Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Leucine can modulate the expression of proteins related to protein degradation signalling under mTOR inhibition in C_2C_{12} cells

Bianca Cristine Favero-Santos[1,2], Maria Cristina Cintra Gomes-Marcondes[1*]

[1] Laboratory of Nutrition and Cancer, Department of Structural and Functional Biology, Biology Institute, University of Campinas, Zip Code 13083862, Campinas, Sao Paulo, Brazil

[2] Obesity and Comorbidities Research Centre, Department of Structural and Functional Biology, Biology Institute, University of Campinas, Campinas, Sao Paulo, Brazil

Correspondence to: cintgoma@unicamp.br

Received January 24, 2018; **Accepted** July 17, 2018; **Published** July 30, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.10.12

Abstract: Many metabolic syndromes lead to energetic disturbs which ends to an intense catabolic state. The branched-chain amino acid leucine shows very positive effects on muscle protein metabolism. However, it is still not clear how leucine acts improving the protein turnover. This study aimed to evaluate in vitro the effects of leucine supplementation in minimising the signalling pathway of protein degradation when mTOR was inhibited. Our studies were conducted in murine C_2C_{12} myotubes exposed to 2mM leucine or 2mM isoleucine in control situation and compared to the inhibition of mTOR by rapamycin. Then, the expression of proteins related to protein synthesis and degradation signalling pathway was obtained by Western Blot. At this concentration, the leucine was sufficient to maintain the expression of proteins evaluated as in control situation. However, when the cells were exposed to rapamycin (80nM), leucine inhibited the expression of SMAD and FoxO3a, showing that leucine was able to modulate the degradation pathway when protein synthesis is compromised. Furthermore, leucine had no effect in modifying the expression of subunits of ubiquitin-proteasome system, showing that leucine had no direct effect in ubiquitin-proteasome system, but acted leading to the phosphorylation of SMAD and FoxO3, which inhibited the activity of transcriptional of these proteins. No similar results were observed in cells exposed to isoleucine under the same experimental protocol, likely showing that leucine has specific action over another branched-chain amino acids. In conclusion, the present study shows that leucine can modulate degradation pathways even under inhibition of mTOR by rapamycin.

Key words: Myotubes C_2C_{12}; Cell culture; Leucine; Protein metabolism.

Introduction

The muscle mass is an important tissue as its homeostasis should be guaranteed by the balance of normal protein turnover, which should provide amino acids to the whole-body protein metabolism (1). In this way, when synthesis pathways signalling, mainly regulated by mTOR, are more stimulated than breakdown pathways, the skeletal muscle gains mass, enhancing in size, hypertrophying this tissue. This hypertrophy process is mainly regulated by mTOR, which is activated by caloric or protein intake, and exercise, releasing insulin, GH, and IGF-1 (2,3). When breakdown signalling pathways are more activated in muscle tissue than synthesis, it seems that an intense catabolic process is leading mainly to protein degradation, decreasing the size of the muscle. Many signalling pathways that lead to muscles protein degradation contribute to atrophy. The list of these signalling pathways includes AKT-FoxO (forkhead family transcription factor), IKK-NF-kB ((*enzyme IκB kinase e nuclear factor*-kappa B *(*NF-*κB) -activation*), JAK-STAT3 (*Janus kinase/*signal *transducers and activators of transcription*), and TGFβ/Myostatin-SMAD2/3 (*tumour/transforming growth factor β/ myostatin e protein* SMAD *pathway*) (4), which are directly related to ubiquitin-proteasome pathway.

Since amino acids can be potent regulators of mTOR, especially mTORC1, which lead the protein synthesis and growth, mainly in skeletal muscle (5–10), the leucine has a special role in this feature. Although, the whole mechanisms by which amino acids influence protein synthesis and also protein degradation are still unclear. Many studies suggest that protein synthesis is increased when the availability of intracellular amino acids enhances (11,12). BCAAs also have inhibitory actions on skeletal muscle proteolysis and can modulate their rate in catabolic states, exerting negative feedback control also in vivo (13). Leucine can play an important role in skeletal muscle cell signalling, and this BCAA can increase protein synthesis and/or decrease muscle proteolysis, independent of the other branched-chain amino acids, isoleucine and valine (14–16). On the other hand, BCCAs supplementation modulated the SMAD signalling in CCI4-induced liver fibrosis, and other study showed that isolate leucine affected the SMAD signalling in skeletal muscle (17,18). Furthermore, studies have shown that leucine decreased the activation of FoxO3a of damaged muscle by cryolesion in rats and also blocked the nuclear accumulation of FoxO3a in skeletal muscle under immobilisation (19,20).

In this way, the principal aim of the present study was to evaluate *in vitro* the effects of leucine supplementation in the signalling pathway of protein degradation when mTOR was inhibited.

Materials and Methods

Reagents

DMEM High Glucose (catalogue number #BR30003-05), fetal bovine serum (FBS, catalogue number #10-bio500), horse serum (catalogue number #11-1270), phosphate-buffered saline (PBS, catalogue number #P4417), antibiotic solution (P/S, catalogue number# BR30111-01), l-leucine (catalogue number #L8912), l-isoleucine (catalogue number #I2752) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rapamycin was purchased from Sigma-Aldrich (catalogue number #082M4018V, St. Louis, MO, USA). Anti-bodies AKT and phospho-AKT$^{(thr308)}$, total mTOR and phospho-mTOR$^{(S2448)}$, p70S6K and phospho-p70S6K$^{(T421/S424)}$ (Cell Signalling, USA) total SMAD2 and phospho- SMAD 2/3 (Sigma-Aldrich, St Louis, USA), total FoxO3a and phospho-FoxO3a (Santa Cruz Biotechnology, EUA), 20S, 19S and 11S (Enzo Life Science, USA). Other biochemical reagents are purchased from Labsynth (Diadema, SP, Brasil); Millipore Corporation (Jaffrey, NH, USA), Promega (Madison, WI, USA) and Sigma-Aldrich (St Luis, MO, USA).

Cell culture and differentiation

C_2C_{12} mouse myoblasts were maintained in DMEM high glucose supplemented with 10% fetal bovine serum, 1% penicillin and 1% glutamine. When cells reached confluence, the medium was switched to DMEM high glucose with 2% horse serum, 1% penicillin and 1% glutamine. Cells were refreshed with new differentiation medium every day, and by the 4th day, the C_2C_{12} cells were fused into myotubes, which corresponded to complete differentiation process (images not shown). C_2C_{12} myotubes were then treated with 80 nM rapamycin and/or 2mM leucine or isoleucine.

C_2C_{12} myoblast cells differentiated into myotubes were distributed into a control group (which will be referred as a non-treated cells)and the following experimental groups: myotubes were treated only with 2mM leucine for one hour (L) or treated with 2mM isoleucine for one hour (I), group which received treatment with 80nM rapamycin for 2 hours (R), and additional groups treated with rapamycin, during 2 hours, associated with leucine or isoleucine for the last 1 hour of rapamycin exposure (LR or IR)

Immunoblotting

C_2C_{12} cells (10×10^5) were seeded as a monolayer on 6-well plates. After the treatment cells were washed twice with phosphate-buffered saline (PBS) and harvested in lysis buffer (Millipore Lysis Buffer), and add 1 mM PMSF, 10 mM EDTA, 1 mM EGTA, and 0.002mg/ml aprotinin, following denaturation in Laemmli sample buffer 2x (5 min, 100 °C). The cell lysate was separated by 10% or 12.5% SDS–PAGE electrophoresis (depending on the protein size) and transferred to nitrocellulose membrane (BioRad, Germany) in 2 hours at a constant voltage (120 V) at 4ºC. The membranes were blocked in 5% non-fat milk in Tris-buffered saline (TBS) com 1% de tween solution for 1 hour. The membranes were then incubated overnight at 4 °C with antibodies against total AKT and phospho-AKT$^{(T308)}$, total mTOR and phospho-mTOR$^{(S2448)}$, p70S6K and phospho-p70S6K$^{(T421/S424)}$ (diluted 1:1000), total SMAD -2 and phospho- SMAD 2/3 (diluted 1:1000), total FoxO3a and phospho-FoXO3a (diluted 1:500), 20S, 19S and 11S (Enzo Life Science, USA). Immunoreactivity was detected by the sequential incubation of membranes with anti-rabbit secondary antibody for 2 hours at room temperature, which was visualised using a chemiluminescence detection system UVITEC (UVItec Cambridge, United Kingdom). The expressions of proteins listed above were estimated versus the constant level of the protein GAPDH (diluted 1:1000).

Activity of proteolysis systems

Measurement of proteasome activity was determined by measuring chymotrypsin-like enzyme activity, the one of the main proteolytic activity of the proteasome β subunits, according to the method of Orino et al. (1991) (21). After collection of treated C_2C_{12} myotubes, these cells were homogenised in cold 0.1M TRIS, pH7.4, sonicated and centrifuged at 10,000xg for 15min at 4ºC. The resulting supernatant was analysed for total protein content (22). Aliquots of homogenate supernatant were accessed, using fluorogenic substrate succinyl-Leu-Leu-Val-Try-7-amino-4-methyl coumarin (Suc LLVY-AMC). The results were expressed as units of fluorescence/µg protein/min (23).

Statistical analysis

Comparison among groups, we used Two-way ANOVA followed by Bonferroni post-test using GraphPad Prism version 6.00 software (GraphPad, USA) and results showed as mean ± standard error (SEM). Significance was defined as $P \leq 0.05$.

Results

Leucine maintained the AKT/mTOR/p70S6K pathway activity

The role of leucine in stimulating protein synthesis in myotubes cells is still unclear (24,25). In this way, we studied whether leucine was capable of enhancing protein synthesis by mTOR, in myotubes pretreated with rapamycin followed by leucine supplementation. In our studied conditions, when the cells were treated only with leucine, we did not observe increases in the mTOR activation (referred by the p-mTOT/total mTOR ratio), neither on p70S6K in non-treated cells (Figure 1B-C; WB images 1D). Leucine accounted for approximately 9.0% of total variance (F=4.92; P-value = 0.0404) in mTOR activation. Although, total protein content or protein synthesis showed no difference among groups (data not shown). Therefore, in the groups treated with rapamycin, the ratio between phospho and total of both mTOR and p70S6K decreased, showing that the inhibition with rapamycin was efficient. Rapamycin accounted for approximately 48.4% of total variance (F=26.3; P value < 0.0001) in mTOR phosphorylation and 55.8% of total variance (F=48.6; P value < 0.0001) in p70S6K phosphorylation. The AKT activity (inferred by p-AKT/total AKT ratio), as expected, was not altered; since it is an upstream protein of mTOR pathway and is not activated by amino acids (Figure 1A) (26,27).

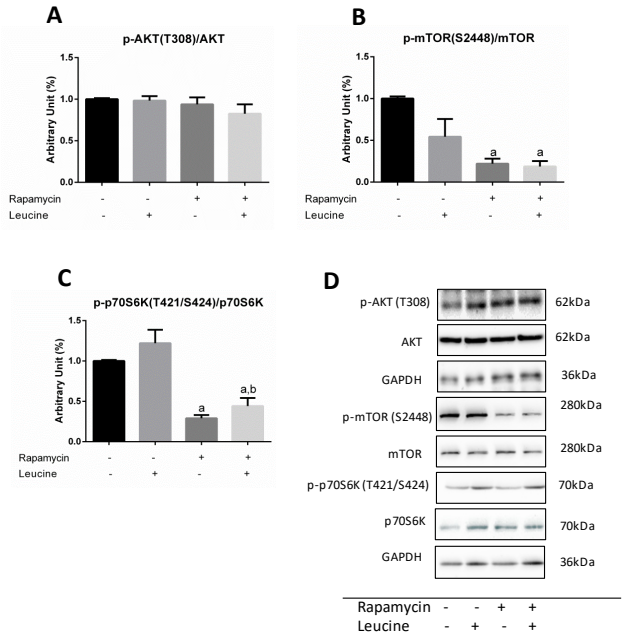

Figure 1. Effects of leucine supplementation in the expression of proteins involved in mTOR signalling pathway. A) AKT phosphorylation and total ratio. B) mTOR phosphorylation and total ratio. C) p70S6K phosphorylation and total ratio. D-E) Representative image from Western Blot analysis. All blots were representative of three independent experiments in triplicates. Statistical difference (a = P < 0.05 versus control; b = P < 0.05 versus leucine) was analysed using Two-Way ANOVA followed by Bonferroni post-test.

Leucine inhibited SMAD 2 and FoxO3a and did not affect the activity of ubiquitin-proteasome system in cells treated with rapamycin

To evaluate whether leucine would be able to modulate degradation pathways and whether mTOR could affect these results, we analysed both SMAD and FoxO3a expressions, since these proteins are transcriptional factors that the activation by post-translational modification was involved with breakdown protein. In our experiments, leucine supplementation did not alter the ratio between the phosphorylated and total expression of both SMAD2 and FoxO3 in cells that received no treatment (Figure 2A, B, Western Blot images 3C). However, the treatment with rapamycin, independent of leucine treatment, promoted a reduction in phosphorylation of SMAD2 (rapamycin and/or leucine association accounted for significant effect leading to 39% and 8% of the total variance respectively; P value < 0,0001; Figure 2A; Thus, under a t-test analysis, we found a significant value (P=0.0495) comparing rapamycin versus rapamycin plus leucine groups). In this same way, the leucine treatment under mTOR inhibition was able to inactivate FoxO3 (inferred by p-FoxO3/total FoxO3 ratio) by the phosphorylation (leucine accounted for approximately 15.26% of total variance (F=4.83; P value=0.0412; Figure 2B). Moreover, it is interesting to note that mTOR may be the key in the crosslink between synthesis and degradation pathways. The leucine treatment associated with rapamycin inhibited SMAD2, being able to inactivate FoxO3 via phosphorylation. This result shows that leucine may exert anti-proteolytic effects in the absence of mTOR.

Besides that, to elucidate the role of leucine in protein metabolism in myotubes, we also evaluated the proteolytic process through the expression of subunits of the ubiquitin-proteasome system and the activity of the chymotrypsin enzyme. In the results, under our experimental conditions, we did not find alteration in the

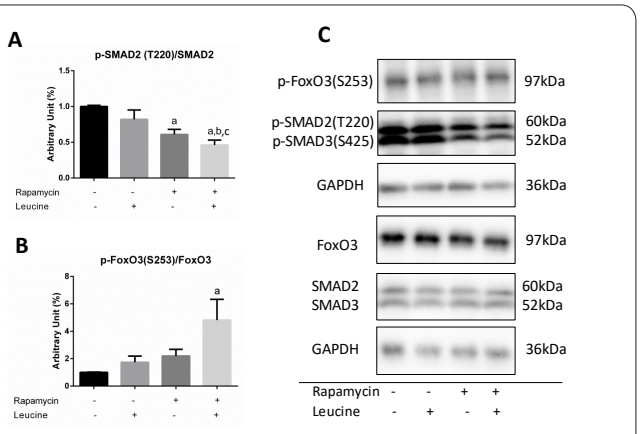

Figure 2. Effects of leucine supplementation on the expression of the proteins involved in degradation pathways. A) SMAD phosphorylation and total ratio. B) FoxO3a phosphorylation and total ratio. C) Representative image from Western Blot analysis. All blots were representative of three independent experiments in triplicates. Statistical difference (a = P < 0.05 vs control; b = P < 0.05 vs leucine) was analysed using Two-Way ANOVA followed by Bonferroni post-test; c = P=0.0495 vs rapamycin after t-test analysis. Besides that, to elucidate the role of leucine in protein metabolism in myotubes, we also evaluated the proteolytic process through the expression of subunits of the ubiquitin-proteasome system and the activity of the chymotrypsin enzyme. In the results, under our experimental conditions, we did not find alteration in the expression of 11S, 19S and 20S proteins (Figure 3A-D, and Western Blot images 3E), nor on the enzyme chymotrypsin activity (Figure 3F). This results showing that even FoxO3 and SMAD was inhibited, it does not act in ubiquitin-proteasome system. Thus, other pathways - as autophagy - should be studied.

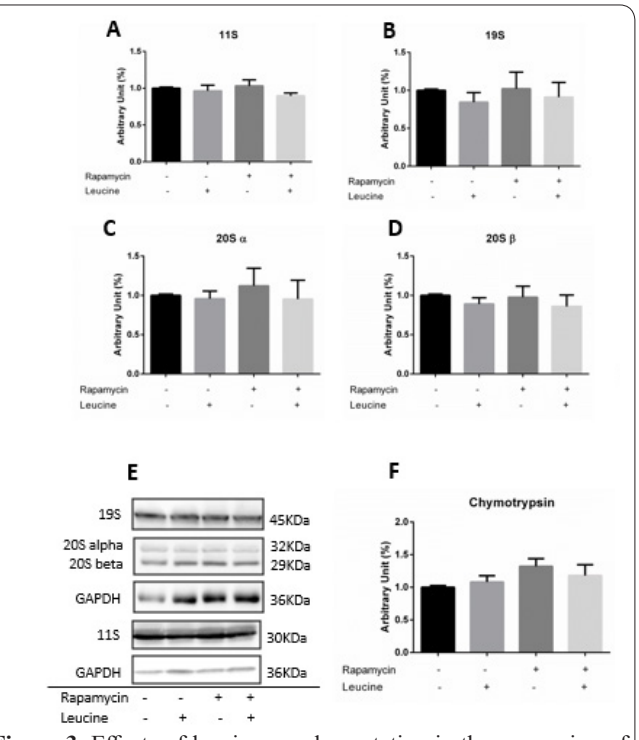

Figure 3. Effects of leucine supplementation in the expression of subunits of the ubiquitin-proteasome system. A) 11S. B) 19S. C) 20S alpha. D) 20S beta. E) Representative image from Western Blot analysis. F) Chymotrypsin activity. All blots were representative of three independent experiments in triplicates

expression of 11S, 19S and 20S proteins (Figure 3A-D, and Western Blot images 3E), nor on the enzyme chymotrypsin activity (Figure 3F). This results showing that even FoxO3 and SMAD was inhibited, it does not act in ubiquitin-proteasome system. Thus, other pathways - as autophagy - should be studied.

Isoleucine had no effect as leucine acted on protein metabolism

To evaluate if the found inhibitory effects were produced only by leucine supplementation or not, we repeated the experiments now treating the cells with isoleucine, and we analysed the expression of the proteins above. The isoleucine supplementation did not alter the expression of any proteins evaluated. Therefore, isoleucine had no effect as verified by leucine action (Figure 4). This results showed that our results were due to the leucine supplementation and not found with other BCAA.

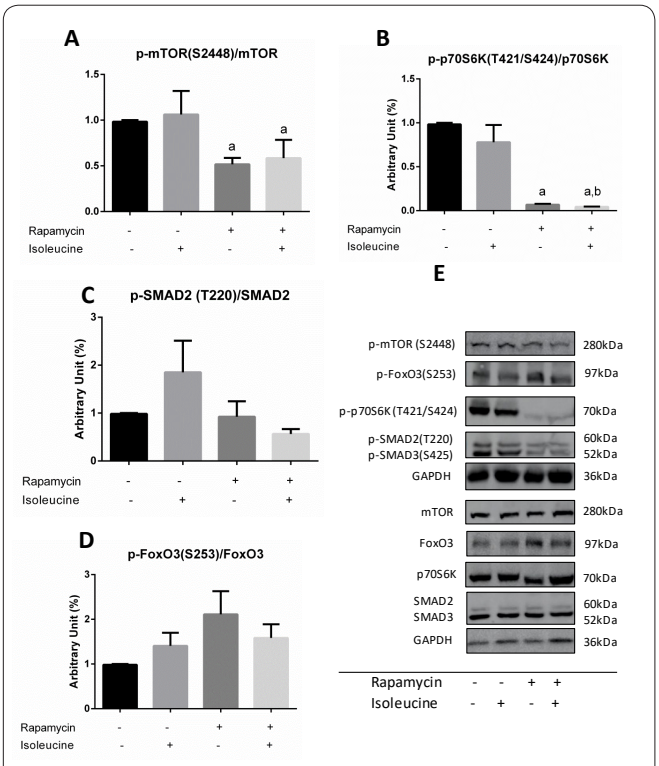

Figure 4. Effects of isoleucine supplementation on protein expression involved in synthesis and degradation pathways. A) mTOR phosphorylation and total ratio. B) p70S6K phosphorylation and total ratio. C) SMAD2 phosphorylation and total ratio. D) FoxO3 phosphorylation and total ratio. E) Representative image from Western Blot analysis. All blots were representative of three independent experiments in triplicates Statistical difference (a = $P < 0.05$ versus control; b = $P < 0.05$ versus isoleucine) was analysed using Two-Way ANOVA followed by Bonferroni post-test.

Discussion

The muscle mass actively participates in the amino acids balance, since no other organ can supply this demand. Therefore, the spare offering amino acids can be used as energetic substrates for the production of ATP (2). Since our main research subject is the lean body mass, we studied in this work whether, in adverse conditions, especially related to catabolic process or anabolic inhibition, the cells could respond better to leucine treatment. Here, our results probably demonstrated that C_2C_{12} myotubes under inhibition of primary nucleus of mTOR, the leucine treatment lonely could prevent or minimise the catabolic process led by SMAD and FoxO3 signalling.

The myotubes when in physiological homoeostasis under anabolic and catabolic balance, the treatment with leucine (in this case, working only one hour of leucine exposure) these myotubes had no changes in cell signalling related to synthesis process and even to catabolic process. In an extensive review written by Saxton and Sabatini, the mTOR signalling is the central core essential to control cell metabolism related to basic cell functions, such as protein synthesis, cell proliferation, etc (28). mTOR (*mechanistic* or *mammalian target of rapamycin*) is a serine/threonine kinase that controls cell growth/activity in response to nutrients (in this case referring especially to leucine), growth factors and energy (9,29). Although there are several substrates of mTORC1, evidence shows that 4EBP1 (eukaryotic initiation factor 4E binding protein 1) and S6K1 are the most important substrates in promoting cell size increase, inducing both cell growth/proliferation and protein synthesis (30). Thus, phosphorylation of S6K (T389/S412) in cells is routinely used as a reflex of the activity of this mTORC1 kinase (31). Many experiments have shown that even leucine modulates mTOR phosphorylation, that activation did not lead necessarily enhanced in protein synthesis (24,25). In our results, we did not find enhance in PI3K/AKT/mTOR by leucine supplementation nor isoleucine in C_2C_{12} cells; these results were similar to those found by Giron and colleagues (24). The authors analysed myoblasts of rat skeletal muscles of the L6.C11 lineage, which were treated with leucine, and verified that these myoblasts did not present a stimulus for increased protein synthesis, attributing this fact to the non-increased phosphorylation of AKT, mTOR and 4EBP1. However, the authors found only increase of p70S6K, as verified in this present work. Thus, even modulating some signalling pathways, leucine did not efficiently increase protein synthesis. Another study demonstrated that 1.5 mM leucine, for 30 minutes, increased the phosphorylation of mTOR, p70S6K and 4EBP1 in C_2C_{12}, under fetal-serum-free DMEM for 1 hour; and the authors verified no increase of phenylalanine incorporation, revealing that phosphorylation of these proteins was not sufficient to increase protein synthesis (25).

In this way, we aimed to study whether leucine supplementation could decrease the breakdown protein in the mTOR-inhibited situation. For this, we observed that the evaluation of expression of two transcriptional proteins – SMAD and FoXO3 – showed some leucine role in this process. FoxO3 is a transcription factor phosphorylated by AKT, which inhibits its action. When phosphorylated, FoxO3 is prevented from migrating to the nucleus and therefore cannot activate protein degradation (4,32). SMAD participates in the signalling pathway of activin receptors (32). Again in control situation (non-treated cells), the leucine appeared to maintain all protein evaluated. However, when cells were treated with rapamycin, both FoxO3 and SMAD were inhibited when treated with leucine. Thus, it was possible to observe that the increase in the phosphorylation of FoxO3,

due to the treatment with leucine and rapamycin, suggests that leucine exerted an anti-proteolytic effect on myotubes under the absence of mTOR. Although, in our results, we observed no alteration in the subunits of the ubiquitin-proteasome system. These results suggested that even with decreased SMAD and FoxO, there was no change in subunits of UPS but could affect any ubiquitin ligases, such as MURF or atrogin or autophagy-related genes.

To evaluate if the results were specific to leucine supplementation, we repeated the all experiment now treating the cells with isoleucine, another BCAA with the same molar mass. The data showed no action of this BCAA since we did not observe inhibition of the SMAD and FoxO3a like as found under leucine supplementation.

Taking all results together, we can conclude that leucine could exert effects on protein degradation, when under a stimulus (Figure 5). In a control situation, leucine supplementation showed no effect on cellular homoeostasis, maintaining proteins of cell signalling under balance. When rapamycin inhibited the mTOR in myotubes, it clearly altered the cell stability. When dealing with a branched-chain amino acid, leucine, our results indicated that leucine acted by shifting the cell balance to a minimising the protein breakdown by inactivating Foxo3a and SMAD pathway (Figure 5).

Thus, we believe that specifically, leucine can modulate signalling pathways related to protein synthesis and also inhibition of protein degradation. Studies point out that VPS34 can be involved in this process, as there is a link between mTOR and VPS34 (20). Further studies are now undergoing to find which protein is related to inhibition of FoxO3a in parallel to the maintenance of AKT or under modulation of the subunits of the ubiquitin-proteasome pathway.

Acknowledgements
The authors thanked for the financial support of CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Cientifico e Tecnológico #302863/2013-3; #302425/2016-9), and FAPESP (grant number #2014/13334-7; #2013/16115-1; #2017/02739-4).

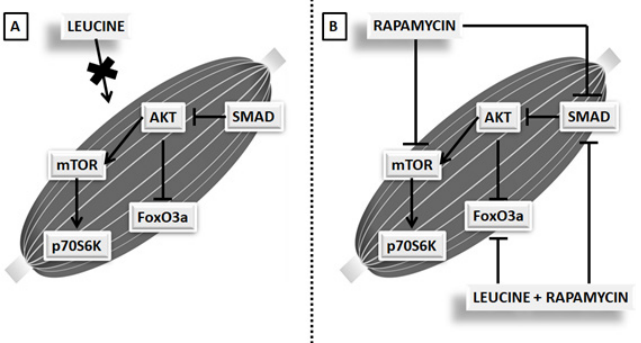

Figure 5. Diagram representation of signalling pathway in C_2C_{12} myotubes which can be modulated by leucine supplementation when mTOR was inhibited. A. C_2C_{12} myotubes under leucine supplementation. B. C_2C_{12} myotubes under leucine supplementation and treated with rapamycin.

Conflict of interest
The authors declare that they have no conflict of interest.

References

1. Wolfe RR. The underappreciated role of muscle in health and disease. American Journal of Clinical Nutrition. 2006. pp. 475–482. doi:84/3/475 (pii)
2. Argilés JM, Campos N, Lopez-Pedrosa JM, Rueda R, Rodriguez-Mañas L. Skeletal Muscle Regulates Metabolism via Interorgan Crosstalk: Roles in Health and Disease. J Am Med Dir Assoc. Elsevier Inc.; 2016; doi:10.1016/j.jamda.2016.04.019
3. Anthony JC, Anthony TG, Kimball SR, Jefferson LS. Signaling pathways involved in translational control of protein synthesis in skeletal muscle by leucine. J Nutr. 2001;131: 856S–860S. Available: http://www.ncbi.nlm.nih.gov/pubmed/11238774
4. Sandri M. Protein Breakdown in Cancer Cachexia. Semin Cell Dev Biol. Elsevier Ltd; 2015; doi:10.1016/j.semcdb.2015.11.002
5. Hara K, Maruki Y, Long X, Yoshino K ichi, Oshiro N, Hidayat S, et al. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell. 2002;110: 177–189. doi:10.1016/S0092-8674(02)00833-4
6. Kim D-H, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, et al. mTOR Interacts with Raptor to Form a Nutrient-Sensitive Complex that Signals to the Cell Growth Machinery. Cell. 2002;110: 163–175. doi:10.1016/S0092-8674(02)00808-5
7. Sarbassov DD, Ali SM, Kim D, Guertin DA, Latek RR, Erdjument-bromage H, et al. Rictor , a Novel Binding Partner of mTOR , Defines a Rapamycin-Insensitive and Raptor-Independent Pathway that Regulates the Cytoskeleton. 2004;14: 1296–1302. doi:10.1016/j
8. Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature. 2006;441: 424–430. doi:10.1038/nature04869
9. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol. 2011;12: 21–35. doi:10.1038/nrm3025
10. Jacinto E, Loewith R, Schmidt A, Lin S, Rüegg MA, Hall A, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol. 2004;6: 1122–1128. doi:10.1038/ncb1183
11. Proud CG. A New Link in the Chain from Amino Acids to mTORC1 Activation. Molecular Cell. 2011. pp. 7–8. doi:10.1016/j.molcel.2011.09.004
12. Proud CG. mTORC1 regulates the efficiency and cellular capacity for protein synthesis. Biochem Soc Trans. 2013;41: 923–6. doi:10.1042/BST20130036
13. Holecek M, Kovarik M. Alterations in protein metabolism and amino acid concentrations in rats fed by a high-protein (casein-enriched) diet - Effect of starvation. Food Chem Toxicol. 2011;49: 3336–3342. doi:10.1016/j.fct.2011.09.016
14. Anthony JC, Yoshizawa F, Anthony TG, Vary TC, Jefferson LS, Kimball SR. Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. J Nutr. 2000;130: 2413–2419.
15. Anthony JC, Lang CH, Crozier SJ, Anthony TG, MacLean D a, Kimball SR, et al. Contribution of insulin to the translational control of protein synthesis in skeletal muscle by leucine. Am J Physiol Endocrinol Metab. 2002;282: E1092–E1101. doi:10.1152/ajpendo.00208.2001
16. Coffey VG, Moore DR, Burd NA, Rerecich T, Stellingwerff T, Garnham AP, et al. Nutrient provision increases signalling and protein synthesis in human skeletal muscle after repeated sprints. Eur J Appl Physiol. 2011;111: 1473–1483. doi:10.1007/s00421-010-

1768-0
17. Rowlands DS, Nelson AR, Raymond F, Metairon S, Mansourian R, Clarke J, et al. Protein-leucine ingestion activates a regenerative inflammo-myogenic transcriptome in skeletal muscle following intense endurance exercise. Physiol Genomics. 2016;48: 21–32. doi:10.1152/physiolgenomics.00068.2015
18. Khedr NF, Khedr EG. Branched chain amino acids supplementation modulates TGF-beta1/Smad signaling pathway and interleukins in CCl4 -induced liver fibrosis. Fundam Clin Pharmacol. England; 2017;31: 534–545. doi:10.1111/fcp.12297
19. Pereira MG, Baptista IL, Carlassara EOC, Moriscot AS, Aoki MS, Miyabara EH. Leucine supplementation improves skeletal muscle regeneration after cryolesion in rats. PLoS One. 2014;9. doi:10.1371/journal.pone.0085283
20. Baptista IL, Silvestre JG, Silva WJ, Labeit S, Moriscot AS. FoxO3a suppression and VPS34 activity are essential to anti-atrophic effects of leucine in skeletal muscle. Cell Tissue Res. Cell and Tissue Research; 2017;369: 381–394. doi:10.1007/s00441-017-2614-z
21. Orino E, Tanaka K, Tamura T, Sone S, Ogura T, Ichihara A. ATP-dependent reversible association of proteasomes with multiple protein components to form 26S complexes that degrade ubiquitinated proteins in human HL-60 cells. FEBS Lett. 1991;284: 206–210. doi:10.1016/0014-5793(91)80686-W
22. Bradford MM. A Rapid and Sensitive Method for the Quantitation Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. 1976;254: 248–254.
23. Gonçalves EM, Salomão EM, Gomes-Marcondes MCC. Leucine modulates the effect of Walker factor, a proteolysis-inducing factor-like protein from Walker tumours, on gene expression and cellular activity in C2C12 myotubes. Cytokine. 2013;64: 343–350. doi:10.1016/j.cyto.2013.05.018
24. Girón MD, Vílchez JD, Salto R, Manzano M, Sevillano N, Campos N, et al. Conversion of leucine to β -hydroxy- β -methylbutyrate by α -keto isocaproate dioxygenase is required for a potent stimulation of protein synthesis in L6 rat myotubes. 2016;1: 68–78. doi:10.1002/jcsm.12032
25. Areta JL, Hawley J a., Ye J-M, Chan MHS, Coffey VG. Increasing leucine concentration stimulates mechanistic target of rapamycin signaling and cell growth in C2C12 skeletal muscle cells. Nutr Res. Elsevier Inc.; 2014;34: 1000–1007. doi:10.1016/j.nutres.2014.09.011
26. Nobukuni T, Joaquin M, Roccio M, Dann SG, Kim SY, Gulati P, et al. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proc Natl Acad Sci. 2005;102: 14238–14243. doi:10.1073/pnas.0506925102
27. Wang X, Proud CG. The mTOR pathway in the control of protein synthesis. Physiology (Bethesda). 2006;21: 362–9. doi:10.1152/physiol.00024.2006
28. Saxton RA, Sabatini DM. Review. Cell. Elsevier Inc.; 2017;168: 960–976. doi:10.1016/j.cell.2017.02.004
29. Tavares MR, Pavan ICB, Amaral CL, Meneguello L, Luchessi AD, Simabuco FM. The S6K protein family in health and disease. Life Sci. Elsevier Inc.; 2015;131: 1–10. doi:10.1016/j.lfs.2015.03.001
30. Fingar DC, Salama S, Tsou C, Harlow E, Blenis J. Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev. 2002;16: 1472–1487. doi:10.1101/gad.995802
31. Weng QP, Kozlowski M, Belham C, Zhang A, Comb MJ, Avruch J. Regulation of the p70 S6 kinase by phosphorylation in vivo. Analysis using site-specifig antiphosphopeptide antibodies. J Biol CHem. 1998;273: 16621–16629.
32. Sartori R, Milan G, Patron M, Mammucari C, Blaauw B, Abraham R, et al. Smad2 and 3 transcription factors control muscle mass in adulthood. Am J Physiol Cell Physiol. 2009;296: C1248–C1257. doi:10.1152/ajpcell.00104.2009.