Regulation of Protein Synthesis by Leucine Starvation Involves Distinct Mechanisms in Mouse C2C12 Myoblasts and Myotubes1,2

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ABSTRACT Leucine modulates protein translation in higher eukaryotes by affecting phosphorylation and the function of proteins that regulate the initiation and/or elongation steps. These include the initiation factor 4E binding protein 1 (4E-BP1), initiation factor 4E (eIF4E), initiation factor 2 (eIF2α), ribosomal S6 kinases (S6K1/2), and elongation factor 2 (eEF2). The alteration of protein translation by leucine starvation was studied during myogenic differentiation using the mouse C2C12 cell line, as well as the role of rapamycin-sensitive mTOR (mammalian target of rapamycin) in the signaling of leucine in myotubes. A time course study showed that 1 h of leucine starvation decreased protein synthesis and S6K1 phosphorylation in myoblasts, whereas 3–5 h of starvation were necessary to induce such an alteration in myotubes. Although S6K1 phosphorylation was reduced in leucine-deprived myotubes, S6K2 and S6 phosphorylation were not affected. In contrast, rapamycin decreased the phosphorylation of S6K2 and S6 in myotubes. It is therefore likely that under the conditions present, the rapamycin-sensitive mTOR was not affected by leucine starvation. S6K1 dephosphorylation may thus be mTOR independent, and the functional mTOR/S6K2 pathway may maintain S6 phosphorylation. An increased phosphorylation of eEF2 in myoblasts and myotubes indicated that global protein synthesis was reduced via a decrease in translation elongation. An increased association between 4E-BP1 and eIF4E, and increased phosphorylation of eIF2α also contributed to decreasing protein synthesis in leucine-starved myoblasts. In contrast, in leucine-starved myotubes, there were no change in the 4E-BP1-eIF4E association or eIF2α phosphorylation, suggesting that these factors were not rate limiting for decreasing protein synthesis in leucine-deprived myotubes. J. Nutr. 136: 1466–1471, 2006.

KEY WORDS: • myogenic differentiation • leucine starvation • protein translation • mTOR • S6K1

Protein turnover in higher eukaryotes is essential for maintaining tissue integrity, growth, and functionality. Both protein synthesis and stability are highly regulated processes that are sensitive to environmental changes, especially variation of essential amino acids levels such as leucine. In response to amino acid deficiency, cells simultaneously decrease their rates of protein synthesis and increase their rates of protein degradation by the induction of macroautophagy (1,2).

Amino acids affect the activity and/or phosphorylation state of translation factors or regulatory proteins linked to the machinery of protein synthesis. Amino acid deficiency correlates with eukaryotic initiation factor (eIF)4 4E-binding protein (4E-BP1) dephosphorylation (3–8). A decrease in phosphorylated 4E-BP1 increases its binding capacity to eIF4E, leading to inhibition of the initiation complex eIF4F formation at the cap structure of mRNAs (9). Furthermore, acid starvation increases uncharged tRNA levels that bind to mGCN2 kinase, resulting in its activation. Active mGCN2 phosphorylates the α-subunit of eIF2 at Ser51, inducing the inhibition of exchange factor eIF2B activity and a defect in the association of (Met)-tRNA with the 40S ribosomal subunit (10). Amino acid deficiency also correlates with S6 kinase (S6K)1 and S6K2 decreased activity (3,4,6–8,11–14). S6K1 (15) and S6K2 (16–18) are 2 ribosomal S6 kinase homologs that exist as 2 isoforms generated from alternative translation start sites. S6K2 carries a nuclear localization signal at its C-terminus (16,19). S6K1 is mainly cytosolic and S6K2 mainly nuclear, but both shuttle between these 2 compartments (20,21).

S6K1 can regulate protein translation by the control of the capacity of peptide chain elongation. Indeed, S6K1 decreases eukaryotic elongation factor 2 kinase (eEF2k) activity by inducing its phosphorylation at Ser366 (22). S6K1 inactivation in response to amino acid starvation increases eEF2k activity and phosphorylation of eEF2 at Thr56, which inhibits the translocation step of peptide chain elongation (23). The relative inefficiency of eEF2 phosphorylation by S6K2 was reported (22). S6K1 could also regulate the translation initiation of specific mRNAs. Indeed, S6K1 was shown to be necessary for the translation of mRNAs that contain polyuridylic acid tracks at their translational start [5′ terminal oligopyrimidineline tract

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4 Abbreviations used: eIF, eukaryotic initiation factor; 4E-BP1, eIF4E-binding protein; eEF, eukaryotic elongation factor; ERK, extracellular-signal regulated kinase; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; S6K, S6 kinase; S6-P, phosphorylated ribosomal S6; TCA, trichloroacetic acid; TOP, terminal oligopyrimidineline tract.
(5′TOP) mRNAs (24), a family that includes transcripts for ribosomal proteins and elongation factors. Initial experiments indicated that S6K1 could act through the phosphorylation of S6, which then associates with 40S ribosomes and recognizes the TOP motif (25). This issue is controversial, with results showing that the translation of TOP mRNAs requires neither S6K1 activity nor S6 phosphorylation (26,27).

Resupplying amino acids reverses the decrease in 4E-BP1 phosphorylation and the decrease in S6K1 activity. The mammalian target of rapamycin (mTOR) is involved in the mediation of the amino acid effect because this effect is blocked by rapamycin, a specific inhibitor of the mTOR kinase (3,8,11,12). A nutrient-regulated complex composed of mTOR, raptor, and G8L (mLST8) was characterized (28–30). Poor nutrient conditions induce a stabilization of the raptor-mTOR association that decreases the mTOR kinase activity (29). S6 kinases contain a TOR signaling motif, necessary for their activation by mTOR (31), that binds raptor (32).

Skeletal muscle is very sensitive to circulating amino acid concentrations and represents a tissue in which leucine regulates S6K1 activity via the rapamycin-sensitive mTOR (1,8). Using the C2C12 muscle cell line, we showed previously that leucine starvation does not induce the dephosphorylation of ribosomal S6 protein in differentiated myotubes (33). Therefore, investigation of the rapamycin-sensitive mTOR and its downstream targets S6K1 and/or S6K2 in leucine-starved myotubes was warranted. This question was addressed in the context of a possible differential regulation of protein translation in response to leucine starvation between nondifferentiated and differentiated muscle cells.

MATERIALS AND METHODS

Materials. Primary and secondary antibodies were purchased from Cell Signaling Technology. Anti-S6K2 was kindly provided by Dr. Ivan Gout (Ludwig Institute for Cancer Research, London, UK). Anti-S6P (directed against the 5 phosphorylated Ser residues at the C-term of ribosomal S6 protein) was kindly provided by Dr. Morris J. Barnbaum (Departament of Medicine, University of Pennsylvania, Philadelphia, PA).

Cell culture and treatments. The C2C12 mouse muscle cell line was from the ATCC. Culture and differentiation of C2C12 cells were performed as previously described (33). Studies were either performed on myoblasts 24 h after initial plating (d 0, ~80% confluence) or during differentiation after 2 (d 2) or 5 (d 5) days. Cells were incubated with either complete DMEM/F12 (Sigma) (Leu+) or medium lacking leucine (Leu−) prepared from DMEM/F12 base (Sigma), supplemented with 5% dialyzed fetal calf serum (d 0), 5% dialyzed horse serum (d 2), and 2% dialyzed horse serum (d 5). Unless specified otherwise, myoblasts were leucine starved for 1 h and myotubes for 5 h. When indicated, rapamycin was included at a final concentration of 100 nmol/L.

35S] Methionine uptake and incorporation. Cells were cultured in 24-well plates (3 x 10^6 cells/cm²). During the last 12 min, L-[^35]S]Met was added (0.5 μCi/well). Cells were rinsed twice with cold PBS and precipitated at 4°C with 10% trichloroacetic acid (TCA). TCA-soluble radioactivity was counted using a TRI-CARB 2100TR (Packard). DNA and protein content were determined from the TCA-insoluble fraction as described in (34) and the radioactivity incorporated into protein was counted using a TRI-CARB 2100TR (Packard). Protein synthesis capacity was evaluated from the TCA-insoluble radioactivity incorporated/μg protein for each time unit. Total methionine uptake was calculated from the addition of TCA-soluble and -insoluble radioactivity/μg protein for each time unit. Results were expressed as the percentage modification in L-[^35]S]Met incorporation into proteins or L-[^35]S]Met uptake relative to 100% of the control. The values were also normalized to DNA content variation among d 0, 2, and 5.

Polysome profiles. For the polysome profiles, 11 mL of 15–45% linear sucrose gradients were prepared in polysome buffer (300 mmol/L KCl, 5 mmol/L MgCl2, 10 mmol/L HEPES, pH 7.4, 1 mmol/L dithiothreitol). Cells were scraped in polysome buffer containing 1% NP-40. The lysate was passed 3 times through a 26-gauge needle and centrifuged for 3 min at 12,000 × g. Equal amounts of lysate (1.2 mg) were loaded onto the gradients that were then centrifuged for 90 min at 4°C in a Beckman SW41 rotor at 185,000 × g. Gradients were collected and absorbance at 254 nm was monitored.

Protein extraction-immunoblotting. Cells were extracted as described (33). Equal amounts of protein were heated for 10 min at 90°C, separated on SDS-PAGE under reducing conditions (35), and transferred to Hybond P. The membranes were blocked (0.2% nonfat dry milk in PBS), exposed to specific antiserum, washed, and exposed to an alkaline phosphatase-conjugated anti-IgG. Western blots performed with antiphospho antibodies were usually stripped in 0.2 mol/L glycine, pH 2.2, for 1.5 h at room temperature and reprobed with the corresponding nonphospho-specific antibodies.

m7-GTP sepharose binding. The procedure used was essentially that described by Elledorova et al. (36). Starting material corresponded to 200 μg of total cell extracts incubated with 20 μL of m7-GTP Sepharose (Sigma). After washing, eluted proteins were detected by immunoblotting with anti-4E-BP1 and anti-eIF4E.

Quantification of immunoblots. Fluorescent AP-immunocomjugates were revealed using the ECF detection reagents (Amersham Life Science), detected using a Phosphor-Imager (Molecular Dynamics), and quantified using ImageQuant software. Data for phosphorylation, or expression, were expressed as a ratio to the total phosphorylation, or expression, of the protein in control cells.

Statistical analysis. Data were expressed as means ± SE and analyzed using Statview. Two-way repeated-measures ANOVA was used to compare the time course data of groups. Classification factors were differentiation level, time, and treatment. For significant F-values, the Student-Newman-Keuls multirange test was employed for post hoc analysis. Differences were considered significant when P < 0.05.

RESULTS

Effect of leucine starvation on protein synthesis during myogenic differentiation. Basal protein synthesis was first compared between C2C12 myoblasts (d 0), cells undergoing differentiation (d 2), and myotubes (d 5). L-[^35]S]Methionine uptake decreased during myogenic differentiation (~69 ± 3.8% P < 0.001 between d 0 and d 2 or 5), but incorporation into proteins was much more affected (~79 ± 4.3% P < 0.001 between d 0 and d 2 or 5) under the same experimental conditions, indicating that basal protein synthesis was reduced in myotubes compared with myoblasts (Supplemental Table1). Although basal protein synthesis was decreased, total protein content increased during myogenic differentiation (31 ± 3.5% between d 0 and 2 and 50 ± 4% between d 0 and 5, P < 0.005).

In C2C12 myoblasts, leucine starvation decreased L-[^35]S] methionine incorporation after 1 h (~21 ± 9%, P < 0.001). At d 2, a significant decrease in L-[^35]S]methionine incorporation was measured after 3 h of leucine starvation (~13 ± 7%, P < 0.001). In C2C12 myotubes at d 5, the leucine deprivation-induced decrease of L-[^35]S]methionine incorporation became significant after 3–5 h of deprivation, but always remained below the values measured in myoblasts (~18 ± 3%, P < 0.001 (Supplemental Table1). We verified that L-[^35]S]methionine uptake was not reduced by leucine starvation at a given stage. Thus, the decrease in protein synthesis in response to leucine deprivation was delayed during myogenic differentiation and less pronounced in myotubes compared with myoblasts.

Regulation of S6K1/2 and S6 by leucine starvation during myogenic differentiation. In C2C12 myoblasts at d 0, 1 h of leucine starvation decreased S6K1 phosphorylation at Thr389 (~62 ± 8%) and Thr421/Ser424 (~48 ± 7%) (Fig. 1A, d 0).
and S6 phosphorylation (−54 ± 5%) (Fig. 1B, d 0). The S6K1 protein level was not affected by leucine starvation at d 0 (Fig. 1A, d 0), but the S6 protein level was decreased (Fig. 1B, d 0). At d 2, 1 h of leucine starvation had no regulatory effects on S6K1 or S6, but after 3 h of deprivation, S6K1 phosphorylation at Thr389 and Thr421/Ser424 and S6/S6-P levels were decreased (Fig. 1A,B, d 2). In C2C12 myotubes at d 5, 1 or 3 h of leucine starvation did not affect S6K1, but after 5 h, S6K1 phosphorylation at Thr389, Thr421/Ser42 was decreased (Fig. 1E), whereas S6 or S6-P levels were still not affected (Fig. 1F). We showed previously a lack of S6-P regulation by leucine starvation in C2C12 myotubes during an 8-h time course study (33).

S6 phosphorylation was thus maintained in leucine-starved myotubes although S6K1 phosphorylation was decreased. More specifically, the phosphorylation of S6 on Ser 240/246, a residue whose phosphorylation is strictly regulated by S6K1 and S6K2 (37), was unchanged in leucine-starved myotubes but decreased in leucine-starved myoblasts (Fig. 2A). Phosphorylation of S6K2 was assayed in myotubes using a gel shift and was unaffected by leucine starvation (Fig. 2B). In contrast with leucine starvation, inhibition of mTOR with rapamycin in myotubes decreased S6K2 phosphorylation and that of S6 on Ser240/244 (Fig. 2A,B). Finally, Erk phosphorylation was assayed in leucine-starved myotubes because activation of this pathway positively regulates S6K2 (38–40). Erk phosphorylation was not increased after 3 or 5 h of leucine starvation (Fig. 2C).

Regulation of translation initiation and elongation by leucine starvation in leucine-starved myoblasts and myotubes. Polysome profiles were established to evaluate the effect of leucine starvation on translation initiation. In the basal state, polysome profiles (Fig. 3A,B, left panel) showed an increase in the monosomal peak heights (accumulation of 80S) and a decrease in the polysomal peak heights between myoblasts and myotubes, in agreement with the results of Smith et al. (41) and the decrease in protein synthesis measured during differentiation. Leucine starvation for 1 h in myoblasts at d 0 increased the 80S peak and decreased polysome peak heights (Fig. 3A). In myotubes at d 5, 5 h of leucine starvation, although decreasing global protein synthesis as determined by L-[35S]methionine incorporation, did not affect the 80S peak, consistent with a lack of translation initiation block (Fig. 3B). In contrast with leucine starvation, treatment of myotubes with rapamycin induced an increase of the 80S peak on the polysome profile (Fig. 3B).

The regulation of eIF4E availability and eIF2α phosphorylation, which are both involved in translation initiation regulation, was then studied. Leucine starvation for 1 h in myoblasts at d 0 increased the fraction of 4E-BP1 bound to eIF4E (40 ± 10%), indicative of a reduction in eIF4E availability to form the eIF4E-eIF4G complex. In myotubes, the amount of 4E-BP1 bound to eIF4E did not increase after 5 h of leucine starvation (Fig. 4A). eIF2α phosphorylation at Ser51 increased over a 5-h time course in myoblasts, whereas it was not regulated in myotubes during the same time course study (Fig. 4B). The latter result was not due to a lack of the eIF2α kinase mGCN2.

![Figure 1](https://academic.oup.com/jn/article-abstract/136/6/1466/4664319/data1.png)

**Figure 1** Regulation of S6K1 and S6 by leucine starvation during myogenic differentiation. (A) Western blot analysis using anti-S6K1 directed against Thr421/Ser424 or Thr389 or directed against total protein. (B) Western blot analysis using antiphospho ribosomal S6 antibody (S6-P) or total protein (S6). Ratios to the total phosphorylation or expression of the proteins (S6K1 and S6) in complete DMEM/F12 are indicated. Values are means ± SEM, n = 4. *Different from Leu +, P < 0.05.

![Figure 2](https://academic.oup.com/jn/article-abstract/136/6/1466/4664319/data2.png)

**Figure 2** Regulation of S6 Ser240/Ser244 (A), S6K2(B), and Erk (C) phosphorylation. (A) Ratios to the total phosphorylation of S6 at Ser240/244 in complete DMEM/F12 are indicated. Values are means ± SEM, n = 4. *Different from Leu +, P < 0.05. (B) Arrows indicate the distinct electrophoretic forms of S6K2.

![Figure 3](https://academic.oup.com/jn/article-abstract/136/6/1466/4664319/data3.png)

**Figure 3** Effect of leucine starvation on polysome profile in myoblasts (A) and myotubes (B). The arrow shows the 80S peak. Representative polysome profiles of at least 3 independent experiments are shown.
which was still expressed in myotubes, although at a lower level compared with myoblasts (Fig. 4C). Finally, the phosphorylation of eEF2 was investigated as an index of the capacity of translation elongation. Leucine starvation increased phosphorylation of eEF2 at Thr56 (40 ± 6%) in both myoblasts and myotubes (Fig. 5).

DISCUSSION

In skeletal muscle (8) or various cell lines (3,4,6,7,11,12), the administration of leucine after fasting or amino acid starvation stimulates protein synthesis, and promotes phosphorylation and activation of S6K1 via the rapamycin-sensitive mTOR. Our previous work (33) showed a constitutive phos-
was sufficient to measure a significant decrease of protein translation and to induce the maximal decrease of S6K1 phosphorylation at Thr389 and Thr421/Ser424. During the myogenic process, longer starvation times are necessary to obtain these responses (~3 h at d 2, 3–5 h at d 5 in myotubes). We have evidence that the differential cellular response to leucine starvation between myoblasts and myotubes may be limited to some adaptation processes. For example, the kinetics and intensity of the leucine starvation–induced increase in protein breakdown are the same in myoblasts and myotubes [(33) and Supplemental Table 2]. Basal protein synthesis was decreased during myogenic differentiation, and protein content was increased due to the accumulation of muscle-specific proteins. At least 2 phenomena contributed to the decrease in basal translation, i.e., the serum depletion that is necessary to induce differentiation and the longer half-lives of muscle-specific proteins. We hypothesized that protein breakdown supplies enough intracellular leucine to support basal protein synthesis during the first hours of leucine starvation in myoblasts.

The fall in the protein synthesis rate together with the unchanged protein degradation rate during the transition from myoblasts to myotubes (Supplemental Tables 1 and 2) also suggests an increase in intracellular leucine that could make myoblasts relatively resistant to leucine deprivation compared with myoblasts. Beugnet et al. (46) showed that an elevation of intracellular amino acid induced by protein synthesis inhibitors allowed CHO-T cells and provided evidence that it is intracellular amino acid levels that regulate mTOR activity. Kobayashi et al. (47) showed a reduction in muscle protein synthesis in response to a hemodilution-induced decrease in plasma amino acids and provided evidence that the signal for change in synthesis appeared to be changes in the plasma amino acid concentrations. It is thus conceivable that elevated intracellular leucine in myotubes could prevent a fall in mTOR activity and that the variation in intracellular concentration could mediate the effect of leucine on protein translation in myotubes by mTOR-independent mechanisms.

The decrease in protein synthesis in leucine-starved myoblasts and myotubes involves distinct mechanisms. Even though there was an increase in eIF2 phosphorylation indicative of a reduction in translation-elongation in both cell types, decreased eIF4E availability and elongation and decreased S6 phosphorylation, which are all involved in the regulation of translation initiation, were observed only in leucine-starved myoblasts. The latter results suggested a blockage of translation initiation only in leucine-starved myoblasts. Polysome analysis showing a shift from polysomes to 80S monomers in leucine-starved myoblasts was indeed consistent with a block of translation initiation in this cell type. In leucine-starved myoblasts, the polysome profile was unaffected. We postulate that both initiation and elongation are equally affected. Indeed, if elongation alone was affected, then a decrease in 80S monosomes together with an aggregation of polysomes would be seen. Thus, it is likely that alterations in eIF4E availability and eIF2α phosphorylation are not rate controlling for global protein synthesis in leucine-starved myoblasts and that leucine deprivation in this cell type affects the function of another protein involved in the regulation of translation initiation. Kobayashi et al. (47) showed that a 40% decrease in plasma amino acid concentration decreased muscle protein synthesis, and this was accompanied by a reduction in the guanine nucleotide exchange activity of eIF2B. This factor, whose activity is controlled via several signal-transduction pathways (48), could be involved in the mediation of leucine starvation effects in myoblasts.

In conclusion, this work establishes key points relevant to a differential adaptation to leucine starvation between C2C12 myoblasts and myotubes associated with the regulation of protein synthesis. Under the conditions used here, there was a lack of regulation of the rapamycin-sensitive mTOR in response to leucine starvation in C2C12 myotubes, suggesting that the regulation of S6K1 phosphorylation may be mTOR independent. In contrast to myotubes, eIF4E availability together with eIF2α phosphorylation are not controlled steps for decreasing global protein synthesis in leucine-starved myoblasts.

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**LITERATURE CITED**


