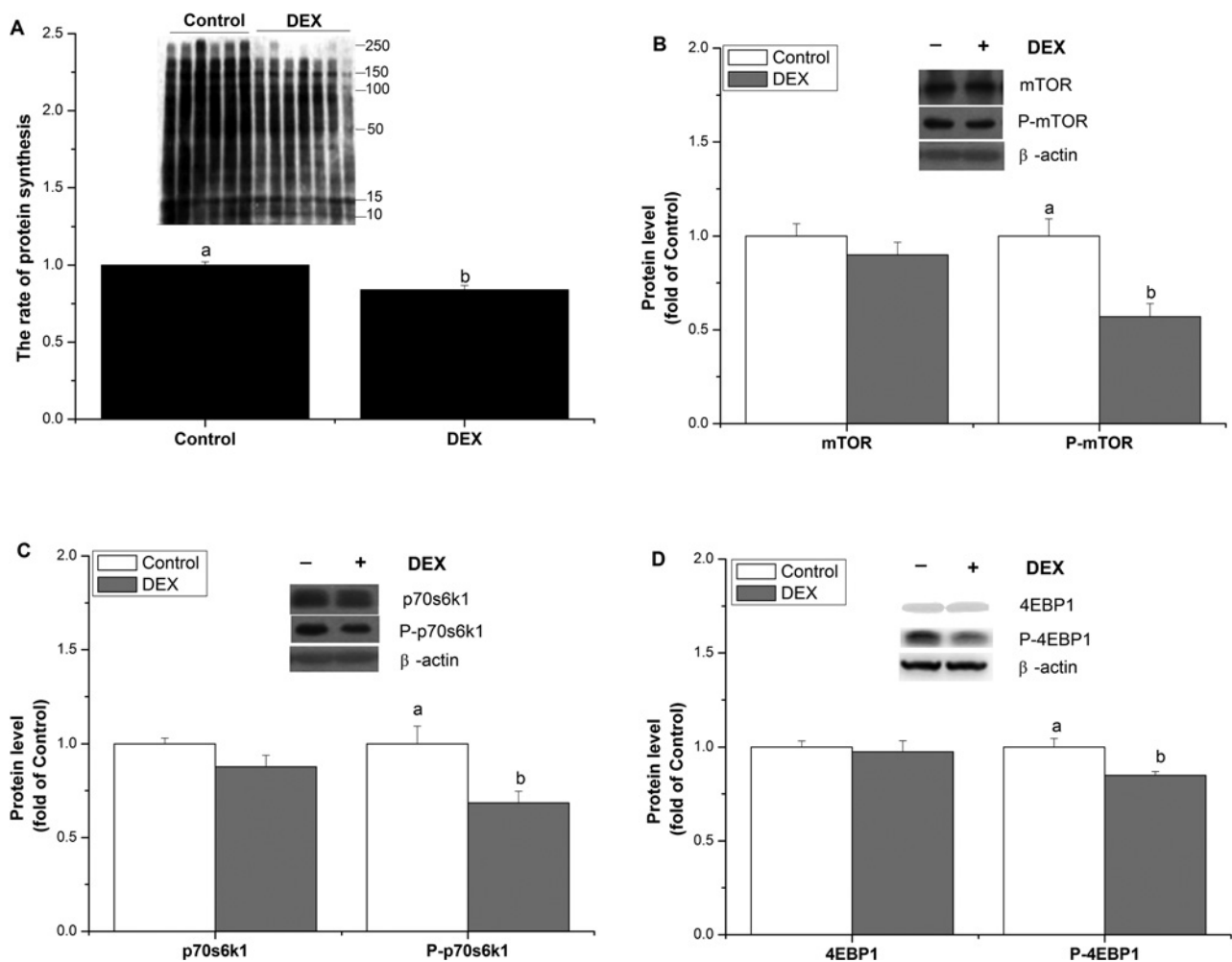
primary effect of treatment was significant, differences between means were assessed by Duncan's multiple range analysis. Means were considered significantly different at $P < 0.05$.

RESULTS

In cultured muscle cells, we demonstrated that the DEX treatment significantly suppressed protein synthesis ($P < 0.05$, Figure 2A), as well as the phosphorylation of mTOR, p70s6k1 and 4EBP1 ($P < 0.05$, Figures 2B–2D), whereas the total protein

Table 1 Gene-specific primers of related genes

Gene name	GenBank number	Primers position	Primers sequences (5'→3')	Product length (bp)
β -Actin	NM_007393	Forward	ACCACACCTTCTACAATGAG	182
		Reverse	ACGACCAGAGGCATACAG	
BCAT2	NM_001243052	Forward	CCTGTTCCCTGGCTTCTATGT	100
		Reverse	GCTTCTTCTGGTTCTTTGGT	
MAP4K3	NM_001290345	Forward	ATTCTGTGGAGGTGGCTCTTTA	176
		Reverse	CGTGACCATTATCCGTTAGGAG	
Rheb	BC012273	Forward	TCTGTGGAAAGTCCTCATTG	115
		Reverse	ACTCTTGACCATTACCGTGAT	
GR	X66367	Forward	CCCATGGAGGTAGCGATTGT	100
		Reverse	TGTAAGGCTGCCAATGTGT	


Figure 2 mTOR inhibition is involved in DEX-suppressed protein synthesis

The effect of DEX (100 μ mol/l for 24 h) on protein synthesis (A), and the protein expression of mTOR, P-mTOR (B), p70s6k, P-p70s6k (C), 4EBP1, P-4EBP1 (D) in C2C12 myoblasts. The protein level was quantified by normalizing total proteins with β -actin, and by normalizing phosphorylated proteins with their total pairs. The values shown are the means \pm S.E.M. ($n = 6$); a, b: means with different letters are significantly different ($P < 0.05$).

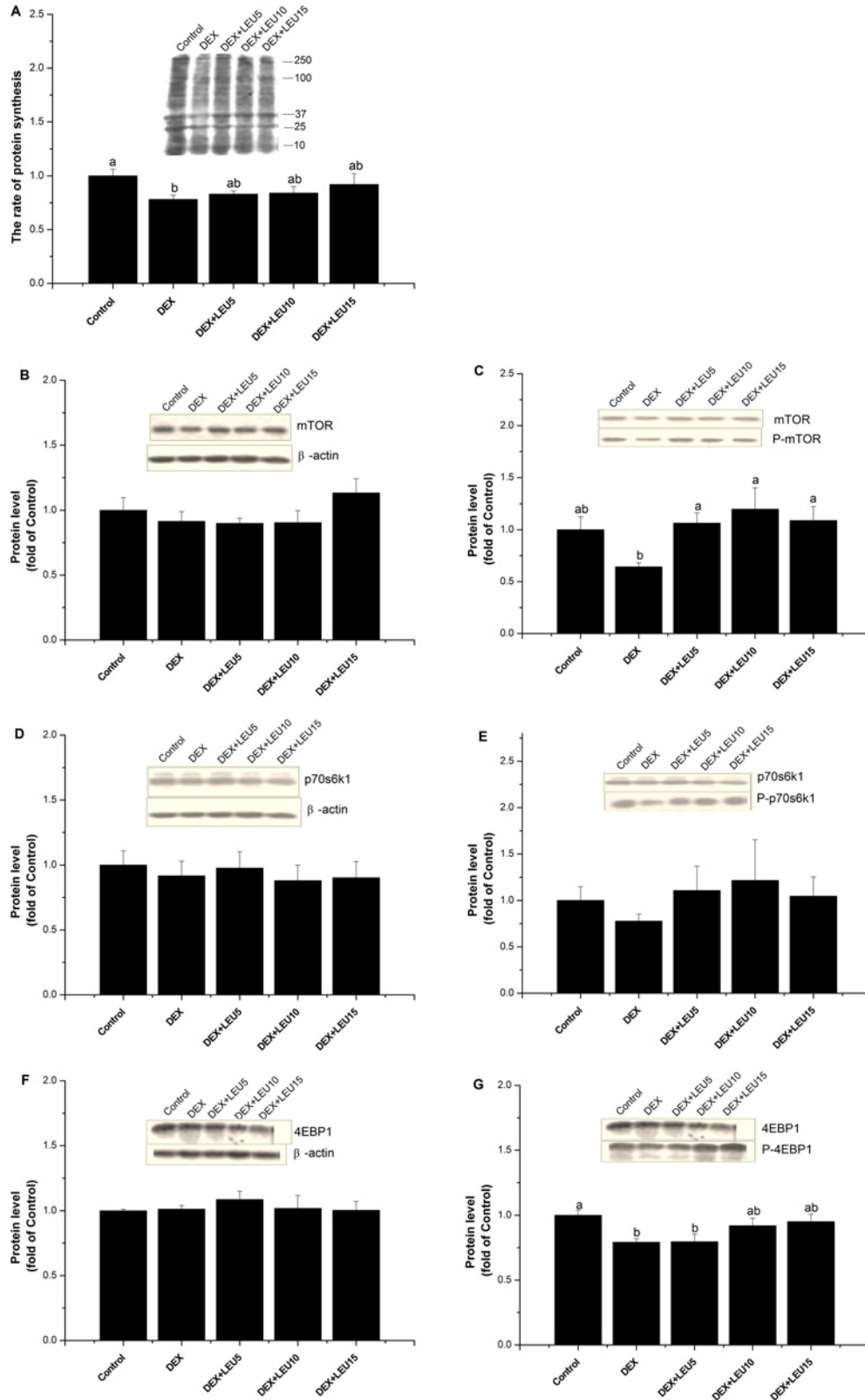


Figure 3 Leucine ameliorated DEX-suppressed protein synthesis by stimulating mTOR and suppressing AMPK pathway

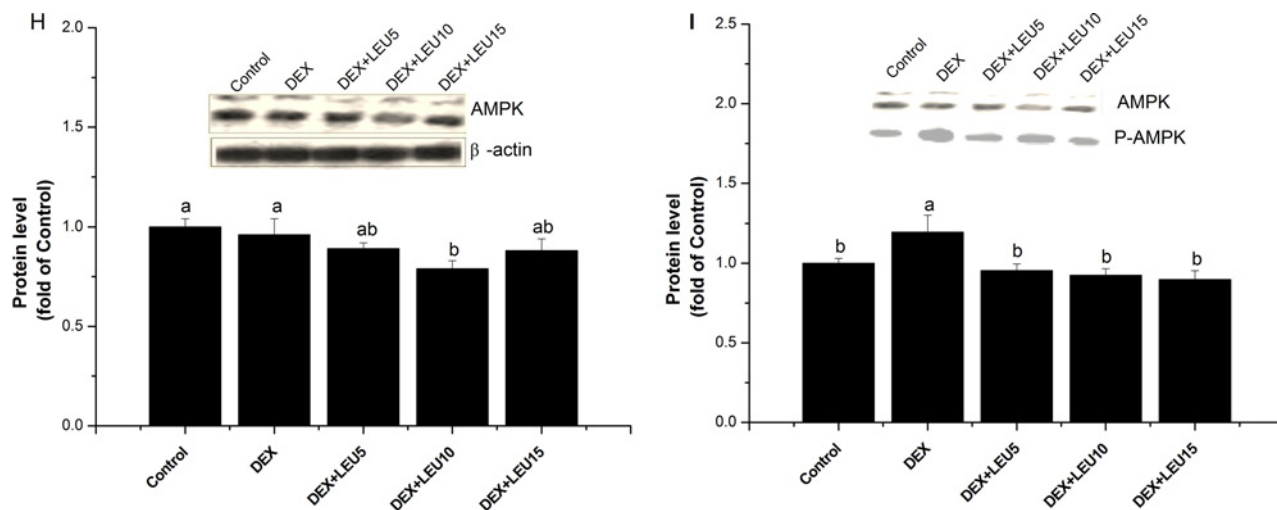


Figure 3 Continued

The effect of DEX (100 μ mol/l for 24 h) and leucine treatment (5 mmol/l or 10 mmol/l or 15 mmol/l for 1 h) on protein synthesis (A), and the protein expression of mTOR (B), P-mTOR (C), p70s6k (D), P-p70s6k (E), 4EBP1 (F), P-4EBP1 (G), AMPK (H) and P-AMPK (I) in C2C12 myoblasts. The protein level was quantified by normalizing total proteins with β -actin, and by normalizing phosphorylated proteins with their total pairs. The values shown are the means \pm S.E.M. ($n = 6$); a, b: means with different letters are significantly different ($P < 0.05$).

expression of mTOR, p70s6k1 and 4EBP1 was not affected by DEX ($P > 0.05$, Figures 2B–2D).

In the presence of DEX and leucine, DEX-suppressed myoblast protein synthesis was restored to normal (Figure 3A). Leucine supplementation completely/partially alleviated the negative effect of DEX alone on both mTOR and 4EBP1 phosphorylation (Figures 3C and 3G). DEX and leucine had no effect on the protein expressions of mTOR, p70s6k1, phosphor-p70s6k1 and 4EBP1 ($P > 0.05$, Figures 3B, 3D, 3E and 3F). DEX alone showed no obvious effect on the total protein level of AMPK ($P > 0.05$), but DEX + leucine (10 mmol/l) treatment reduced AMPK protein expression compared with the control ($P < 0.05$, Figure 3H). DEX exposure significantly enhanced the phosphorylation of AMPK compared with the control group ($P < 0.05$), and this impact exerted by DEX alone was restored to the control level after supplementation with leucine (Figure 3I). These results suggest that the inhibiting effect of leucine on AMPK phosphorylation, at least partially, is due to the reduced total AMPK protein.

Compared with the control, glucocorticoid receptor (GR) mRNA level was increased by DEX alone ($P < 0.05$), as well as DEX + leucine treatment ($P < 0.05$, Figure 4B). DEX treatment had no significant influence on the mRNA expressions of β -actin, branched-chain amino transferase 2 (BCAT2), mitogen-activated protein kinase kinase kinase 3 (MAP4K3) and RAS homologue enriched in brain (Rheb) compared with the control ($P > 0.05$, Figures 4A, 4C, 4D and 4E). Compared with the DEX alone, BCAT2, MAP4K3 and Rheb mRNA levels were increased in the DEX + leucine (5 mmol/l) group ($P < 0.05$) but restored in the DEX + leucine (10 mmol/l) group (Figures 4C–4E).

DISCUSSION

In the present study, we assessed the direct effect of leucine on muscle protein synthesis in the presence of GCs. The results show that the leucine supplementation could relieve the suppression effect of GCs on protein synthesis by evoking mTOR/p70s6k pathway. We firstly demonstrate that AMPK is also involved in the regulation of GCs and leucine on muscle protein synthesis. Proposed model of GCs and leucine action on protein synthesis in C2C12 myoblasts is shown in Figure 5.

DEX retards myoblast protein synthesis

Muscle growth is largely due to the balance of muscle protein synthesis and degradation. In the present study, we determined the protein synthesis rate of cultured C2C12 myoblasts by using puromycin to label the newly-synthesized polypeptides [21]. We demonstrated that DEX-suppressed protein synthesis, in line with previous findings *in vivo* [4–6]. This may explain the involvement of GCs in catabolism/anabolism disorders, which are associated with a number of pathological conditions, including muscle atrophy [24–26].

mTOR inhibition is involved in DEX-suppressed protein synthesis

mTOR acts as a critical mediator that controls protein synthesis at the transcriptional and translational levels, by sensing and integrating signals from nutrients and energy. mTOR activation up-regulates the translational machinery and promotes protein

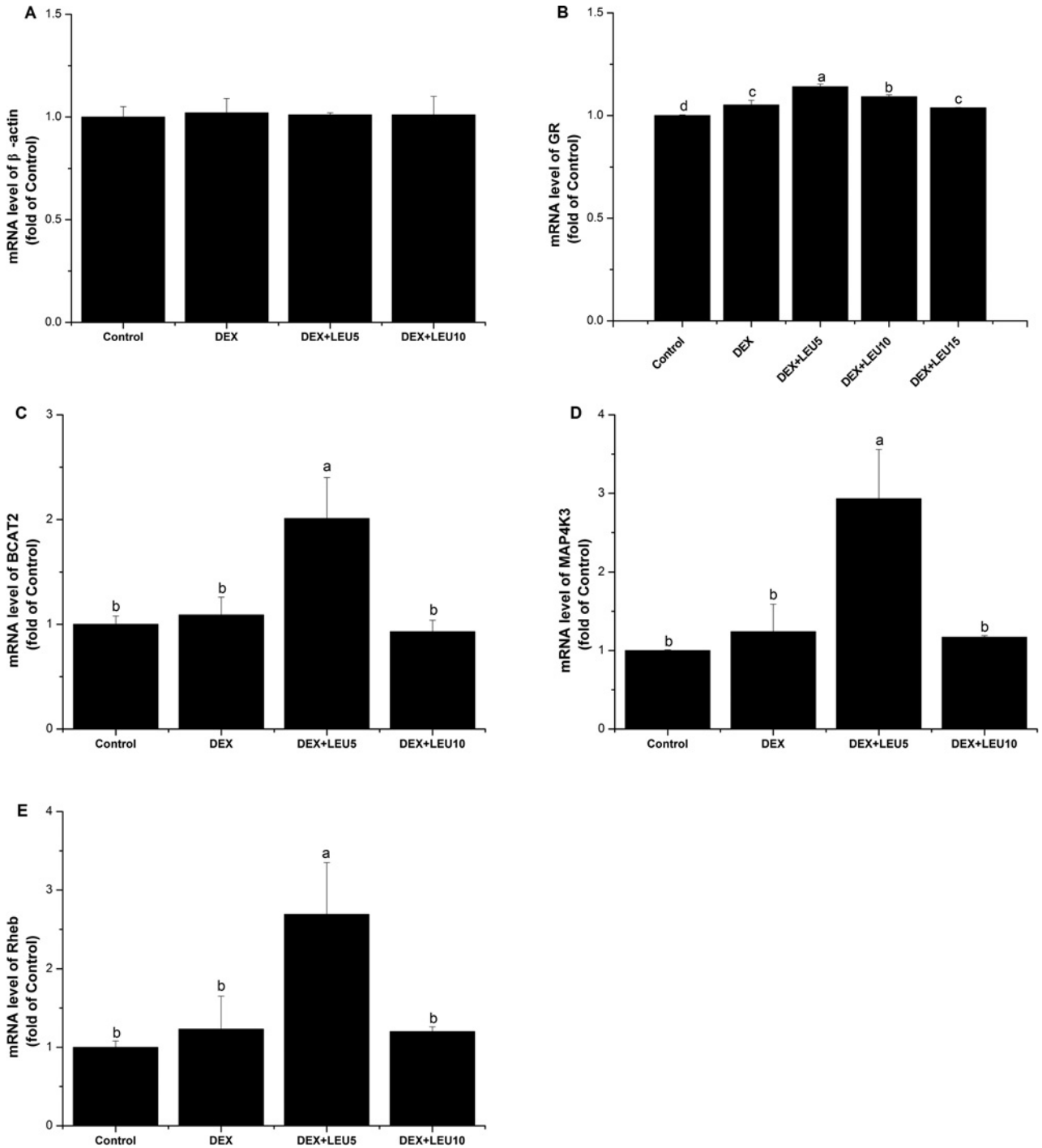


Figure 4 Expressions of regulators sensitive to GCs and leucine

The effect of DEX ($100 \mu\text{mol/l}$ for 24 h) and leucine treatment (5 mmol/l or 10 mmol/l or 15 mmol/l for 1 h) on the mRNA expression of β -actin (A), GR (B), BCAT2 (C), MAP4K3 (D) and Rheb (E) in C2C12 myoblasts. The values shown are the means \pm S.E.M. ($n = 6$); a, b, c, d: means with different letters are significantly different ($P < 0.05$).

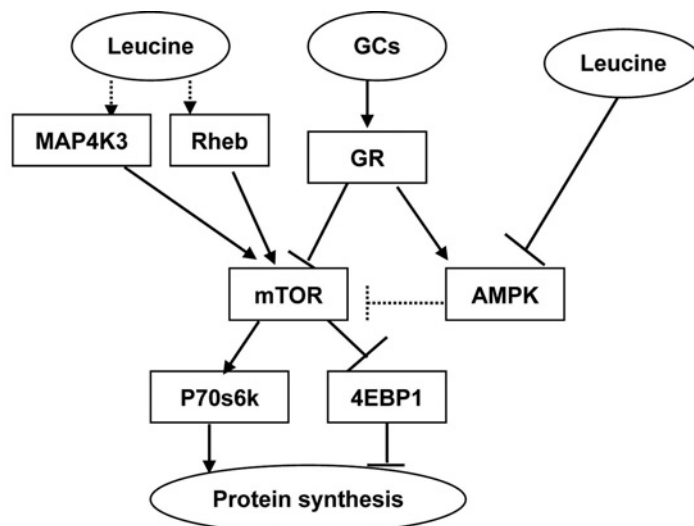


Figure 5 Proposed model of GCs and leucine action on protein synthesis in C2C12 myoblasts (→ stimulatory; ⊥ inhibitory; findings reported previously [13, 14, 47–49, 55])

Activation of GCs suppresses myocellular protein synthesis via inhibiting mTOR and stimulating AMPK; leucine relieves GCs-induced inhibition on protein synthesis by evoking mTOR and suppressing AMPK pathway.

translation [27]. Previous studies have demonstrated the role of GCs in the modulation of mTOR signalling and in the regulation of protein synthesis. Rannels et al. [7] revealed that the inhibition of protein synthesis by GCs is most probably associated with the inhibition of mRNA translation initiation. GCs suppressed mTOR pathway by dephosphorylating p70s6k and 4EBP1 in skeletal muscle cells [6,28–30], which are currently the two best-known downstream effectors of mTOR signalling, and control the protein synthetic pathway [11]. In the present study, the decreased phosphorylation of mTOR and p70s6k and 4EBP1 by DEX indicated that DEX-suppressed muscle protein synthesis by inhibiting the mTOR signalling pathway. The observation is consistent with the results obtained by Long et al. [31], who reported that DEX inhibits the stimulation of muscle protein synthesis and the phosphorylation of p70s6k.

Leucine ameliorated DEX-suppressed protein synthesis by stimulating mTOR pathway

mTOR regulates multiple cellular functions, including translation, in response to nutrients, especially BCAA. Bolster et al. [32] and Deldicque et al. [33] reported that amino acids stimulate muscle protein synthesis partially through the activation of the mTOR pathways. Kimball and Jefferson [13] also revealed that BCAA mediates translational control of protein synthesis. Leucine and other members of the BCAA family are the dominant players in the amino acids-induced regulation of p70s6k [34–36]. Although insulin alone can increase muscle protein synthesis in animals, amino acids (particularly leucine) appear to have much more potent anabolic effect [13,37–39]. The administration of leucine after fasting or amino acids starvation stimulates protein synthesis and promotes the phosphorylation and activation of

S6K1 via the rapamycin sensitive mTOR in skeletal muscle [37]. GCs could inhibit mTOR activity [40]. Therefore, we tested that whether leucine supplementation could relieve the inhibition of mTOR by GCs. The present result indicated that DEX-induced myoblast protein synthesis was restored to 83–92% of normal by leucine supplementation. Meanwhile, leucine supplementation completely/partially removed the inhibition of DEX on mTOR and 4EBP1 phosphorylation. The result suggests that leucine relieves the negative effect of DEX on protein synthesis by evoking mTOR pathway.

The effect of GCs is mediated by the ligand-dependent activation of GR. Upon binding GCs, the activated GR acts as a transcription factor, translocating into the nucleus and controlling the level of target gene expression and modulating intracellular signalling pathways [41–43]. GR is mandatory for muscle atrophy in response to GCs excess both *in vitro* [44] and *in vivo* [45]. On the contrary, muscle-specific GR-knockout mice are resistant to the atrophy induced by GCs [46]. GCs inhibit mTOR activity via GR [40]. Herein, we found that GR mRNA abundance was elevated by DEX. Moreover, leucine supplementation further up-regulated GR mRNA level, suggesting that increased abundant mRNA level of GR is a feedback effect of GC and leucine.

BCAT2, a mitochondrial enzyme catalysing the first reaction in the catabolism of BCAA [46], is a critical determinant of cellular BCAA content in skeletal muscle. GCs inhibit mTOR activity by evoking the activity of BCAT [40]. In the present study, BCAT2 mRNA level was not influenced by DEX but was increased by leucine supplementation at a dose of 5 mmol/l. The result may imply that BCAT2 is not the only way involved in the regulation of GCs and leucine on mTOR. The increase in BCAT2 mRNA level with 5 mmol/l leucine was lost with 10 mmol/l leucine, suggesting a substrate inhibition characteristic of BCAT2.

MAP4K3 is an upstream amino acids-sensitive regulator of mTORC1 signalling [47]. In primary human fibroblasts, knock-down of MAP4K3 resulted in a significant attenuation in leucine-induced mTORC1 signalling [48]. Rheb is also known to be critical elements in the pathway that links amino acids availability to mTORC1 activation [49]. In the present study, the mRNA expression of Rheb and MAP4K3 were enhanced when 5 mmol/l leucine was supplemented, compared with DEX alone. However, this transcriptional stimulation of Rheb and MAP4K3 was not observed after 10 mmol/l leucine supplementation. The irregularity of leucine action on mRNA expression indirectly confirmed that leucine may exert its action on Rheb and MAP4K3 via a post-transcriptional regulation. This speculation is consistent with studies of Yan et al. [50] and Tee et al. [51] who reported that Rheb farnesylation and MAP4K3 phosphorylation were required for mTORC1 activation. Further works on translational and post-translational modification are required.

AMPK is involved in the activation of mTOR by leucine

AMPK modulates metabolism for cellular energy demand by responding to changes in the AMP/ATP ratio [52–54]. AMPK suppresses protein synthesis in rat skeletal muscle through the down-regulation of mTOR signalling [55]. AMPK activation can phosphorylate both TSC2 and Raptor, resulting in the depression of mTORC1 signalling [13,14]. AMPK appears to provide an overriding switch that links p70s6k regulation to cellular energy metabolism [15]. In the present study, the increased phosphorylation level of AMPK by DEX treatment indicated that the stimulated AMPK pathway by DEX. Leucine supplementation, however, down-regulated the phosphorylation of AMPK. The result suggests that AMPK synergizes mTOR underlying in the regulation of GCs and leucine on muscle protein synthesis. These novel observations of the synergy effect of AMPK and mTOR pathways are consistent with the results of Lang et al. [56] and Du et al. [17], who reported that leucine stimulates mTOR, at least partially, through the inactivation of AMPK.

In conclusion, both mTOR and AMPK pathways are involved in the DEX-induced suppression of protein synthesis in muscle cells. Leucine supplementation relieves DEX-induced inhibition on protein synthesis by evoking mTOR and suppressing AMPK pathway.

AUTHOR CONTRIBUTION

Hai Lin conceived and designed the experiments. Xiao Wang and Xin Yang performed the experiments. Xiao Wang, Xin Yang and Ru Wang analysed the data. Hong Jiao, Zhi Song and Jing Zhao contributed reagents/materials/analysis tools. Xiao Wang and Hai Lin wrote the paper. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Basic Research Program of China [grant number 2012CB124706].

REFERENCES

- 1 Matthews, S.E. (1999) Proteins and amino acids. Modern Nutrition and Health and Diseases, 9th edn. (Shils, M.E., Olson, J.A., Shike, M. and Ross, A.C., eds), pp. 11–48, Williams & Wilkins, Baltimore
- 2 Hoffman, E.P and Nader, G.A. (2004) Balancing muscle hypertrophy and atrophy. *Nat. Med.* **10**, 584–585 [CrossRef PubMed](#)
- 3 Munck, A., Guyre, P.M. and Holbrook, N.J. (1984) Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocr. Rev.* **5**, 25–44 [CrossRef PubMed](#)
- 4 Rannels, S.R. and Jefferson, L.S. (1980) Effects of glucocorticoids on muscle protein turnover in perfused rat hemi-corpus. *Am. J. Physiol.* **238**, E564–E572 [PubMed](#)
- 5 Southorn, B.G., Palmer, R.M. and Garlick, P.J. (1990) Acute effects of corticosterone on tissue protein synthesis and insulin sensitivity in rats *in vivo*. *Biochem. J.* **272**, 187–191 [CrossRef PubMed](#)
- 6 Shah, O.J., Kimball, S.R. and Jefferson, L.S. (2000a) Acute attenuation of translation initiation and protein synthesis by glucocorticoids in skeletal muscle. *Am. J. Physiol.* **278**, E76–E82
- 7 Rannels, D.E., Rannels, S.R., Li, J.B., Pegg, A.E., Morgan, H.E. and Jefferson, L.S. (1980) Effects of glucocorticoids on peptide chain initiation in heart and skeletal muscle. *Adv. Myocardiol.* **1**, 493–501 [PubMed](#)
- 8 Wullschleger, S., Loewith, R. and Hall, M.N. (2006) TOR signaling in growth and metabolism. *Cell* **124**, 471–484 [CrossRef PubMed](#)
- 9 Bentzinger, C.F., Romanino, K., Cloetta, D., Lin, S., Mascarenhas, J.B., Oliveri, F., Xia, J., Casanova, E., Costa, C.F., Brink, M. et al. (2008) Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy. *Cell Metab.* **8**, 411–424 [CrossRef PubMed](#)
- 10 Risson, V., Mazelin, L., Roceri, M., Sanchez, H., Moncollin, V., Corneloup, C., Richard-Bulteau, H., Vignaud, A., Baas, D., Defour, A. et al. (2009) Muscle inactivation of mTOR causes metabolic and dystrophin defects leading to severe myopathy. *J. Cell. Biol.* **187**, 859–874 [CrossRef PubMed](#)
- 11 Hay, N. and Sonenberg, N. (2004) Upstream and downstream of mTOR. *Genes. Dev.* **18**, 1926–1945 [CrossRef PubMed](#)
- 12 Wang, H., Kubica, N., Ellisen, L.W., Jefferson, L.S. and Kimball, S.R. (2006) Dexametasonone represses signaling through the mammalian target of rapamycin in muscle cells by enhancing expression of REDD1. *J. Biol. Chem.* **281**, 39128–39134 [CrossRef PubMed](#)
- 13 Kimball, S.R. and Jefferson, L.S. (2006) Signaling pathways and molecular mechanisms through which branched-chain amino acids mediate translational control of protein synthesis. *J. Nutr.* **136**, 227S–231S [PubMed](#)
- 14 Gwinn, D.M., Shackelford, D.B., Egan, D.F., Mihaylova, M.M., Mery, A., Vasquez, D.S., Turk, B.E. and Shaw, R.J. (2008) AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol. Cell* **30**, 214–226 [CrossRef PubMed](#)
- 15 Kimura, N., Tokunaga, C., Dalal, S., Richardson, C., Yoshino, K., Hara, K., Kemp, B.E., Witters, L.A., Mimura, O. and Yonezawa, K. (2003) A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway. *Genes Cells* **1**, 65–79 [CrossRef](#)
- 16 Wilson, G.J., Layman, D.K., Moulton, C.J., Norton, L.E., Anthony, T.G., Proud, C.G., Rupassara, S.I. and Garlick, P.J. (2011) Leucine or carbohydrate supplementation reduces AMPK and eEF2 phosphorylation and extends postprandial muscle protein synthesis in rats. *Am. J. Physiol.* **301**, E1236–E1242 [CrossRef PubMed](#)
- 17 Du, M., Shen, Q.W., Zhu, M.J. and Ford, S.P. (2007) Leucine stimulates mammalian target of rapamycin signaling in C2C12 myoblasts in part through inhibition of adenosine monophosphate-activated protein kinase. *J. Anim. Sci.* **85**, 919–927 [CrossRef PubMed](#)



- 18 Foucaud, L., Niot, I., Kanda, T. and Besnard, P. (1998) Indirect dexamethasone down-regulation of the liver fatty acid-binding protein expression in rat liver. *Biochim. Biophys. Acta* **1391**, 204–212 [CrossRef PubMed](#)
- 19 Schmidt, E.K., Clavarino, G., Ceppi, M. and Pierre, P. (2009) SUnSET, a nonradioactive method to monitor protein synthesis. *Nat. Methods* **6**, 275–277 [CrossRef PubMed](#)
- 20 Nakano, K. and Hara, H. (1979) Measurement of the protein-synthetic activity *in vivo* of various tissues in rats by using [³H] Puromycin. *Biochem. J.* **184**, 663–668 [CrossRef PubMed](#)
- 21 Goodman, C.A., Mabrey, D.M., Frey, J.W., Miu, M.H., Schmidt, E.K., Pierre, P. and Hornberger, T.A. (2011) Novel insights into the regulation of skeletal muscle protein synthesis as revealed by a new nonradioactive *in vivo* technique. *FASEB J.* **25**, 1028–1039 [CrossRef PubMed](#)
- 22 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685 [CrossRef PubMed](#)
- 23 Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2- $[\Delta\Delta Ct]$ method. *Methods* **25**, 402–408 [CrossRef PubMed](#)
- 24 Schakman, O., Gilson, H. and Thissen, J.P. (2008a) Mechanisms of glucocorticoid-induced myopathy. *J. Endocrinol.* **197**, 1–10 [CrossRef](#)
- 25 Schakman, O., Kalista, S., Bertrand, L., Lause, P., Verniers, J., Ketelslegers, J.M. and Thissen, J.P. (2008b) Role of Akt/GSK-3 β /beta-catenin transduction pathway in the muscle anti-atrophy action of insulin-like growth factor-I in glucocorticoid-treated rats. *Endocrinology* **149**, 3900–3908 [CrossRef](#)
- 26 Zheng, B., Ohkawa, S., Li, H., Roberts-Wilson, T.K. and Price, S.R. (2010) FOXO3a mediates signaling crosstalk that coordinates ubiquitin and atrogin-1/MAFbx expression during glucocorticoid-induced skeletal muscle atrophy. *FASEB J.* **24**, 2660–2669 [CrossRef PubMed](#)
- 27 Schmeizle, T. and Hall, M.N. (2000) TOR, a central controller of cell growth. *Cell* **103**, 253–262 [CrossRef PubMed](#)
- 28 Shah, O.J., Kimball, S.R. and Jefferson, L.S. (2000b) Glucocorticoids abate p70S6k and eIF4E function in L6 skeletal myoblasts. *Am. J. Physiol.* **279**, E74–E82
- 29 Shah, O.J., Anthony, J.C., Kimball, S.R. and Jefferson, L.S. (2000c) Glucocorticoids oppose translational control by leucine in skeletal muscle. *Am. J. Physiol.* **279**, E1185–E1190
- 30 Shah, O.J., Kimball, S.R. and Jefferson, L.S. (2000d) Among translational effectors, p70(S6k) is uniquely sensitive to inhibition by glucocorticoids. *Biochem. J.* **347**, 389–397 [CrossRef](#)
- 31 Long, W., Wei, L. and Barrett, E.J. (2001) Dexamethasone inhibits the stimulation of muscle protein synthesis and PHAS-I and p70S6-kinase phosphorylation. *Am. J. Physiol.* **280**, E570–E575
- 32 Bolster, D.R., Jefferson, L.S. and Kimball, S.R. (2004) Regulation of protein synthesis associated with skeletal muscle hypertrophy by insulin-, amino acid- and exercise-induced signaling. *Proc. Nutr. Soc.* **63**, 351–356 [CrossRef PubMed](#)
- 33 Deldicque, L., Theisen, D. and Francaux, M. (2005) Regulation of mTOR by amino acids and resistance exercise in skeletal muscle. *Eur. J. Appl. Physiol.* **94**, 1–10 [CrossRef PubMed](#)
- 34 Patti, M.E., Brambilla, E., Luzi, L., Landaker, E.J. and Kahn, C.R. (1998) Bidirectional modulation of insulin action by amino acids. *J. Clin. Invest.* **101**, 1519–1529 [CrossRef PubMed](#)
- 35 Xu, G., Kwon, G., Marshall, C.A., Lin, T.A., Lawrence, J.C. and McDaniel, M.L. (1998) Branched-chain amino acids are essential in the regulation of phasi and p70s6 kinase by pancreatic beta-cells- a possible role in protein translation and mitogenic signaling. *J. Biol. Chem.* **273**, 28178–28184 [CrossRef PubMed](#)
- 36 Kimball, S.R., Shantz, L.M., Horetsky, R.L. and Jefferson, L.S. (1999) Leucine regulates translation of specific mRNAs in L6 myoblasts through mTOR-mediated changes in availability of eIF4E and phosphorylation of ribosomal protein S6. *J. Biol. Chem.* **274**, 11647–11652 [CrossRef PubMed](#)
- 37 Anthony, J.C., Yoshizawa, F., Anthony, T.G., Vary, T.C., Jefferson, L.S. and Kimball, S.R. (2000) Leucine stimulates translation initiation in skeletal muscle of post absorptive rats via a rapamycin sensitive pathway. *J. Nutr.* **130**, 2413–2419 [PubMed](#)
- 38 O'Connor, P.M., Bush, J.A., Suryawan, A., Nguyen, H.V. and Davis, T.A. (2003) Insulin and amino acids independently stimulate skeletal muscle protein synthesis in neonatal pigs. *Am. J. Physiol.* **284**, E110–E119
- 39 Crozier, S.J., Kimball, S.R., Emmert, S.W., Anthony, J.C. and Jefferson, L.S. (2005) Oral leucine administration stimulates protein synthesis in rat skeletal muscle. *J. Nutr.* **135**, 376–382 [PubMed](#)
- 40 Shimizu, N., Yoshikawa, N., Ito, N., Maruyama, T., Suzuki, Y., Takeda, S., Nakae, J., Tagata, Y., Nishitani, S., Takehana, K. et al. (2011) Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle. *Cell Metab* **13**, 170–182 [CrossRef PubMed](#)
- 41 Herrlich, P. (2001) Cross-talk between glucocorticoid receptor and AP-1. *Oncogene* **20**, 2465–2475 [CrossRef PubMed](#)
- 42 Karin, M. and Chang, L. (2001) AP-1-glucocorticoid receptor crosstalk taken to a higher level. *J. Endocrinol.* **169**, 447–451 [CrossRef PubMed](#)
- 43 Hafezi-Moghadam, A., Simoncini, T., Yang, Z., Limbourg, F.P., Plumier, J.C., Rebsamen, M.C., Hsieh, C.M., Chui, D.S., Thomas, K.L., Prorock, A.J. et al. (2002) Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. *Nat. Med.* **8**, 473–479 [CrossRef PubMed](#)
- 44 Zhao, W., Qin, W., Pan, J., Wu, Y., Bauman, W.A. and Cardozo, C. (2009) Dependence of dexamethasone-induced Akt/FOXO1 signaling, upregulation of MAFbx, and protein catabolism upon the glucocorticoid receptor. *Biochem. Biophys. Res. Commun.* **378**, 668–672 [CrossRef PubMed](#)
- 45 Watson, M.L., Baehr, L.M., Reichardt, H.M., Tuckermann, J.P., Bodine, S.C. and Furlow, J.D. (2012) A cell-autonomous role for the glucocorticoid receptor in skeletal muscle atrophy induced by systemic glucocorticoid exposure. *Am. J. Physiol.* **302**, E1210–E1220
- 46 Gray, S., Wang, B., Orihuela, Y., Hong, E.G., Fisch, S., Haldar, S., Cline, G.W., Kim, J.K., Peroni, O.D., Kahn, B.B. and Jain, M.K. (2007) Regulation of gluconeogenesis by Krüppel-like factor 15. *Cell Metab.* **5**, 305–312 [CrossRef PubMed](#)
- 47 Findlay, G., Yan, L., Procter, J., Mieulet, V. and Lamb, R.F. (2007) A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling. *Biochem. J.* **403**, 13–20 [CrossRef PubMed](#)
- 48 Schriever, S.C., Deutsch, M.J., Adamski, J., Roscher, A.A. and Ensenauer, R. (2013) Cellular signaling of amino acids towards mTORC1 activation in impaired human leucine catabolism. *J. Nutr. Biochem.* **24**, 824–831 [CrossRef PubMed](#)
- 49 Long, X., Ortiz-Vega, S., Lin, Y. and Avruch, J. (2005) Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency. *J. Biol. Chem.* **280**, 23433–23436 [CrossRef PubMed](#)
- 50 Yan, Y., Flinn, R.J., Wu, H., Schnur, R.S. and Backer, J.M. (2009) hVps15, but Not Ca²⁺/CaM, is required for the activity and regulation of hvps34 in mammalian cells. *Biochem. J.* **417**, 747–755 [CrossRef PubMed](#)

- 51 Tee, A.R., Manning, B.D., Roux, P.P., Cantley, L.C. and Blenis, J. (2003) Tuberous sclerosis complex gene products, tuberin and hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr. Biol.* **13**, 1259–1268 [CrossRef PubMed](#)
- 52 Kim, J., Solis, R.S., Arias, E.B. and Cartee, G.D. (2004) Postcontraction insulin sensitivity: relationship with contraction protocol, glycogen concentration, and 5'AMP-activated protein kinase phosphorylation. *J. Appl. Physiol.* **96**, 575–583 [CrossRef PubMed](#)
- 53 Hawley, S.A., Pan, D.A., Mustard, K.J., Ross, L., Bain, J., Edelman, A.M., Frenguelli, B.G. and Hardie, D.G. (2005) Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab.* **2**, 9–19 [CrossRef PubMed](#)
- 54 Woods, A., Dickerson, K., Heath, R., Hong, S.P., Momcilovic, M., Johnstone, S.R., Carlson, M. and Carling, D. (2005) Ca^{2+} /calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metab.* **2**, 21–33 [CrossRef PubMed](#)
- 55 Bolster, D.R., Crozier, S.J., Kimball, S.R. and Jefferson, L.S. (2002) AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. *J. Biol. Chem.* **277**, 23977–23980 [CrossRef PubMed](#)
- 56 Lang, C.H., Frost, R.A., Deshpande, N., Kumar, V., Vary, T.C., Jefferson, L.S. and Kimball, S.R. (2003) Alcohol impairs leucine-mediated phosphorylation of 4E-BP1, S6K1, eIF4G, and mTOR in skeletal muscle. *Am. J. Physiol.* **285**, E1205–E1215 [CrossRef](#)

Received 2 April 2016/27 April 2016; accepted 28 April 2016

Version of Record published 28 April 2016, doi 10.1042/BSR20160096
