RESEARCH ARTICLE

# Leucine induces myofibrillar protein accretion in cultured skeletal muscle through mTOR dependent and -independent control of myosin heavy chain mRNA levels

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**Scope:** Nutritional intervention during muscle wasting aims to attenuate net muscle protein loss. Branched chain amino acids, especially leucine, are able to stimulate the anabolic mammalian target of rapamycin (mTOR) signalling cascade and protein synthesis. It has been suggested that muscle myofibrillar protein expression is more responsive to amino acid supplementation compared to cytoplasmic proteins, although accretion of myofibrillar proteins has not extensively been investigated. We hypothesized that leucine specifically increases myofibrillar protein synthesis in skeletal muscle.

**Methods and results:** This hypothesis was investigated in C2C12 skeletal muscle cells using physiologically relevant culture conditions. Leucine supplementation specifically increased myofibrillar protein accretion, including myosin heavy chain-slow and -fast and myosin light chain 1 and -3 in C2C12 cells. Neither total protein content, nor de novo protein synthesis was affected, despite leucine-induced increased 4E-BP1 and S6K1 phosphorylation. Leucine supplementation did not affect myogenesis, measured by creatine kinase activity and myoblast fusion, either. Remarkably, leucine-induced increased myofibrillar protein accretion was accompanied by elevated MyHC mRNA levels, which involved mTOR-dependent and -independent regulation of *MyHC-4* and *MyHC-7* gene-expression, respectively.

**Conclusion:** This study clearly demonstrates myofibrillar and not generic protein accretion in skeletal muscle following leucine supplementation, and suggests this involves pre-translational control of MyHC expression by leucine.

**Keywords:**
Amino acids / Hypertrophy / Myosin / Protein accretion

## 1 Introduction

Preservation of skeletal muscle mass is instrumental to maintain physical fitness and quality of life during aging and in disease. Muscle mass homeostasis is determined by the balance between muscle protein synthesis and degradation. Muscle protein synthesis can be stimulated with physiological or pharmacological agents. Physiological anabolic stimuli like the branched chain amino acids (BCAA) and leucine particularly utilize a signalling route which overlaps with the distal part of the insulin like growth factor I (IGF-I) signalling

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**Abbreviations: AA**, amino acids; **BCAA**, branched chain amino acids; **DM**, differentiation medium; **4E-BP1**, eukaryotic translation initiation factor 4E binding protein 1; **GM**, growth medium; **mTOR**, mammalian target of rapamycin; **MCK**, muscle creatine kinase; **MyHC**, myosin heavy chain; **S6K1**, p70 ribosomal S6 kinase 1

 

pathway, namely the mammalian target of rapamycin (mTOR) cascade (mTORC1) [1].

Downstream targets of mTORC1 eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and p70 ribosomal S6 kinase 1 (S6K1) control translation initiation and translation capacity, thereby mainly determining protein synthesis rate. P-4E-BP1 and p-S6K1 are generally measured as markers of mTORC1 activity and increased p-4E-BP1 and p-S6K1 have not only been described in tissue culture and rodent models [1–3], but in studies using human subjects as well. Liu et al. demonstrated increased S6K1 and 4E-BP1 phosphorylation in muscle biopsies of healthy subject after short term BCAA infusion [4]. Leucine infusion alone also increases S6K1 phosphorylation in human skeletal muscle [5]. Although these studies suggested increased protein synthesis in response to leucine, net protein accretion was not measured in these studies.

Conversely, Glynn et al. [6] recently demonstrated that essential amino acids ingestion increases muscle protein synthesis, without improving net protein balance, despite a leucine dose-dependent raise in mTORC1 signalling. Therefore, it is still not proven that activation of the mTORC1 pathway by leucine enhances general muscle protein accretion. Interestingly, it has been suggested that compared to cytoplasmic protein concentrations, myofibrillar protein levels are more responsive to amino acids (AA) and feeding [7, 8]. Myofibrillar proteins complex constituents include the myosin heavy and light chains (MyHCs and MyLCs, respectively). Depending on the contractile and metabolic properties of muscle fibers specific myosin isoforms are expressed, e.g. *MyHC-4* in fast-twitch, glycolytic fibers, whereas *MyHC-7* is expressed in slow-twitch muscle fibers characterized by oxidative metabolism. However, the molecular basis for a preferential response of myofibrillar protein synthesis to AA stimulation remains to be elucidated.

The C2C12 muscle cell line has been extensively applied to study mechanisms of protein turnover. However, little attention has been paid to ascertain that culture media approach physiological concentrations of nutrients. Furthermore, it has recently been demonstrated that leucine and glutamine have antagonistic effects on mTOR phosphorylation and on the downstream targets 4E-BP1 and S6K1 [2]. This raises the question whether the routinely used culture conditions are optimal or even suitable to investigate the anabolic properties of AA, as it may hamper the translation to the *in vivo* relevance of such findings.

In this study we hypothesized that leucine supplementation specifically increases myofibrillar protein content in skeletal muscle. In cultured C2C12 skeletal muscle cells using physiologically relevant culture conditions we demonstrate that leucine supplementation specifically induced accretion of the myofibrillar proteins MyHC-slow and -fast and MyLC-1 and -3 in C2C12 cells. Neither total protein content, nor de novo protein synthesis was affected, despite increased 4E-BP1 and S6K1 phosphorylation after leucine stimulation. Leucine supplementation did not affect myogenesis, measured by creatine kinase activity and myoblast fusion, either. Remarkably, myofibrillar protein accretion was accompanied by increased MyHC mRNA levels in response to leucine, which involved mTOR-dependent and -independent regulation of *MyHC-4* and *MyHC-7* gene-expression, respectively.

## 2 Materials and methods

### 2.1 Cell culture

The murine skeletal muscle cell line C2C12 obtained from the American Type Culture Collection (ATCC number CRL1772), was cultured in growth medium (GM), composed of DMEM (Dulbecco's modified Eagle medium) containing antibiotics (50 units/mL penicillin and 50 μg/mL streptomycin, all from Invitrogen, Grand Island, NY), 9% (v/v), fetal bovine serum (FBS) (European Union approved, PAA, Cölbe, Germany) and 1 g/L glucose, unless stated otherwise. Differentiation medium (DM) was prepared from DMEM diluted with HBSS (Invitrogen, Grand Island, NY) supplemented with $NaHCO_3$ (Sigma-Aldrich, Saint Louis, MO) (from here on called HBSS+) to yield culture media of similar composition containing 5–100% of AA levels supplemented 1% heat-inactivated FBS and antibiotics. Physiological levels of AA were reached at ∼15% of unmodified medium. Cells were grown on matrigel (BD Biosciences, Bedford, MA) coated dishes (1:50 in DMEM). Cells were plated at $10^4/cm^2$ and cultured in GM for 24 h, before induction of differentiation. When applicable, leucine (Sigma-Aldrich), IGF-I (Sigma-Aldrich) or insulin (Sigma-Aldrich) were added directly after induction of differentiation and again 24 h thereafter when the cells were provided with fresh DM. During signalling studies leucine, IGF-I, rapamycin (Sigma-Aldrich) and PP242 (Sigma-Aldrich) were dissolved in HBSS+ and added after 3 days of differentiation. There were no additives, such as streptomycin, in the HBSS+ to rule out any unknown effects on the investigated mechanisms.

### 2.2 Muscle creatine kinase activity

Biochemical myogenic differentiation was assessed via determination of muscle creatine kinase (MCK) activity. After treatments, cells were washed twice in cold PBS, lysed in 0.5% Triton/PBS, and scraped off the dish with a rubber policeman. Whole cell lysates were centrifuged for 2 min at 16 000 × *g*, and the supernatant was stored in separate aliquots at −20°C for determination of total soluble protein content or MCK activity. MCK activity was measured using a spectrophotometry-based kit from Sigma Diagnostics (St. Louis, MO). Total soluble protein content was assessed using the BCA kit according to manufacturer's protocol (Pierce).

### 2.3 Western blot analysis

Protein abundance of myosin heavy chain fast (MyHC-f), MyHC-slow (MyHC-sl), myosin light chain 1 (MyLC-1),

 

**Table 1.** Amino acid concentrations in 100% DMEM

| Amino acid | Concentration (μM) |
|---|---|
| Glycine | 400 |
| Arginine hydrochloride | 398 |
| Cystine 2HCl | 201 |
| Glutamine | 3970 |
| Histidine hydrochloride-$H_2O$ | 200 |
| Isoleucine | 802 |
| Leucine | 802 |
| Lysine hydrochloride | 798 |
| Methionine | 200 |
| Phenylalanine | 400 |
| Serine | 400 |
| Threonine | 798 |
| Tryptophan | 784 |
| Tyrosine | 398 |
| Valine | 803 |

MyLC-3, p-mTOR, p-4E-BP1 and p-S6K1 was evaluated using Western blot analysis. Adherent cells were washed in PBS, and whole cell lysates were prepared by the addition of lysis buffer (20 mM Tris, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM DTT, 1 mM $Na_3VO_4$, 1 mM PMSF, 10 μg/mL leupeptin, and 1% (v/v) aprotinin). Whole cell lysates were incubated on ice for 30 min, followed by 30 min of centrifugation at 16 000 × *g*. A aliquot of the supernatant was saved for soluble protein determination, and 4× Laemmli sample buffer (0.25 M Tris·HCl, pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 0.4 M DTT, and 0.04% (w/v) bromphenol blue) was added to the supernatant, followed by boiling of the samples for 5 min and storage at −20°C. Total soluble protein was assessed using the BCA kit (Pierce, Rockford, IL) according to the manufacturer's instructions, and equal amounts of protein were loaded per lane and separated on a SDS-page gel or on a criterion gel (4–12% Bis-Tris, Bio-Rad) in XT MOPS Running Buffer (Bio-Rad), followed by transfer to a 0.45-μm nitrocellulose membrane (Bio-Rad) by electro-blotting. The membrane was blocked for 1 h at room temperature in 5% (w/v) non-fat dry milk. Nitrocellulose blots were washed in PBS-Tween20 (0.05% v/v), followed by overnight incubation (4°C) with a primary antibody; MyHC-f (M4276, Sigma-Aldrich, Saint Louis, MO), MyHC-sl (M8421, Sigma-Aldrich), MyLC 1 and 3 (F310, MyLC1/3 Developmental Studies Hybridoma Bank, Iowa City, IA), p-mTOR Ser2448 (2971, Cell Signalling, Danvers, MA), total mTOR (2983, Cell Signalling), p-4E-BP1 Thr37/46 (9459, Cell Signalling), total 4E-BP1 (9452, Cell Signalling), p-S6K1 Thr389 (9206, Cell Signalling) or total S6K1 (2708, Cell signalling). After three wash steps of 20 min each, the blots were probed with a peroxidase-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) and visualized using SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Images were obtained on a molecular imager (ChemiDoc XRS, Bio-Rad) and total band intensity was quantified with Quantity One 4.5.0 software.

### 2.4 $^{35}$S-methionine incorporation assay

Three day differentiated myoblasts were maintained under serum and AA free conditions for 6 h prior to administration of $^{35}$S-methionine (>600 Ci/mmol, Perkin Elmer, Groningen, The Netherlands) supplemented medium and the appropriate stimuli. After 3 h cells were washed twice with cold PBS and lysed with 0.5% Triton/PBS. Protein in cell lysates was precipitated o/n at 4°C with 20% trichloroacetic acid. Proteins were spun down at 4°C for 5 min at 14 000 rpm. Pellets were washed twice with 5% trichloroacetic acid. Proteins were dissolved in 0.3 M NaOH after which 1.5 M HCl was added. The protein suspension was diluted in 4 mL scintillating liquid (Opti-Fluor, Perkin Elmer) and incorporated $^{35}$S-methionine was measured in a scintillation counter (Wallac 1414, Perkin Elmer).

### 2.5 RNA isolation, cDNA synthesis and Q-PCR analysis

Total RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform extraction method (Ambion LtD, Ijssel, The Netherlands). cDNA was made with the Transcription First Strand cDNA Synthesis Kit (Roche, Almere, The Netherlands). Transcription levels of *MyHC-7*, *MyHC-4*, *MuRF1* and *Atrogin-1* (Table 2: primer information) were determined on the Bio-Rad iCycler apparatus (Biorad, Hercules, CA) using a two-step PCR program followed by a melting curve (performed by heating from 60°C to 95°C in increments of 0.5°C). Gene expression levels were derived from the standard curve based on the Ct values with the MyiQ analysis software (Biorad) and normalized to the geometric average of four out of five reference genes (*β-actin, GAPDH, tubulin, calnexin and cyclophilin A*) by the geNorm software [9].

**Table 2.** Q-PCR primer information

| GENE | Forward primer | Reverse primer |
|---|---|---|
| Myosin heavy chain 7 | 5′-CCAAGGGCCTGAATGAGGAG-3′ | 5′-GAGTCTGGACCTCGGAAACG-3′ |
| Myosin heavy chain 4 | 5′-ACAAGCTGCGGGTGAAGAGC-3′ | 5′-GCAAGAAACAGTGACAGGAC-3′ |
| *murf1* XM_485456 | 5′-CTTCCTCTCAAGTGCCAAGCA-3′ | 5′-TACCTGATGAAATGAGAGAATCTTGTG-3′ |
| *atrogin-1* | 5′-CAGCAGCTGAATAGCATCCAGAT-3′ | 5′-CGAATGTTGACTTGTAGTACGTCT-3′ |

 

### 2.6 Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons among experimental groups were performed by ANOVA and a $p$-value $\leq 0.05$ was considered to be statistically significant. Data are representative for at least three individual experiments.

## 3 Results

### 3.1 Myotube formation but not total protein levels or creatine kinase activity are affected by amino acid concentrations in differentiation medium

In order to answer our research question we first investigated the influence of AA concentrations in tissue culture medium on myotube formation, since standard DMEM contains supra-physiological AA levels [1011], which may affect the differentiation process. C2C12 cells were differentiated for 5 days in culture media that had a lower AA content compared to control medium. The AA concentrations in standard DMEM are stated in Table 1. The contribution of 1% FBS to AA levels in standard DM is <1% of total AA levels in standard DM. Physiological concentrations of AA corresponded to conditions containing ~15% of the AA concentration present in standard medium. Figure 1A shows that myotube formation was sub-optimal in standard medium and improved when the levels of AA were lowered to more physiological concentrations. As myogenesis is accompanied by protein accretion and the induction of muscle specific protein expression [12], total protein content and MCK activity were determined. Neither total protein (Fig. 1B) nor MCK activity (Fig. 1C) were increased in muscle cells following differentiation for 5 days in medium containing decreased AA concentrations compared to standard medium.

### 3.2 Physiological culture conditions support the anabolic effects of insulin-like growth factor

To assure that an AA content of 10% AA in DM is still sufficient to facilitate anabolic responses, C2C12 cells were supplemented with IGF-I. Data depicted in Fig. 1 show that total protein content (D) and MCK activity (E) increased in IGF-I supplemented myoblasts differentiating in medium containing 4.5 g/L glucose and 10% AA. This indicates that 10% AA in DMEM is adequate to facilitate IGF-I (5 nM) mediated accretion of total and MCK protein – a marker of myogenesis – in C2C12 myoblasts. A glucose concentration of 4.5 g/L is however considered hyperglycaemic. C2C12 cells were differentiated in the presence of IGF-I in medium containing 1 g/L glucose and 10% AA to assure that a more physiological relevant glucose concentration of 1 g/L still allows detection of anabolic effects. Decreasing the concentration of glucose did not affect the anabolic response to IGF-I, measured by total protein levels (Fig. 1D) nor did it affect differentiation, measured by CK activity (Fig. 1E).

These physiologically more relevant culture conditions eliminate the possibility of masking effects of anabolic components in general and leucine in particular, caused by an excess amount of BCAA or hyperglycemic levels of glucose already present in culture media. Furthermore, the concentration of glutamine in particular is in the physiological range, minimizing potential inhibitory effects of glutamine on anabolic signalling in response to leucine [2].

### 3.3 Myosin heavy chain slow and -fast and myosin light chain 1 and -3 content are increased in myotubes after leucine stimulation

Following optimization of our culture protocol, we investigated the protein levels of muscle specific proteins MyHC-sl and MyHC-f in C2C12 cells differentiated in the presence of additional leucine (Fig. 2A). A clear dose-dependent increase in MyHC-sl (Fig. 2B) and MyHC-f protein (Fig. 2C) expression after leucine supplementation was observed. In addition to MyHC-sl, MyHC-f, MyLC-1 (Fig. 2D) and MyLC-3 (Fig. 2E) protein abundance were increased in a dose responsive manner after leucine supplementation.

### 3.4 Myofibrillar protein accretion in response to leucine is observed in presence of physiological amino acid concentrations

To address the importance of physiological relevant culture conditions, MyHC-sl, MyHC-f, MyLC-1 and MyLC-3 content was determined following leucine supplementation and compared between myotubes differentiated in the presence of 10% AA in DM and myotubes differentiated in standard DM containing 100% AA. Figure 2B–E (right side) clearly shows that in contrast to our optimized conditions, leucine does not increase, or even decreases myofibrillar protein content of myotubes differentiated in medium with supra-physiological AA concentrations compared to supra-physiological control conditions.

### 3.5 Leucine does not increase total protein accretion or de novo protein synthesis, despite mTOR, 4E-BP1 and S6K1 phosphorylation

To address whether leucine supplementation caused a generic anabolic response, total protein levels and *de novo* protein synthesis were determined. Although IGF-I significantly increased total protein content (Fig. 3A) and acute *de novo* protein synthesis (Fig. 3B), this was not observed for leucine. To confirm the previously reported ability of leucine to activate the mTOR signalling pathway, short-term stimulation

 

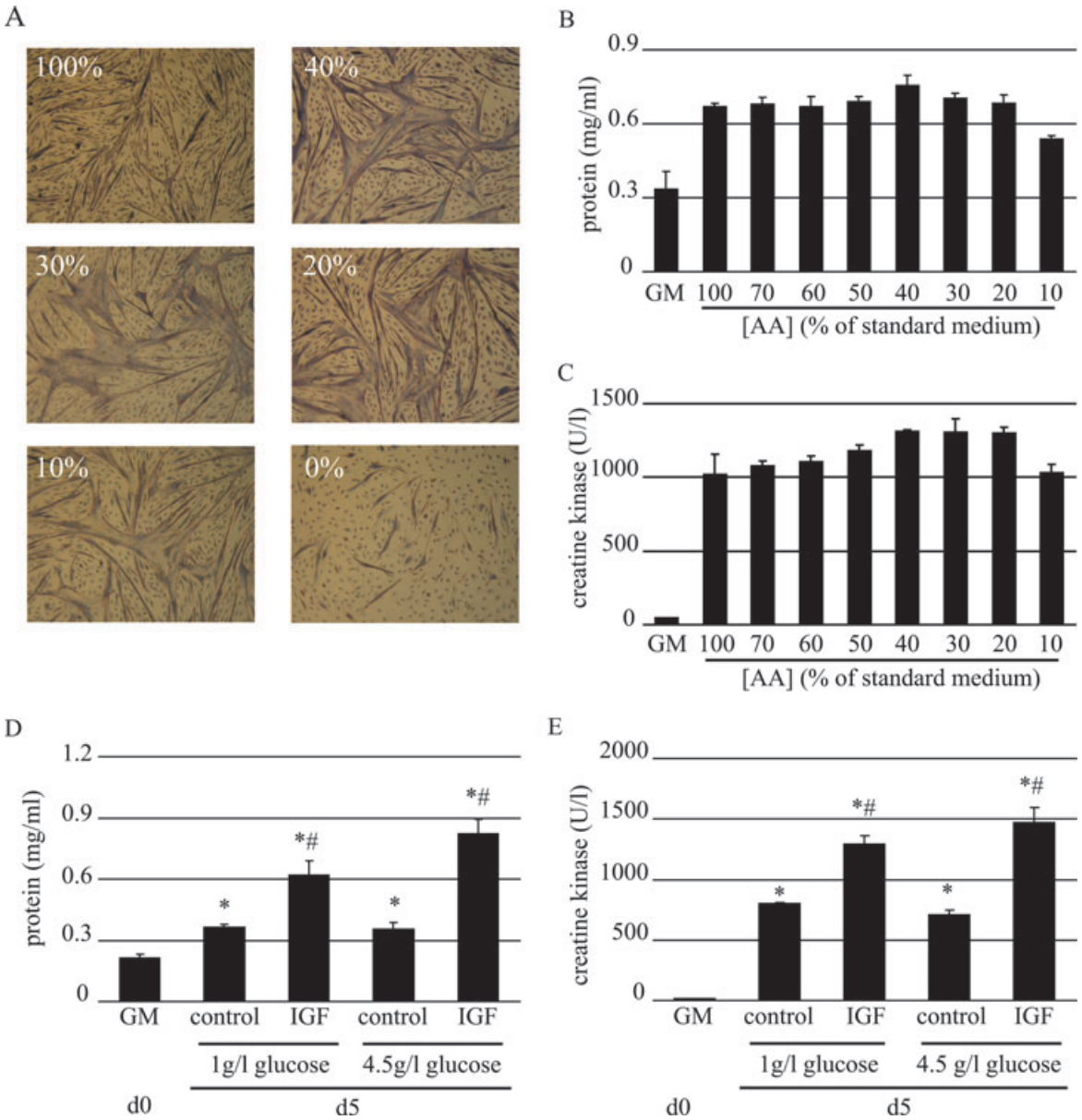


**Figure 1.** Myotube formation but not total protein levels or creatine kinase activity are affected by amino acid concentrations in differentiation medium. (A) C2C12 cells were 5 days differentiated in standard differentiation medium (100% DMEM + 1% FBS) containing supra-physiological AA concentrations or differentiation medium (DM) containing decreasing levels of amino acids and stained with May-Grünwald-Giemsa. Cells were differentiated in DM containing decreasing levels of AA. Total cell protein content (B) and MCK (C) were determined after 5 days of differentiation in cell lysates. * = Significant from 100% DMEM. C2C12 cells were differentiated for 5 days in 10% AA in DM containing 4.5 g/L or 1 g/L glucose in the absence or presence of 5 nM IGF-I. Medium was refreshed 24 h after induction of differentiation and every 48 h there after. Differentiation in DM containing 1 g/L glucose did not alter (D) total protein content or (E) MCK activity compared to differentiation in DM containing 4.5 g/L glucose. * Significant from GM, # Significant from respective control.

experiments were performed on 3-day differentiated myoblasts cultured with the adapted culture routine. This time point was chosen, since it is half way the differentiation process leading to increased myosin content. In addition, the phosphorylation of the investigated AA-residues of 4E-BP1 and S6K1 are crucial activation steps necessary for downstream event [13–15]. Stimulation with leucine increased phosphorylation of mTOR and downstream targets 4E-BP1 and S6K1 as depicted in Fig. 3C. Representative blots are shown in the right panel of Fig. 3C. A low, by itself non-anabolic concentration of insulin may be required to facilitate anabolic effects of leucine. Non- or slightly anabolic doses of

 

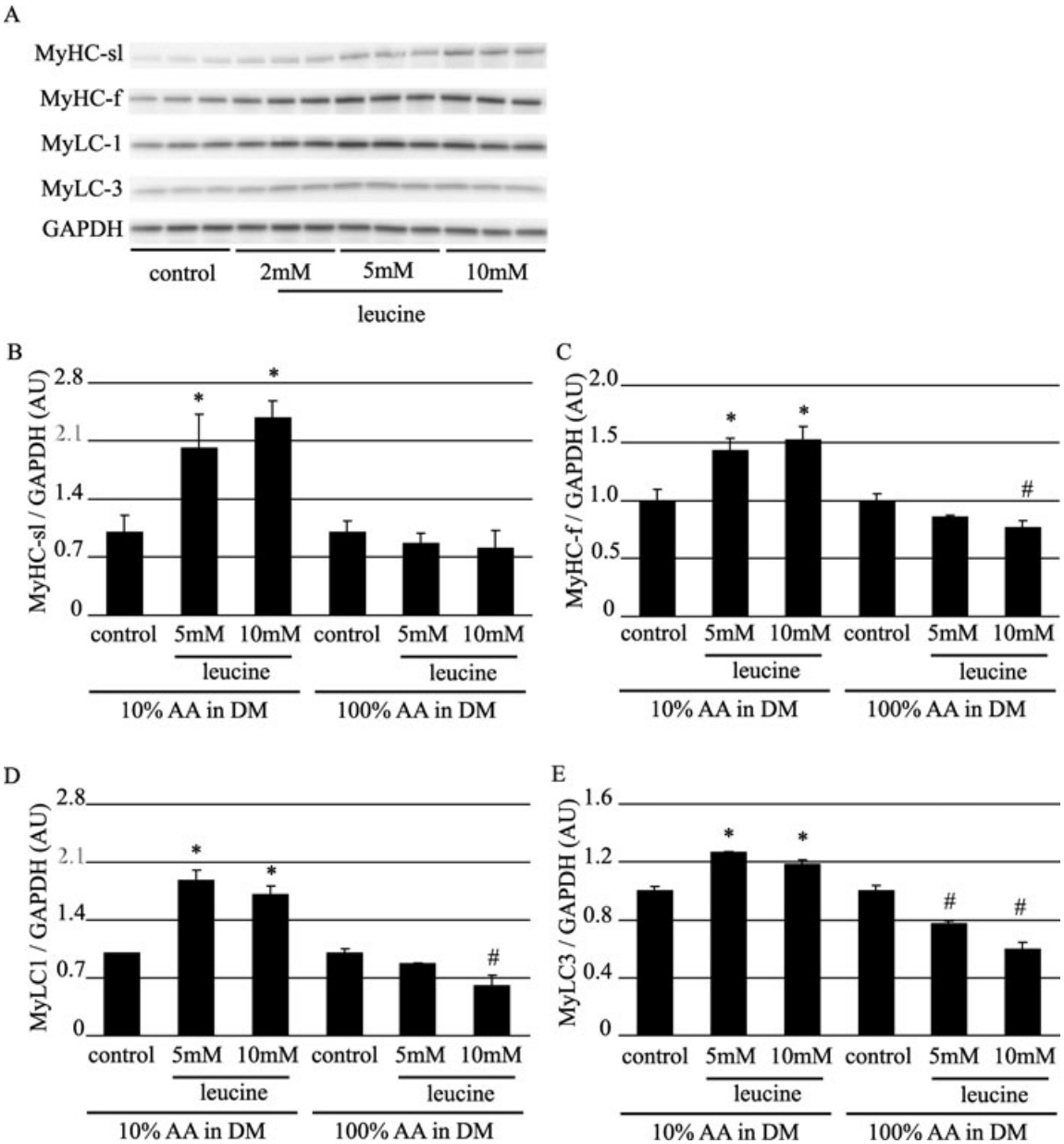


**Figure 2.** Leucine only increases myosin heavy chain and myosin light chain content under physiological amino acid conditions. C2C12 cells were differentiated for 5 days in 10% AA in DM or 100% AA in DM in the absence or presence of 5 or 10 mM leucine. Medium and leucine were refreshed every 48 h. Whole cell lysates were prepared and protein content was determined. Equal amounts of protein were loaded. (A) MyHC-sl, MyHC-f, MyLC-1 and MyLC-3 levels showed a dose dependent increase after leucine supplementation. Representative blots of myofibrillar proteins. (B) MyHC-sl, (C) MyHC-f, (D) MyLC1 and (E) MyLC3 levels were determined and normalized for GAPDH levels. MyHC-sl, MyHC-f, MyLC1 and MyLC3 increased after leucine stimulation in the presence of 10% AA in DM. Leucine supplementation in 100% AA in DM demonstrated a decrease in MyHC-f, MyLC1 and MyLC3 content. * Significant from 10% AA in DM control. # Significant from 100% AA in DM control.

insulin, determined in a titration experiment, did not lead to increased total cell protein content when combined with leucine either (data not shown). Altogether, these data suggest that myofibrillar protein accretion in response to leucine is not a consequence of a generic anabolic response.

### 3.6 Leucine does neither increase myogenesis nor mediators of proteolysis

To investigate whether stimulation of MyHC and MyLC content was explained by improved myogenesis in general, MCK

 

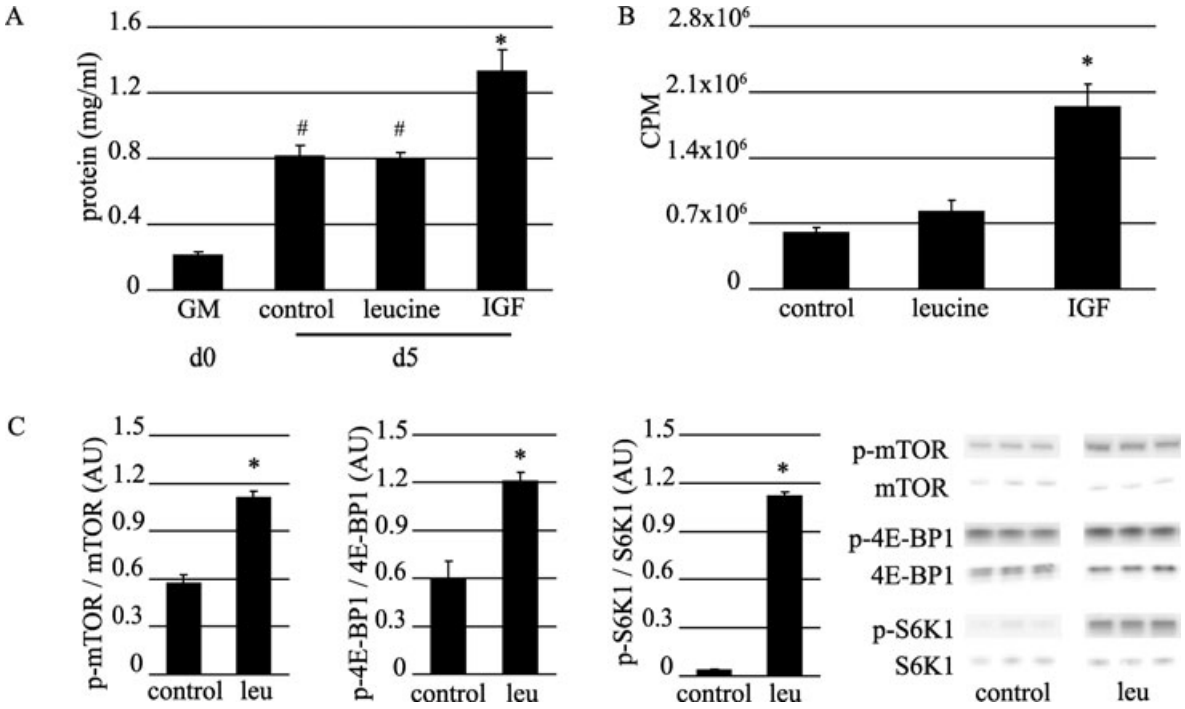


**Figure 3.** Leucine does not increase total protein accretion or de novo protein synthesis, despite mTOR, 4E-BP1 and S6K1 phosphorylation. (A) C2C12 cells were differentiated for 5 days in 10% AA in DM in the absence or presence of 5 mM leucine. Medium and leucine were refreshed every 48 h. Cells were lysed and total protein content was determined. Leucine did not increase total cell protein content of C2C12 cells. (B) C2C12 cells were AA and growth factor starved for 6 h in HBSS+ after 3 days of differentiation in 10% AA in DM, followed by stimulation in $^{35}$S-methionine supplemented 10% AA in DM for 3 h. Total de novo protein synthesis was determined in whole cell protein precipitates, measured by $^{35}$S-methionine incorporation. $^{35}$S-methionine incorporation was only significantly increased by IGF-I stimulation. C2C12 cells were AA and growth factor starved for 6 h in HBSS+ to reduce AA induced phosphorylation events [27, 28] after 3 days of differentiation in 10% AA in DM, followed by 30 min leucine (5 mM) stimulation. Whole cell lysates were prepared and protein content was determined. Equal amounts of protein were loaded and (C) mTOR (Ser2448), 4E-BP1 (Thr37/46) and S6K1 (Thr389) phosphorylation levels were determined and normalized for total mTOR, 4E-BP1 and S6K1 levels. Representative blots are shown on the right. * Significant from control. # Significant from Growth Medium (GM).

activity was measured in myotubes that had differentiated for 5 days in the presence of supplemented leucine. An increase in MCK activity could not be measured in the leucine supplemented myotubes compared to control (Fig. 4A). Myoblast fusion was not affected (Fig. 4B) either after 5 days of leucine supplementation. Protein accretion is the net result of protein synthesis relative to protein degradation. The E3-ligase MuRF1 has been described to specifically target myofibrillar proteins for degradation via de ubiquitin-proteasome system [16, 17]. *MuRF1* and *Atrogin-1* expression levels were determined in 2 day differentiated myoblast supplemented with leucine for 48 h to investigate if the increased myofibrillar protein content could be due to decreased breakdown. Slight increases in *MuRF1* (Fig. 4C) and *Atrogin-1* (Fig. 4D) mRNA expression levels were detectable after leucine stimulation compared to control. These data suggest that decreased proteolysis or increased myogenesis are unlikely explanations for the specific myofibrillar protein accretion observed in response to leucine.

### 3.7 Increased *MyHC-4* but not *MyHC-7* mRNA levels in response to leucine are dependent of mTOR activity

To investigate the contribution of increased *MyHC* gene expression levels to increased MyHC protein levels after 5 days of supplementation during differentiation, MyHC gene expression was determined in 2 days differentiated myoblasts that were supplemented with leucine for 48 h. Increased mRNA levels of *MyHC-7* (MyHC-sl isoform; Fig. 5A) and *MyHC-4* (MyHC-f isoform; Fig. 5B) were detected after leucine supplementation. To confirm that leucine-induced increased *MyHC* gene expression was independent of muscle cellular differentiation status, 5 day fully differentiated myotubes were supplemented with leucine for 48 h. Figure 5C and D show, respectively, leucine-induced *MyHC-7* and *MyHC-4* gene expression in fully differentiated myotubes.

Since it has been demonstrated before that mTOR related processes can diverge during and after muscle cell

 

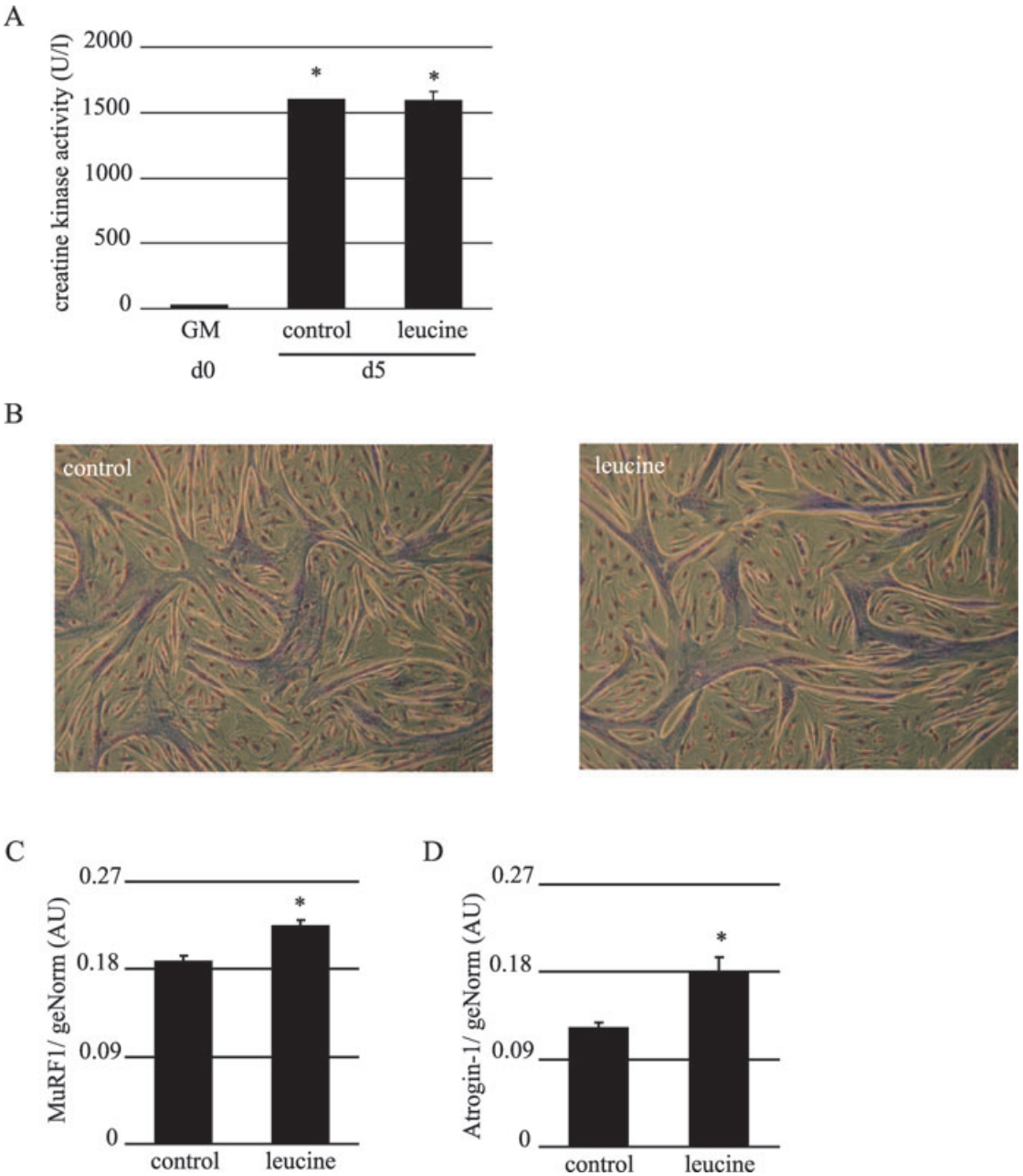


**Figure 4.** Leucine does not increase myogenesis nor mediators of proteolysis. C2C12 cells were differentiated for 5 days in 10% AA in DM in the absence or presence of 5 mM leucine. Medium and leucine were refreshed every 48 h. Cells were lysed and creatine kinase activity was measured or myotubes were fixed and stained with May-Grünwald-Giemsa. (A) Leucine did not stimulate creatine kinase activity compared to control (B) nor did it stimulate myoblast fusion. C2C12 cells were differentiated for 4 days in the absence or presence of 5 mM leucine. Medium and leucine were refreshed every 48 h. Total RNA was extracted and cDNA synthesized. mRNA expression levels of *MuRF1* and *Atrogin-1* were determined. (C) *MuRF1* and (D) *Atrogin-1* mRNA levels were significantly up-regulated after leucine stimulation compared to control. * Significant from control.

differentiation [18], the requirement of mTOR signalling for leucine-induced increases in *MyHC* mRNA levels was first investigated in 3 day differentiated C2C12 cells. C2C12 cells were stimulated with leucine with or without the mTOR inhibitor rapamycin. Leucine-induced mTOR phosphorylation was inhibited by rapamycin below control levels (Fig. 5E). Although leucine-induced 4E-BP1 phosphorylation (Fig. 5F) was still slightly increased over control in presence of rapamycin, induction of p-S6K1 by leucine was completely prevented by rapamycin (Fig. 5G). Representative blots are

 

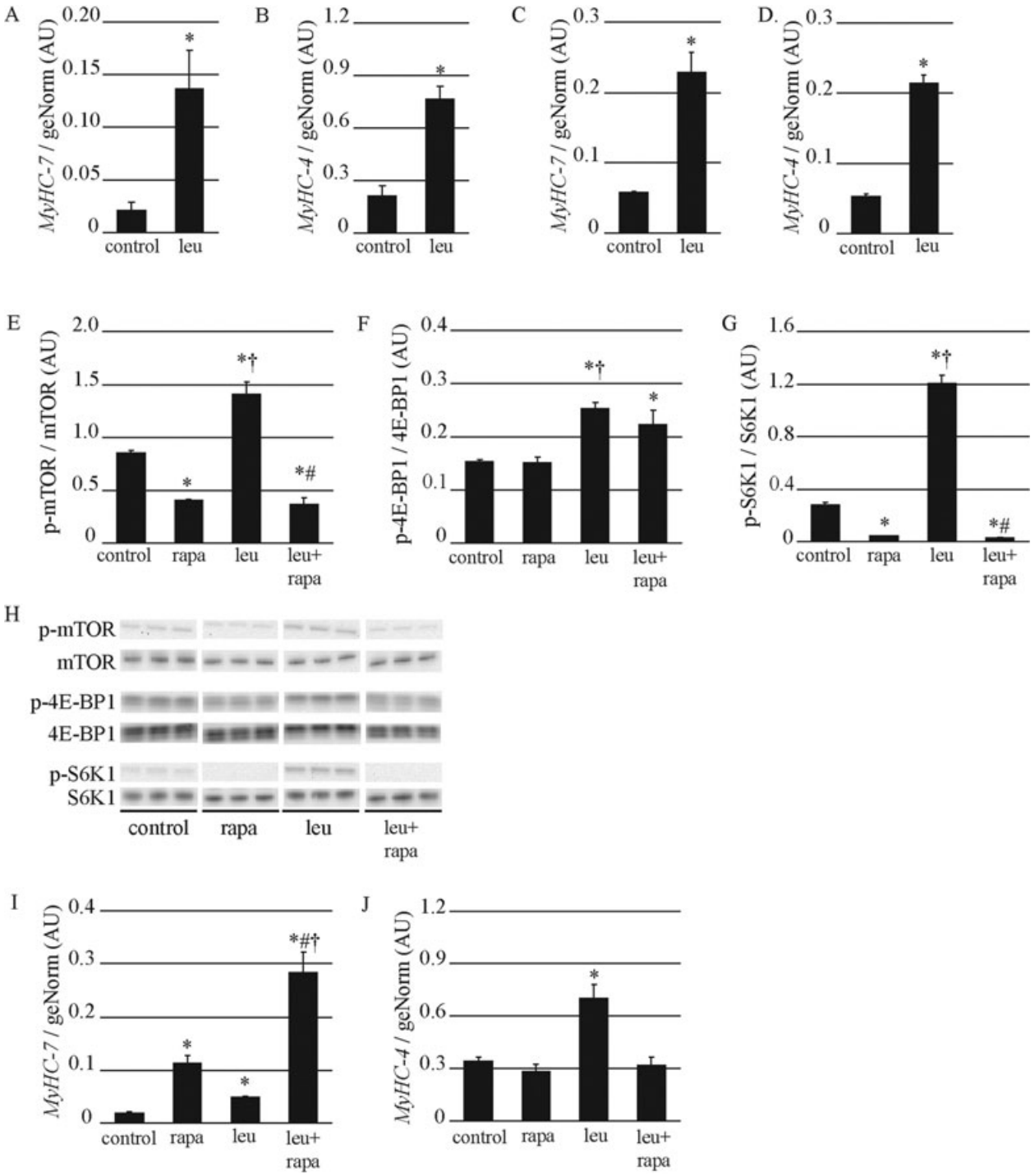


**Figure 5.** Leucine increases M*y*HC-*7* but not *MyHC-4* mRNA levels independent of mTOR activity. C2C12 cells were differentiated for 2 days and subsequently supplemented with 5 mM leucine for 48 h. mRNA expression levels of (A) *MyHC-7* (slow isoform) and (B) *MyHC-4* (fast isoform) were determined. Leucine responsiveness of (C) *MyHC-7* and (D) *MyHC-4* was confirmed by stimulation of fully differentiated myotubes with leucine for 48 h. C2C12 cells were AA and growth factor starved for 6 h in HBSS+ after 3 days of differentiation in 10% AA in DM, followed by 30 min rapamycin (1 μM) and additional 30 min stimulation with leucine (5 mM). Whole cell lysates were prepared and protein content was determined. Equal amounts of protein were loaded and (E) mTOR (Ser2448), (F) 4E-BP1 (Thr37/46) and (G) S6K1 (Thr389) phosphorylation levels were determined and normalized for total mTOR, 4E-BP1 and S6K1 levels. (H) Representative blots. C2C12 cells were differentiated for 2 days and subsequently supplemented with 5 mM leucine for 48 h with or without rapamycin (1 μM). (I) *MyHC-7* but not (J) *MyHC-4* gene expression was further enhanced when cells were co-stimulated with leucine and rapamycin. * Significant from control. † Significant from rapamycin. # Significant from leucine.

 

shown in Fig. 5H. Replication of leucine-induced mTOR related signalling experiments in fully differentiated myotubes resulted in similar findings, namely leucine-induced increases in p-mTOR, p-4E-BP1 and p-S6K1 (data not shown). Inhibition of mTOR signalling using rapamycin did not affect basal *MyHC-4* transcripts, but blocked leucine-induced increases in *MyHC-4* mRNA levels (Fig. 5J). Surprisingly, basal *MyHC-7* expression was increased in response to mTOR blockade, and a synergistic induction of *MyHC-7* mRNA levels was observed when leucine was combined with mTOR inhibitor rapamycin (Fig. 5I). Similar experiments were performed with mTOR inhibitor PP242. Identical to rapamycin, PP242 did prevent leucine-induced increases of *MyHC*-4 but not *MyHC-7* mRNA levels (data not shown). These experiments reveal that leucine increases MyHC expression at the pre-translational level, in an mTOR dependent and independent manner for MyHC-f and MyHC-sl, respectively.

## 4 Discussion

In the present studies we demonstrate that leucine directly induces myofibrillar protein accretion in skeletal muscle cells, and that this involves a pre-translational mechanism, which is in part independent of mTOR activity.

It has been postulated that myofibrillar proteins are more responsive to feeding and AA [7, 8] compared to cytoplasmic proteins. Therefore, to investigate whether direct effects of leucine in skeletal muscle are involved, cultured skeletal muscle cells were used to address this notion. Skeletal muscle cell lines like C2C12 mouse myocytes are commonly used to study regulation of muscle differentiation, muscle hypertrophy and atrophy. In order to unravel mechanisms underlying preferential accretion of myofibrillar proteins with leucine supplementation, we first present an improved C2C12 culture routine that not only allows investigation of signalling events, but muscle specific and total protein accretion in response to an anabolic stimulus during differentiation of C2C12 myoblasts as well. It is of great importance to stress that, although the more physiological relevant AA concentrations are sub-optimal, they are not insufficient and allow an anabolic response of the cells as demonstrated by the response to IGF stimulation depicted in Fig. 1D and 1E. Moreover, total protein content increased in response to IGF-I to a same extent, regardless of whether cells were cultured in 10% AA or 30% AA-containing DM (Supporting Information Fig. S1A). Accretion of myofibrillar proteins MyHC-sl, MyHC-f, MyLC-1 and MyLC-3 was observed when C2C12 myoblasts were supplemented with 2, 5 or 10 mM leucine during differentiation (Fig. 2A). Leucine plasma concentrations in human subjects of 1.7 mM have been observed after ingestion of mixed drinks containing carbohydrates, protein and additional leucine [19], emphasizing the relevance of our experimental setup.

Several studies have determined post-absorptive physiological plasma AA levels in mice and rats, which are comparable with human plasma AA levels under physiological conditions. These studies report average AA concentrations of 0.01–0.5 mM, depending on the AA investigated and total AA levels of approximately 3 mM [10, 11]. In routinely used culture medium for the mouse C2C12 cell line the concentrations of AA are however ten times higher and range from 0.2 mM up to almost 4 mM for glutamine. The concentration of glutamine seems even disproportionably high. It is tempting to speculate that it is the glutamine concentration that inhibits myoblast fusion and biochemical differentiation in routinely used culture medium containing supra-physiological levels of AA, since glutamine is shown to decrease the phosphorylation state of mTOR and downstream targets 4E-BP1 and S6K1 [2] and increase the expression of E3-ligase Atrogin-1 [20]. Growth factors like IGF-I can stimulate C2C12 differentiation [21] as demonstrated in Fig. 1. To confirm that leucine-induced MyHC accretion is growth factor-independent, experiments were repeated in medium supplemented with dialyzed FBS. The myofibrillar protein accretion stimulating properties of leucine were persistent in medium supplemented with dialyzed FBS (data not shown).

Our data indicate that mechanistical studies on signalling parameters [2, 3, 5] may have little predictive value for effects of leucine supplementation over a long(er) period on total protein content and muscle anabolism: despite leucine-induced increased phosphorylation of mTOR and downstream targets 4E-BP1 and S6K1, leucine stimulation did not increase de novo protein synthesis nor increase total protein content, suggesting the absence of general muscle anabolism. Our data are supported by a recently published study in which there was no increase in incorporated $^{35}$S-methionine after 3 h of leucine stimulation of C2C12 myotubes [2]. During our $^{35}$S-methionine incorporation experiments leucine was administered in 10% AA in DM. This medium supports maximal stimulation of mTOR, 4E-BP1 and S6K1 phosphorylation (data not shown) by AA and growth factor starvation. It is therefore tempting to speculate that AA-induced de novo protein synthesis was already at a maximal capacity and additional leucine was not able to further increase protein synthesis. In line with this thought, data from a recent human intervention study demonstrate that ingestions of 10 g EAA specifically enriched in leucine content does not further enhance muscle protein synthesis compared to an isonitrogenous 10 g EAA high quality protein control [6].

The novel finding that MyHC as well as MyLC protein conten, but neither MCK protein expression nor myoblast fusion, increases in skeletal muscle cells after leucine supplementation rules out a general stimulatory effect on myogenesis, but suggests differential regulation of myofibrillar protein expression and total protein accretion in response to leucine. Leucine supplementation did not decrease but rather slightly increased expression of the E3-ligases MuRF1 and Atrogin-1, which are required for muscle proteolysis [22, 23]. This further strengthens the thought that the accretion of myofibrillar proteins in response to leucine is due to a synthesis regulating

 

mechanism rather than an inhibition of myofibrillar protein breakdown [16, 17]. Since the observations did not concern a general stimulation of protein accretion or myogenesis, we assessed whether increases in mRNA transcript levels could explain the specific accretion of myofibrillar protein in response to leucine. Our data for the first time reveal pre-translational regulation of myofibrillar protein expression by leucine, as mRNA levels encoding MyHC-slow and -fast proteins were increased. These findings were confirmed in fully differentiated myotubes (Fig. 5C and D) and imply that leucine responsiveness of MyHC genes is muscle cell differentiation status independent. We furthermore excluded general AA insufficiency as a possible explanation for these findings. The optimal AA concentration for myoblast differentiation and MyHC-f content (data not shown) is 30% AA in DM (Fig. 1A and C). We therefore confirmed the leucine-induced increase of *MyHC-7* (Supporting Information Fig. S1B) and *MyHC-4* (Supporting Information Fig. S1C) gene expression over control as demonstrated in Fig. 5B in cells that had differentiated in DM containing 30% AA. In addition, no increase in MyHC-sl protein content was observed following equimolar supplementation with arginine (Supporting Information Fig. S1D) further emphasizing that the observed effects of leucine supplementation are not merely the consequence of increased AA availability.

Transcriptional regulatory responses to leucine have been documented previously. Sun et al. demonstrated leucine-induced mRNA expression of the leptin receptor in C2C12 myotubes [24], and others revealed leucine-induced alterations in metabolism-related gene expression in rainbow trout hepatocytes [25]. However, apart from the limited number of studies implying pre-translational regulation of gene expression by leucine, no information is available on whether this requires signalling through the currently best described leucine-sensitive pathway, the mTOR-4E-BP-1/p70S6K1 signalling module. Therefore, mTOR-activity dependence of leucine-induced changes in MyHC mRNA levels was evaluated. The MyHC transcripts displayed a differential sensitivity towards mTOR blockade, revealing an mTOR activity-dependent induction of *MyHC-4* by leucine, which encodes a MyHC isoform mostly present in muscle fibers characterized by fast-twitch contractile properties and glycolytic metabolism. In contrast, *MyHC-7* is expressed in slow twitch muscle fibers characterized by oxidative metabolism, and these transcript levels were induced by leucine in an mTOR-independent manner. Leucine-induced rapamycin sensitive stimulation of muscle proteins has already been suggested to be in part mTOR independent [1]. We are however the first to actually demonstrate leucine-induced up-regulation of MyHC gene expression. Moreover, we demonstrate here that this can, dependent on the MyHC isoform, occur in the absence of mTOR signalling. Our findings are in line with a study by Bentzinger et al., as a MyHC-f to -slow shift was observed in skeletal muscle of mTORC1 -/- mice, indicating the mTOR-independent expression of *MyHC-7* [26].

In conclusion, this study shows for the first time that leucine supplementation stimulates myofibrillar protein rather than generic protein accretion in skeletal muscle, and implies that mTOR- dependent and -independent regulation by leucine controls MyHC mRNA expression levels.

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*The authors have declared no conflict of interest.*

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