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Original Paper

Myotube Protein Content Associates with Intracellular L-Glutamine Levels

Diogo Antonio Alves de Vasconcelos[a,b] Pieter Giesbertz[c] Gilson Masahiro Murata[a] Diego Ribeiro de Souza[d] Jarlei Fiamoncini[e] Daniella Duque-Guimaraes[a,f] Carol Góis Leandro[b] Sandro Massao Hirabara[d] Hannelore Daniel[c] Rui Curi[a,d] Tania Cristina Pithon-Curi[d]

[a]Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil, [b]Post-graduate Program in Nutrition, Physical Activity and Phenotypic Plasticity, Federal University of Pernambuco, Vitoria de Santo Antao, Brazil, [c]Nutritional Physiology, Technische Universität München, München, Germany, [d]Interdisciplinary Post-graduate Program in Health Sciences, Cruzeiro do Sul University, Sao Paulo, Brazil, [e]FoRC – Food Research Center, Department of Food and Experimental Nutrition, School of Pharmaceutical Sciences, University of Sao Paulo, Sao Paulo, Brazil, [f]Institute of Metabolic Science, University of Cambridge, Cambridge, United Kingdom

Key Words
Amino acids • β-aminoisobutyric acid • Glutamine metabolism • Skeletal muscle cells • Protein breakdown

Abstract
Background/Aims: Skeletal mass loss is reported in several catabolic conditions and it has been associated with a reduced intracellular L-glutamine content. We investigated the association of intracellular L-glutamine concentration with the protein content in skeletal muscle cells. ***Methods:*** We cultivated C_2C_{12} myotubes in the absence or presence of 2 (reference condition), 8 or 16 mM L-glutamine for 48 hours, and the variations in the contents of amino acids and proteins measured. We used an inhibitor of L-glutamine synthesis (L-methionine sulfoximine - MSO) to promote a further reduction in intracellular L-glutamine levels. Amino acids contents in cells and media were measured using LC-MS/MS. We measured changes in phosphorylated Akt, RP-S6, and 4E-BP1contents in the absence or presence of insulin by western blotting. ***Results:*** Reduced intracellular L-glutamine concentration was associated with decreased protein content and increased protein breakdown. Low intracellular glutamine levels were also associated with decreased p-Akt contents in the presence of insulin. A further decrease in intracellular L-glutamine caused by glutamine synthetase inhibitor reduced protein content and levels of amino acids generated from glutamine metabolism and increased bAib still further. Cells exposed to high medium glutamine levels did not have any change in protein content but exhibited increased contents of the amino acids derived from L-glutamine metabolism. ***Conclusion:*** Intracellular L-glutamine levels per se play a role in the control of protein content in skeletal muscle myotubes.

Diogo Antonio Alves de Vasconcelos
Post-graduate Program in Nutrition, Physical Activity, and Phenotypic Plasticity, Federal University of Pernambuco, Alto do Reservatorio, 55608-680, Vitoria de Santo Antao, Pernambuco (Brazil)
Tel. (00 55 81) 21268463, Fax (00 55 81) 21268473, E-Mail vasconcelos.daa@gmail.com

Introduction

A loss of skeletal mass is associated with catabolic conditions such as diabetes, cancer, sepsis, and fasting, and it links to a reduced intracellular L-glutamine content in skeletal muscle [1-6]. In muscle wasting states, proteins are degraded raising intracellular levels of essential amino acids (EAAs), such as L-leucine, L-isoleucine, L-valine, L-phenylalanine, and L-methionine [6, 7]. Proteolysis is also associated with an increase in transcript levels of EAAs transporters that secondarily leads to a further elevation of these amino acids uptake (GAAC – General Amino Acids Control) by the cells [8]. Elevated levels of the branched-chain amino acids (BCAAs), L-leucine, L-isoleucine, and L-valine lead to elevated levels of the corresponding branched-chain keto acids (BCKAs), α-ketoisocaproic acid, α-keto-β-methylvaleric acid, and α-ketoisovaleric acid, respectively, and in turn of the intermediates of the Krebs cycle such as α-ketoglutarate and oxalacetate [9, 10]. Oxaloacetate is also generated from L-aspartate via aspartate aminotransferase whereas α-ketoglutarate also originates from L-glutamate through glutamate aminotransferase or glutamate dehydrogenase [11, 12]. Asparagine synthetase can generate L-asparagine from L-aspartate as a precursor [11]. BCAAs are the primary source of nitrogen for the synthesis of L-glutamine and L-alanine in skeletal muscle [10, 13].

L-Glutamate donates nitrogen for the formation of L-ornithine, L-aspartate, L-alanine, L-arginine, L-proline, and even L-glutamine [10, 14, 15]. Glutamine synthetase catalyzes the conversion of L-glutamate to L-glutamine [3]. When the intracellular L-glutamine level is high, phosphate-dependent glutaminase forms L-glutamate that generates α-ketoglutarate, via glutamate dehydrogenase, to be oxidized into the Krebs cycle [11, 12]. L-Glutamine provides nitrogen for the synthesis of nucleotides, nucleic acids, and other amino acids [10, 15]. Glutamine is a critical factor to control cell cycle and protein metabolism in tumor cells [11, 16]. However, what controls intracellular glutamine levels and how it links to protein homeostasis remains to be clarified.

Signaling pathways, such as mTORC1 regulate protein turnover [17, 18]. Serine 473 phosphorylated Akt indirectly (through TSC complex) activates mTORC1 that in turn causes phosphorylation of the downstream proteins RPS6 and 4E-BP1 that up-regulate protein synthesis [17]. The L-glutamine export through the plasma membrane leads to the uptake of leucine, which activates mTOR1 signaling pathway increasing protein synthesis and decreasing autophagy [16]. Products of L-glutamine metabolism such as α-ketoglutarate, aspartate, and asparagine, activate mTOR1 signaling [11, 12]. Intracellular L-glutamine deprivation induces endoplasmic reticulum (ER) stress and pro-inflammatory chemokine production via the mTOR-JNK pathway leading to depletion of the Krebs cycle intermediates [19]. L-Glutamine starvation activates GAAC pathway that up-regulates amino acid transporters and increases EAA uptake [20]. Both L-glutamine and L-leucine directly regulate mTORC1 pathway; however, by different mechanisms [21]. Leucine activates mTORC1 pathway via leucyl-tRNA synthetase [22]. Intracellular L-glutamine levels control protein metabolism in several cell types, including breast-sarcoma, osteosarcoma, kidney cells, and fibroblasts [11, 16, 19, 21]. This issue, however, remains to be addressed in skeletal muscle cells. L-Glutamine can reduce muscle atrophy induced by TNF-α via p38 MAPK in myotubes [23].

In the last decade, the modulating effects of glutamine on protein metabolism in several cell types were documented [11, 12, 16, 19-21]. We cultivated C_2C_{12} myotubes in the absence or presence of 2 (reference condition), 8 or 16 mM L-glutamine for 48 hours, and the variations in the contents of amino acids and proteins measured. We used an inhibitor of L-glutamine synthesis (L-methionine sulfoximine - MSO) to promote a further reduction in intracellular L-glutamine levels. Reduced intracellular glutamine levels led to decreased protein content, associated with increased protein degradation and decreased p-Akt content in the presence of insulin. Large L-glutamine concentration, in turn, was not able to change protein content; however, it promoted an elevation in glutamine metabolism. Intracellular

L-glutamine level per se does play a significant role in protein turnover in skeletal muscle myotubes.

Materials and Methods

Myotubes (C_2C_{12}) culture conditions

C_2C_{12} cells were cultured in DMEM with 5.5 mM glucose and 2 mM L-glutamine at 37°C and 5% CO_2 atmosphere conditions. DMEM was supplemented with fetal bovine serum (FBS) at 10% for the growth phase (48 hours) and with horse serum [24] at 2% for the differentiation phase (96 hours). After 5 days of the cell differentiation protocol, myotubes were cultured in medium with horse serum [24] at 2% for 48 hours in different conditions: absence of glutamine, absence or presence of the inhibitor of glutamine synthetase (L-methionine sulfoximine - MSO at 0.1 mM) [25] or in the presence of 2, 8 or 16 mM glutamine. Only medium (DMEM plus 2% HS) without cells was incubated for 48 hours in different L-glutamine concentration conditions to monitor amino acids degradation. Myotubes were washed and scraped off from the plates to measure intracellular amino acids and protein contents. Myotubes and medium without cells were collected separately for the measurement of the amino acid composition and calculation of amino acid cell uptake and export.

Quantitative analysis of amino acids in myotubes and culture medium

We used LC-MS/MS system (Agilent Technologies 1200 Series, Agilent Technologies GmbH, Germany) to measure amino acids composition of C_2C_{12} myotubes supernatant (DMEM from 48 h-cultured myotubes) and medium (48 h-cultured DMEM without cells). Myotubes were scraped off the plates using 80% aqueous methanol solution at 20°C. Samples were transferred into a tube, dried in a centrifugal vacuum concentrator (SPD 111V SpeedVac, Thermo Savant, Germany), and stored at -80°C. The *aTRAQ*™ kit *(*Reagent Kit 200 Assay, Applied Biosystems*,* Foster City, CA, EUA*)* was used for derivatization as previously described [26]. Amino acid mass analysis was performed using multiple reaction-monitoring mode systems [9]. The Analyst® 1.5 software (Applied Biosystems, Foster City, CA, EUA) was used to determine amino acids concentrations. The amino acids norleucine and norvaline were used for quality control of workflow and labeling efficiency. Amino acid concentrations were normalized to total protein concentration as determined by the Bradford assay [27].

Calculations of uptake and export of amino acids

The content of each amino acid measured in the supernatant of cultured myotubes was subtracted from the content in the medium without myotubes kept for the same period in culture conditions to calculate uptake and export of amino acids in cultivated cells. A positive value indicates that the concentration of the amino acid in medium without cell is higher than the concentration in the supernatant of cultured myotubes; therefore, the amino acid was uptaken by the cells. We found a negative value when the concentration of the amino acid in medium without cells is lower than that in the supernatant of cultured myotubes, and so indicates cell export.

Western blotting analysis

C_2C_{12} myotubes were scraped off the plates and homogenized in lysis buffer containing protease and phosphatase inhibitors. The same procedure is in our previous studies [6, 28, 29]. Antibodies were diluted (1:1000) in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% BSA (bovine serum albumin). The primary antibodies used were: p-Akt (Ser^{473}) - rabbit – monoclonal (Cell Signaling; 1:1000 #9271S); p-RPS6 ($Ser^{240/244}$) - rabbit – monoclonal (Cell Signaling; 1:1000 #61H9); and p-4E-BP1 ($Thr^{37/46}$) - rabbit – monoclonal (Cell Signaling; 1:1000 #2855S). Membranes were incubated overnight with primary antibodies, followed by 1 h incubation with the corresponding secondary antibody (1:5000) linked to horseradish peroxidase. After a final wash step, membranes incubated with a substrate for peroxidase and chemiluminescence enhancer (ECL Western Blotting System Kit, GE Healthcare, Little Chalfont, Buckinghamshire, England). Luminescent band intensities were quantified using optical densitometry (ImageJ 1.37, Wayne Rasband, NIH, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/). Results were normalized to sample total protein content as determined by Ponceau S staining and presented as arbitrary units. In our

previous studies [6, 28, 29] and in the others [28-31] the use of the same procedure was reported. Previous *in vitro* studies reported no changes in the content of total insulin signaling proteins [32-36] and highlighted changes in the phosphorylated proteins.

Insulin stimulating effect

We used C_2C_{12} myotubes cultivated in various L-glutamine concentration conditions (no addition or 2, 8 or 16 mM) during 48 hours and treated with insulin (100 nM) in the last hour of the culture period [16, 37]. We determined the contents of phosphorylated Akt, RP-S6, and 4E-BP1 in cells cultivated in the absence or presence of insulin by western blotting.

Statistical analysis

The GraphPad Prism 5 software (Graph Pad Software, Inc., San Diego, CA, USA) was used to perform statistical analysis. Results of 0 mM and 0 mM plus MSO addition conditions were compared using Student's t-test. We compared the results of no addition (0 mM) and the addition of L-glutamine at 8 or 16 mM with results of 2 mM L-glutamine (reference condition). The latter is the usual concentration of L-glutamine in cell culture medium [11]. Findings of different L-glutamine concentration conditions (absence – 0 mM or in the presence of 2, 8 or 16 mM), treated or not with insulin, were analyzed using one-way ANOVA and Tukey post-test. The α adopted was 0.05. We confirmed that the data were normally distributed and excluded outliers by applying Grubbs's test.

Results

Protein content in C_2C_{12} myotubes

Total protein content was very similar in cells cultured with 2, 8 or 16 mM L-glutamine, whereas a reduction in protein content by 54% occurred in cells cultivated in medium without L-glutamine (Fig. 1A). Total contents of the following amino acids, that can be generated from proteolysis (Fig. 1B), were higher in cells cultivated in the absence of L-glutamine (0 mM) as compared with 2 mM: L-leucine (by 85%), L-isoleucine (by 92%), L-valine (by 115%), L-phenylalanine (by 125%), L-threonine (by 76%), L-tryptophan (by 121%), L-histidine (by 124%), L-tyrosine (by 131%), L-lysine (by 126%), L-serine (by 76%), and glycine (by 144%) (Table 1). Concentrations of amino acids and derivatives that are not further metabolized such as β-aminoisobutyric acid (by 168%), taurine (by 141%), o-phosphoethanolamine (by

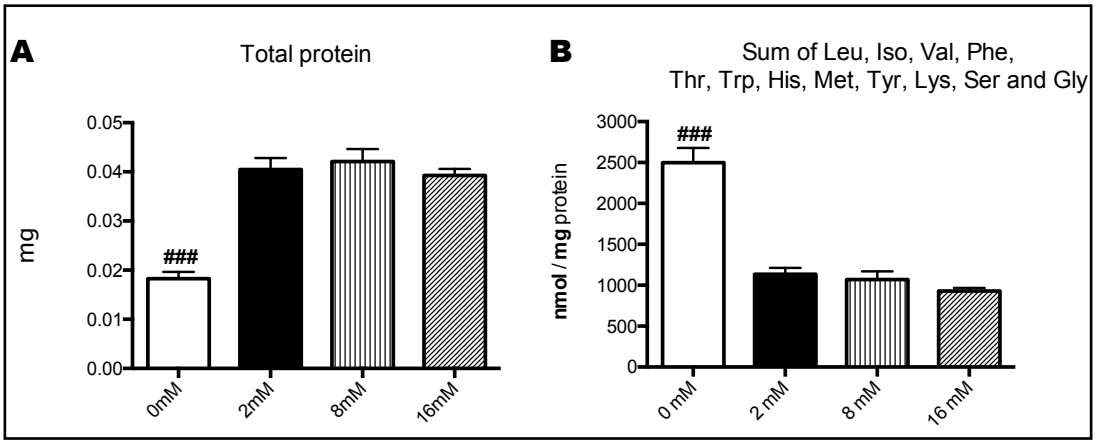

Fig. 1. Total protein and amino acids contents in C_2C_{12} myotubes cultivated in various glutamine concentrations (0, 2, 8 or 16 mM) for 48 hours. (A) Total protein and (B) total amino acids (Sum of Leu, Iso, Val, Phe, Thr, Trp, His, Met, Tyr, Lys, Ser, and Gly) contents. The experimental groups are: (0 mM) no glutamine addition in the medium; 2 mM (reference condition), 8 mM or 16 mM L-glutamine concentration in the medium. We presented the findings as to the mean ± SEM of three independent experiments. Results were analyzed using one-way ANOVA and Tukey post-test. ($^{###}p<0.001$) as compared with 2 mM glutamine.

158%) and the dipeptide carnosine (by 207%) also showed significant elevations in levels in the absence of medium glutamine. Findings in cells cultured in 8 or 16 mM L-glutamine as compared to 2 mM suggest that no significant differences in uptake or export of amino acids by the cells took place (Table 1).

Glutamine metabolism

The content of amino acids generated from L-glutamine (defined as the sum of Glu, Ala, Asp, Asn, Pro, and Arg) decreased by 53% in the absence of L-glutamine as compared to 2 mM whereas, at 8 or 16 mM L-glutamine in medium, these amino acids remained, in essence, unchanged (Fig. 2). In contrast, L-glutamine increased by 273% and 445%, respectively, by elevation of glutamine levels in the medium from 2 mM to 8 or 16 mM. In the absence of glutamine in medium, intracellular levels decreased by 94% when compared with 2 mM (Table 2 and Supplementary Fig. S1A – for all supplemental material see www.cellphysiolbiochem.com). Levels of L-glutamate remained unaltered in cells exposed to 8 or 16 mM L-glutamine but exhibited increased cell uptake, whereas a reduction by 74% and a raised uptake occurred in the absence of medium L-glutamine (Table 2 and Supplementary Fig. S1B).

Table 1. Intracellular amino acids levels in C_2C_{12} myotubes cultivated in various glutamine concentrations (0, 2, 8 or 16 mM) during 48 hours. Amino acids uptake (positive value = [amino acid in medium without myotubes] – [amino acid in medium with myotubes] > 0) or export (negative value = [amino acid in medium without myotubes] – [amino acid in medium with myotubes] < 0) were calculated. The table refers to the following amino acids and derivatives: L-leucine, L-isoleucine, L-valine, L-phenylalanine, L-threonine, L-tryptophan, L-histidine, L-methionine, L-tyrosine, L-lysine, L-serine and L-glycine; β-aminoisobutyric acid, taurine, o-phosphoethanolamine and the dipeptide carnosine. The experimental groups are: (0 mM) without glutamine in the medium; 2 mM (reference condition), 8 mM or 16 mM L-glutamine in the medium. Level of amino acids in intracellular are expressed as nmol/mg of protein and in the medium as μM. The results are presented as mean ± SEM of three independent experiments. The results were analyzed using one-way ANOVA and Tukey post-test. #p<0.05 versus 2 mM glutamine

Amino acid	0 mM	2 mM	8 mM	16 mM
Leucine	75.6 ± 5.9 #	40.7 ± 5.0	41.4 ± 4.6	32.9 ± 1.6
Uptake				
Export	5782 ± 511.4 #	2009 ± 135.5	1324 ± 203.2	2306 ± 526.4
Isoleucine	65.9 ± 5.2 #	34.2 ± 4.5	34.0 ± 3.6	27.7 ± 0.9
Uptake				
Export	4728 ± 345.8 #	2053 ± 206.0	1429 ± 62.0	1532 ± 442.8
Valine	114.0 ± 11.6 #	52.8 ± 5.2	50.3 ± 6.6	41.9 ± 2.8
Uptake				
Export	4020 ± 514.1 #	1571 ± 292.2	180.7 ± 431.7	1037 ± 317.3
Phenylalanine	65.27 ± 7.206 #	29.0 ± 3.4	29.4 ± 2.9	23.86 ± 1.2
Uptake				
Export	105.8 ± 588.5	465.6 ± 159.2	-402.9 ± 220.1	292 ± 414.2
Threonine	350.1 ± 34.2 #	198.8 ± 17.6	156.1 ± 9.9	127.1 ± 3.3
Uptake				
Export	-477.3 ± 331.7	904.3 ± 267.9	-354.7 ± 658.8	304.5 ± 715.4
Tryptophan	18.5 ± 2.0 #	8.3 ± 0.7	8.4 ± 0.7	7.1 ± 0.4
Uptake				
Export	1.0 ± 39.2	96.7 ± 5.7	-61.3 ± 73.0	110.3 ± 54.6
Histidine	36.52 ± 3.4 #	16.25 ± 1.4	15.80 ± 1.4	13.36 ± 0.7
Uptake				
Export	-244.9 ± 330.1	-36.8 ± 100.4	-856.1 ± 453.8	371 ± 151.4
Methionine	20.0 ± 4.1	12.4 ± 1.8	9.8 ± 2.3	8.5 ± 0.7
Uptake				
Export	-66.8 ± 1369	648.8 ± 477.6	628.4 ± 520.5	763.8 ± 351.8
Tyrosine	18.5 ± 2.0 #	8.3 ± 0.7	8.4 ± 0.7	7.1 ± 0.4
Uptake				
Export	-79.5 ± 341.6	311.4 ± 92.1	-300.1 ± 227.5	114.7 ± 375.8
Lysine	121.8 ± 11.3 #	53.8 ± 5.0	45.29 ± 6.6	33.2 ± 0.8
Uptake				
Export	934.8 ± 170	988.7 ± 77.0	-338.7 ± 437.3	678.8 ± 421.3
Serine	124.2 ± 10.1 #	70.6 ± 4.1	81.9 ± 13.2	68.4 ± 6.7
Uptake				
Export	8321 ± 514.4 #	3280 ± 239.8	1135 ± 1242	2396 ± 63.5
Glycine	1440 ± 97.6 #	589.8 ± 40.7	566.7 ± 54.3	520.6 ± 21.1
Uptake				
Export	-7397 ± 733.0 #	-1807 ± 197.2	-2628 ± 524.5	-1942 ± 249.8
Derivatives from amino acids				
bAib	574.9 ± 43.4 #	214.0 ± 17.7	224.3 ± 4.6	224.1 ± 5.4
Taurine	838.8 ± 33.4 #	347.1 ± 24.4	362.1 ± 32.5	388.1 ± 19.3
PEtN	127.3 ± 14.6 #	49.2 ± 3.9	38.1 ± 3.2	26.0 ± 0.3
Carnosine	37.6 ± 7.6 #	12.3 ± 0.7	11.9 ± 2.0	11.0 ± 0.4

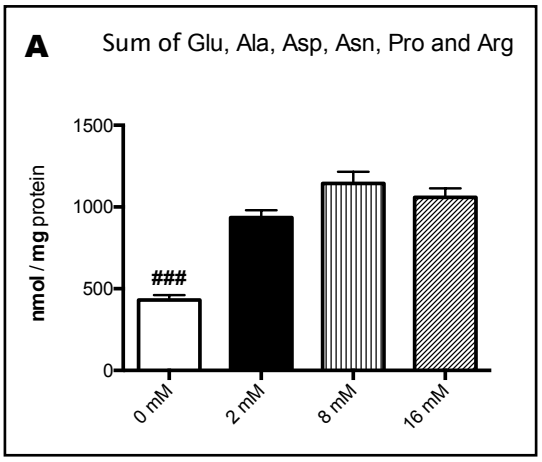

Fig. 2. Contents of amino acid produced from glutamine metabolism in C_2C_{12} myotubes cultivated in various glutamine concentrations (0, 2, 8 or 16 mM) during 48 hours. The Fig. refers to the sum of amino acids derived from glutamine metabolism (Gln, Glu, Asp, Asn, Ala, Pro, Orn, and Arg). The experimental groups are: (0 mM) no glutamine addition in the medium; 2 mM (reference condition), 8 mM or 16 mM L-glutamine concentration in the medium. The values are as the mean ± standard error of three independent experiments. Results were analyzed using one-way ANOVA and Tukey post-test. ($^{\#}p<0.001$) compared with 2 mM glutamine.

L-Glutamine at 8 or 16 mM in the culture medium only slightly increased L-alanine and L-aspartic acid intracellular concentrations but significantly raised L-asparagine (by 55% at both concentrations) and L-proline (8 mM - by 117%; 16 mM - by 179%; both had increased export) contents as compared to 2 mM. The absence of L-glutamine in the medium led to a reduction in intracellular concentration of L-aspartic acid (by 47%) and L-alanine (by 60%) and an increase in L-ornithine and L-arginine contents (both by 146%) but had no significant effect on L-proline and L-asparagine concentrations (Table 2).

Table 2. Intracellular amino acids contents were measured in C_2C_{12} myotubes cultivated in various glutamine concentration conditions (no addition, 2, 8 or 16 mM) for 48 hours. Amino acids uptake (positive value = [amino acid in medium without myotubes] – [amino acid in medium with myotubes] > 0) or export (negative value = [amino acid in medium without myotubes] – [amino acid in medium with myotubes] < 0) were calculated. Table refers to the following amino acids: L-glutamine, L-glutamate, L-alanine, L-aspartate, L-asparagine, L-alanine, L-proline, L-ornithine and L-arginine. The experimental groups are: (0 mM) no glutamine addition to the medium; 2 mM (reference condition), 8 mM or 16 mM L-glutamine concentration in the medium. Level of amino acids in intracellular are expressed as nmol/mg of protein and in the medium as μM. The findings are presented as mean ± SEM of three independent experiments. Results were analyzed using one-way ANOVA and Tukey post-test. ($^{\#}p<0.05$) as compared with 2 mM glutamine

Amino acid	0 mM	2 mM	8 mM	16 mM
Glutamine	11.5 ± 1.2 #	206.4 ± 27.8	770 ± 24.9 #	1125 ± 57 #
Uptake				
Export	-894.2 ± 51.7	6418 ± 341.1	-9843 ± 5170	-30870 ±16142
Glutamate	148.7±14.3 #	580.5 ± 32.6	662.9 ± 32.6	571.4 ± 38.4
Uptake				
Export	253.2 ± 3.1 #	161.7 ± 5.2	398.8 ± 13.6 #	848.0 ± 19.9 #
Aspartate	64.0 ± 5.7 #	121.9 ± 5.8	141.9 ± 6.9	141.8 ± 5.1
Uptake				
Export	61.1 ± 17.7	44.2 ± 11.3	33.0 ± 7.5	42.5 ± 6.6
Asparagine	17.1 ± 2.5	17.3 ± 0.7	27.0 ± 1.8 #	27.1 ± 2.1 #
Uptake				
Export	-55.6 ± 9.2	-240.4 ± 17.8	-590.0 ± 175.7	-650.6 ± 81.3
Alanine	58.9 ± 4.7 #	144.9 ± 8.9	198.5 ± 24.6	194.3 ± 11.5
Uptake				
Export	-2829 ± 577.2	-4963 ± 38.0	-8696 ± 637.9 #	-10010 ± 864.4 #
Proline	51.6 ± 6.5	33.5 ± 1.9	72.7 ± 7.2 #	93.7 ± 7.6 #
Uptake				
Export	-535.3 ± 15.2	-304.5 ± 37.6	-1171 ± 210.2 #	-1641 ± 280.1 #
Ornithine	16 ± 1.1 #	6.5 ± 0.4	14.1 ± 3.9	11.9 ± 1.2
Uptake				
Export	-293.1 ± 31.4	-162.7 ± 8.1	-515.5 ± 234.3	-347.5 ± 58.9
Arginine	75.1 ± 5.1 #	30.5 ± 2.9	26.6 ± 3.1	18.3 ± 1.2
Uptake				
Export	-195.6 ± 301.6	1064 ± 147.6	-4.5 ± 438.9	1330 ± 367.6

Pronounced reduction of intracellular glutamine level by inhibition of glutamine synthesis

C_2C_{12} myotubes cultured in the absence of L-glutamine and with the addition of MSO did not show any change in the total amino acid content and amino acids generated from protein breakdown (data not shown). However, the addition of MSO in the absence of glutamine reduced by 22%

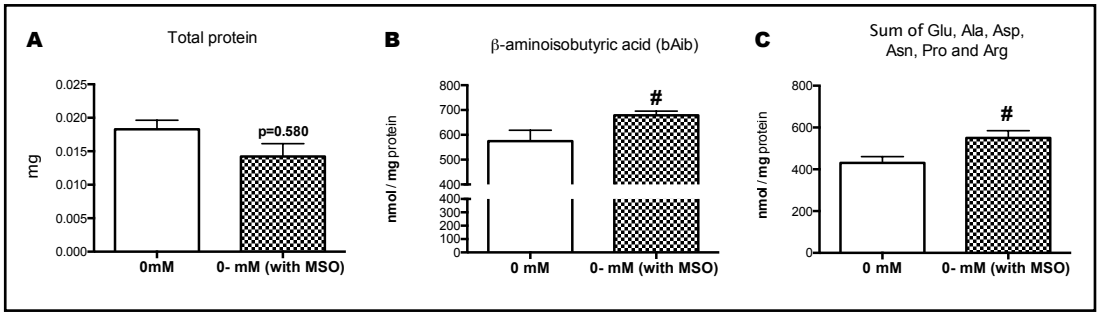

Fig. 3. Contents of total protein, β-aminoisobutyric acid and total amino acids produced from glutamine metabolism in C_2C_{12} myotubes cultivated in the absence of glutamine (no addition and no addition plus glutamine synthase inhibitor) for 48 hours. Fig.s refer to the following measurements: (A) total protein, (B) total amino acids produced from glutamine/glutamate metabolism (Sum of Glu, Asp, Asn, Ala, Pro, Orn, and Arg concentrations), and (C) β-aminoisobutyric acid. The experimental groups are: (0 mM) no glutamine addition in the medium and (0 mM with glutamine synthase inhibitor) no glutamine addition in the medium with the glutamine synthase inhibitor (L-methionine sulfoximine – MSO at 0.1 mM). Results are presented as mean ± standard error and were analyzed using Student's t-test ($^{\#}p<0.05$).

the total protein content (p=0.58) and increased by 18% the levels of β-aminoisobutyric acid and by 25% the amino acids derived from L-glutamine metabolism (Fig. 3). These changes occurred concomitant to a decrease in intracellular L-glutamine content (by 35%, p=0.05; decreased export) (Fig. 4A). The mentioned condition (no glutamine + MSO) also led to an increased concentration of L-glutamate (by 45%; a decreased uptake), L-alanine (by 31%; increased export), L-aspartate (by 42%), and L- ornithine (by 79%) (Fig. 4A) but it did not change L-asparagine level (increased export) (Fig. 4B). A high correlation between intracellular L-glutamine concentration and its derived amino acids (L-glutamate, L-alanine, L-aspartate, L-asparagine, and L-proline contents) existed (Fig. 4, 5 and Supplementary Fig. S1A).

Contents of phosphorylated Akt, RP-S6, and 4E-BP1 in myotubes cultivated in the absence or presence of insulin

We investigated the effects of intracellular L-glutamine levels on protein synthesis signaling (phosphorylated Akt, RP-S6, and 4E-BP1) by western blotting analysis (Supplementary Fig. S2). Insulin did not raise phosphorylated Akt content in the absence of extracellular glutamine (0 mM) (Fig. 6A). In opposition, in the presence of 2, 8 or 16 mM L-glutamine, insulin increased the content of phosphorylated Akt by 75%, 78%, and 67%, respectively, as compared to the control group. At 16 mM L-glutamine and in the presence of insulin, the phosphorylated Akt content was 84% higher than in the reference condition (2 mM) (Fig. 6A). The p-RPS6 content was increased by insulin in all conditions; in the absence (by 92%) and at 2 (by 109%), 8 (by 154%) or 16 mM (by 208%) L-glutamine (Fig. 6B). There was no change in the p-4EBP1 content in the conditions of this study (data not shown).

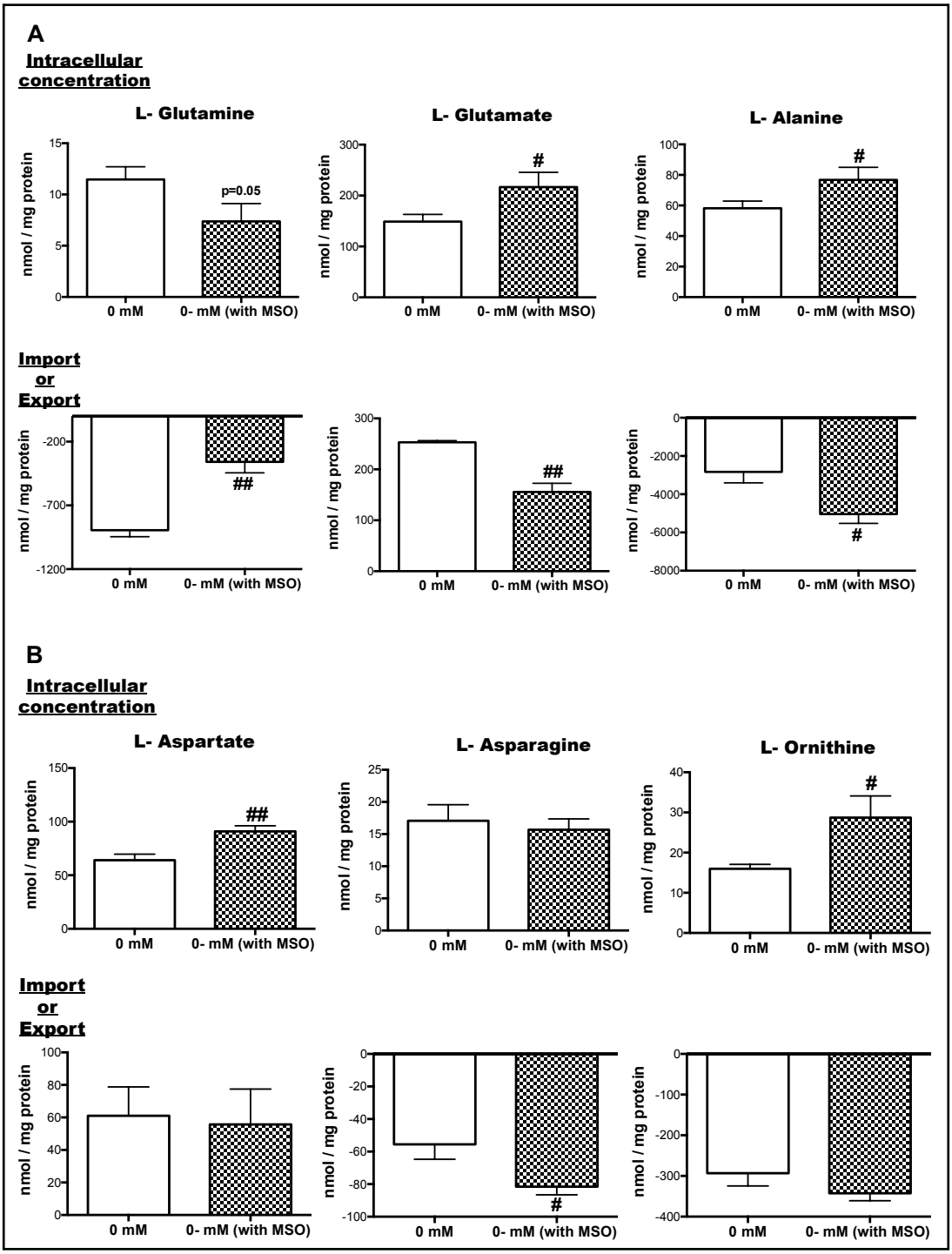

Fig. 4. Intracellular amino acid contents, amino acid uptake (positive) or export (negative) measured in C_2C_{12} myotubes cultivated in the absence of glutamine (no addition and no addition plus glutamine synthase inhibitor) for 48 hours. Fig.s refer to the following measurements: (Above) intracellular content of amino acids. (below) Uptake (positive) or export (negative) of amino acids in cells; (A) L-glutamine, L-glutamate and L-alanine; (B) L-aspartate, L-asparagine, and L-ornithine. The experimental groups are: (0 mM) no glutamine addition in the medium; (0 mM with glutamine synthase inhibitor) no glutamine addition in the medium with the glutamine synthase inhibitor (L-methionine sulfoximine – MSO at 0.1 mM). Results are presented as mean ± standard error and were analyzed using one-way ANOVA and Tukey post-test. ($^{\#}p<0.05$; $^{\#\#}p<0.01$) compared with 2 mM glutamine.

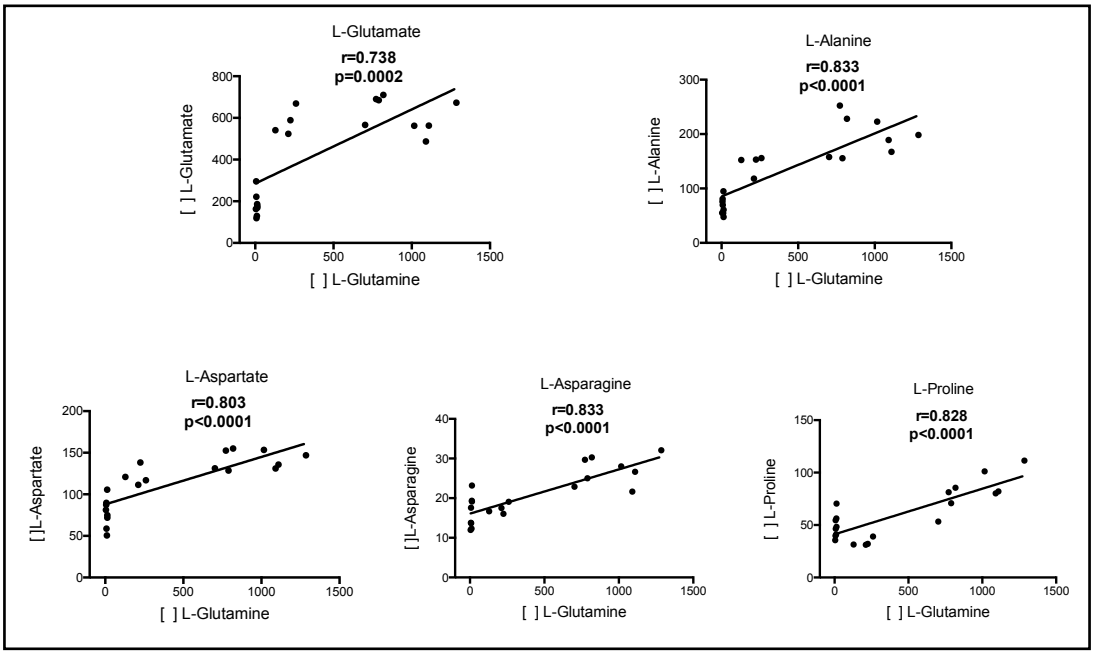

Fig. 5. Correlation between intracellular L-glutamine levels and contents of amino acids produced from glutamine metabolism in C_2C_{12} myotubes cultivated in the absence of glutamine (no addition and no addition plus glutamine synthase inhibitor) for 48 hours. Fig.s refer to the following amino acids: L-glutamine, L-alanine; L-aspartate, L-asparagine, and L-proline. The experimental groups are: (0 mM) no glutamine addition in the medium; (0 mM with glutamine synthase inhibitor) no glutamine addition in the medium with the glutamine synthase inhibitor (L-methionine sulfoximine – MSO at 0.1 mM). Results were analyzed using Pearson correlation (Pearson r>0.07).

Discussion

Intracellular L-glutamine level is involved in the control of protein synthesis [11, 16, 19-21] in several cell types *in vitro*. Similar studies were lacking in skeletal muscle cells. C_2C_{12} myotubes were exposed to different (0, 2, 8, and 16 mM) L-glutamine concentrations for 48 h. Levels of total proteins, amino acids, amino acid metabolism derived products and phosphorylated Akt, RP-S6, and 4E-BP1 were then determined.

In the absence of L-glutamine, a decrease in total protein content and an increase in amino acids indicators of protein breakdown, Leu, Iso, Val, Phe, Thr, Trp, His, Tyr, Lys, Ser, and Gly [10], and also bAib, taurine, PtEN, and carnosine [38-41], were found. Glutamine at 2 mM concentration and higher abolished these effects and elevated phosphorylated Akt content in the presence of insulin. Amino acids derived from glutamine, namely glutamate, aspartate, asparagine, alanine, and proline, revealed elevated levels under increased glutamine concentrations.

In the absence of extracellular glutamine combined with glutamine synthesis inhibition by MSO, we found an even more pronounced decrease of protein content. Although this treatment did not change significantly the levels of amino acids released in the proteolysis, β-aminoisobutyric acid concentrations increased, mimicking a catabolic condition [42]. These findings support the proposition that intracellular L-glutamine concentration plays a role in the control of protein content in myotubes. These changes were associated with accumulation of glutamine metabolism derived amino acids (glutamate, alanine, aspartate, and ornithine). The protein content is not rescued in human glioblastoma cells when asparagine replaces glutamine in the culture medium [12].

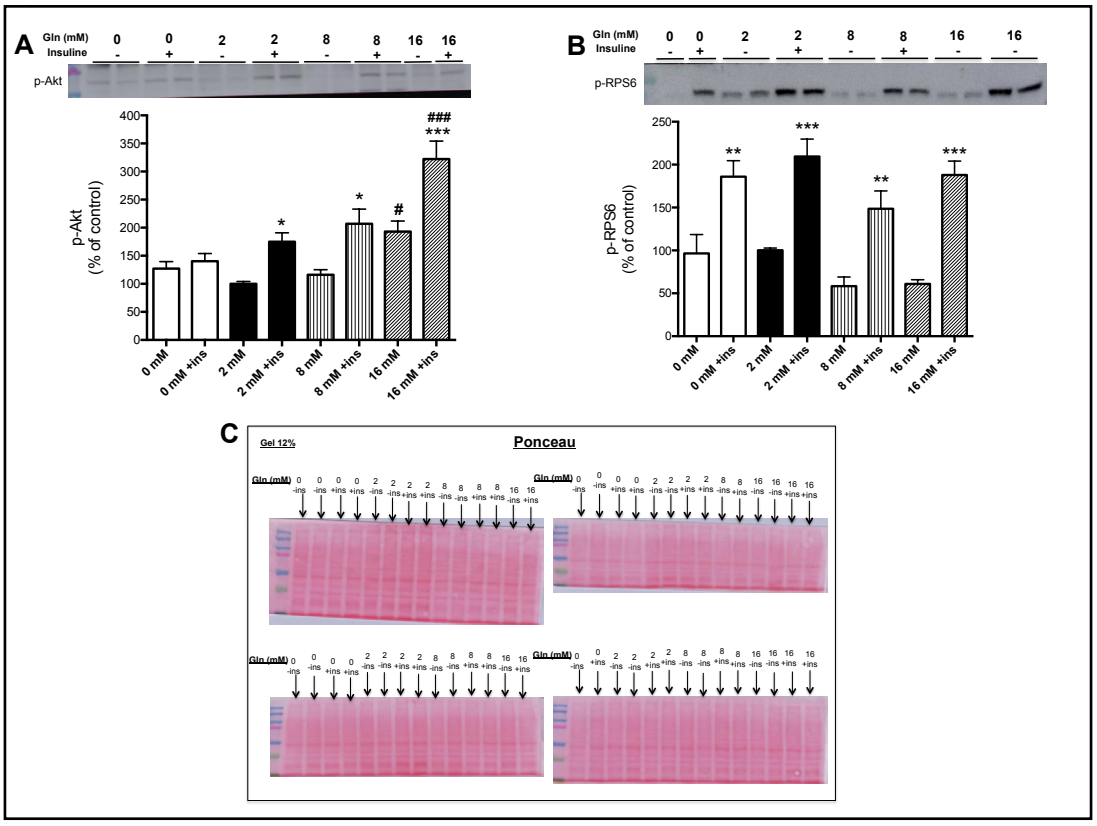

Fig. 6. Contents of phosphorylated Akt and RPS6 in C_2C_{12} myotubes cultivated in various glutamine concentration conditions (no addition or 2, 8 or 16 mM glutamine) for 48 hours stimulated with insulin (100nM) in the last hour. (A) p-Akt and (B) p-RPS6 contents. (C) Ponceau S staining of nitrocellulose membranes after protein transfer from 12% polyacrylamide gels. The experimental groups are: (0 mM) no glutamine addition in the medium; 2 mM (reference condition), 8 mM or 16 mM L-glutamine in the medium. The findings are as the mean ± SEM of three independent experiments. Results were analyzed using one-way ANOVA and Tukey post-test. (*p<0.05; **p<0.01; ***p<0.001) compared with the respective group without insulin; ($^{\#}$p<0.001) compared with 2 mM glutamine; ($^{\#\#\#}$p<0.001) compared with 2 mM glutamine plus insulin. Outliers were excluded by applying Grubbs's test. The western blotting images of the results are in the Supplementary Fig. S2.

L-Glutamine is an anaplerotic amino acid and feeds into various metabolic pathways associated with nitrogen and energy homeostasis [10, 12, 14]. A reduced intracellular L-glutamine content was associated with increased protein degradation as indicated by raised levels of the amino acids indicators of proteolysis in skeletal muscle (Leu, Iso, Val, Phe, Thr, Trp, His, Tyr, Lys, Ser, and Gly). However, these amino acids can be further metabolized [10]. There might be an increased uptake of L-leucine, L-isoleucine, L-valine, and L-serine associated with a reduced L-glutamine concentration as a compensatory mechanism to maintain intracellular essential amino acids concentrations. Accordingly, EAA uptake transporters were reported to have increased mRNA-levels in low glutamine levels [8]. The only glycine exhibited significant export from myotubes when glutamine concentration in the medium raised. The products of amino acids, bAib, taurine, and PEtN and the dipeptide carnosine [38-41, 43], which are not metabolized in muscle cells, also increased but were not exported. The amino acids indicators of proteolysis are donors of carbon and nitrogen groups to the synthesis of other amino acids. Herein, the intracellular concentrations of the amino acids were associated with changes in L-glutamine metabolism, uptake, and export.

At low intracellular L-glutamine concentration, phosphorylated RPS6 content, which is a downstream target of the mTOR signaling, was increased in the presence of insulin.

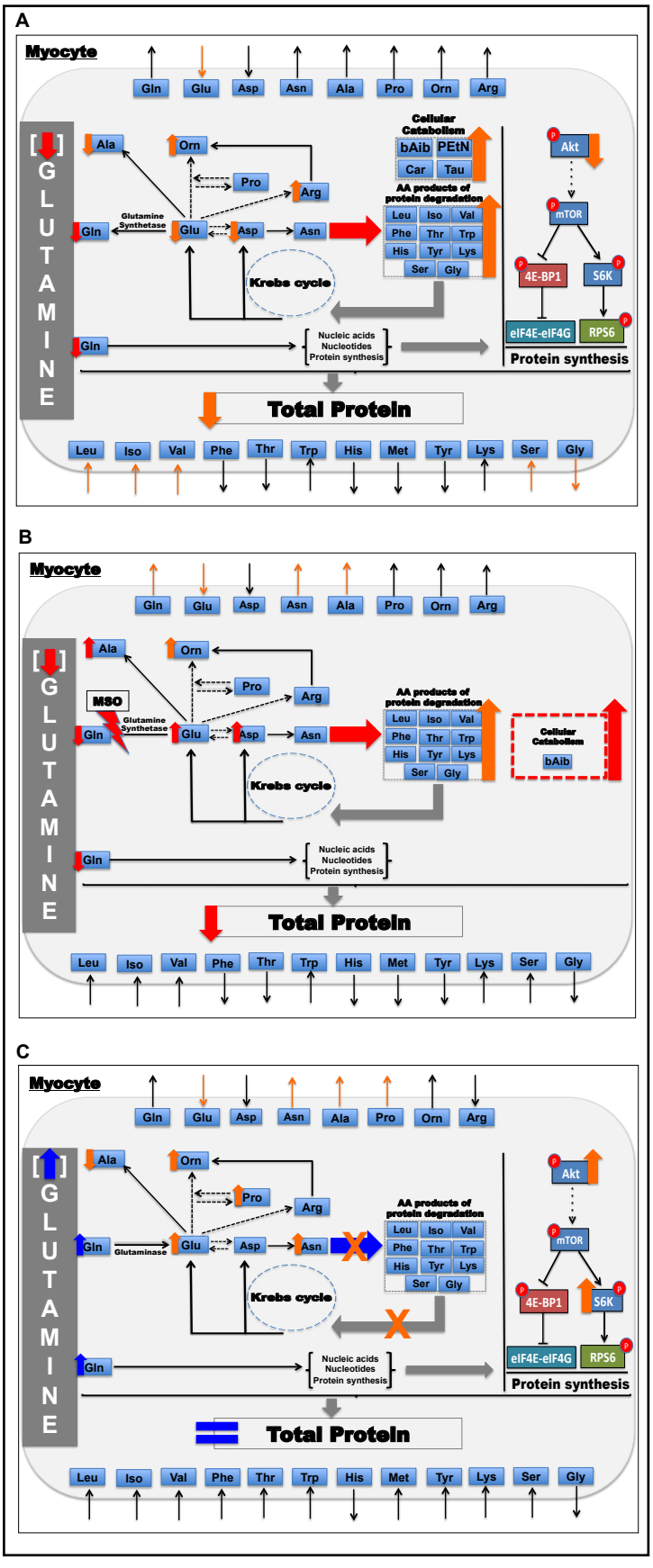

Fig. 7. Summary of the effects of intracellular glutamine level (A – reduced glutamine; B – reduced glutamine and inhibition of glutamine synthesis - schemes above) (C- increased glutamine - scheme below) on protein turnover in myotubes. Intracellular L-glutamine contents do change protein turnover. Black arrows indicate actions that did not have significance result; Orange arrows indicate actions that have significant result; dashed black arrows indicate actions that may occur in the myotubes.

This finding contrasts with observations in HeLa cells [16]. L-Glutamine has been reported to modulate other signaling processes such as the p38 MAPK pathway [23]. Although not assessed herein, the mTORC2-Akt-Foxo pathway is also involved in the control of protein homeostasis [18, 44-46]. These findings led us to postulate that low glutamine concentration reduces protein content partially by inducing protein breakdown, and Akt pathway is presumably involved in this effect in myotubes. One limitation of this study is that we did not measure the content of total Akt to allow the phosphorylated Akt to total Akt protein ratio calculation. Previous studies reported that under the conditions of this study, there is no change in total insulin signaling proteins, whereas there is a marked increase in the phosphorylated proteins forms [16, 32-35].

Increased intracellular L-glutamine levels correlated well with the cellular levels of amino

acids such as Glu, Asp, Asn, Ala, and Pro produced from glutamine metabolism [12, 15], and this was associated with increased phosphorylated Akt levels. However, we could not find evidence for an increased EAA uptake in contrast to what was reported by others in HeLa cells [16]. The high L-glutamine levels provided nitrogen and carbon groups to form L-glutamate, L-aspartate, L-alanine, and L-proline and increased the phosphorylated Akt levels but the period of treatment was probably not enough to enhance the total protein content significantly.

The inhibition of L-glutamine synthesis caused a further reduction in intracellular glutamine level, decreased the cellular protein content and increased levels of bAib that is a product of L-valine or thymine degradation (nucleobases in the nucleic acid of DNA) and is not further metabolized in muscle cells [39, 43]. We observed this catabolic scenario in low intracellular glutamine level [42]. The raised levels of EAA provide sufficient substrates for the Krebs cycle or via α-ketoglutarate for the synthesis of L-glutamate and L-glutamine. Blocking intracellular glutamine formation by MSO in a medium without glutamine led to the accumulation of L-glutamate, L-alanine, L-aspartate, and L-ornithine. The accumulation of these amino acids, however, does attenuate cellular protein catabolism as reported by others [12] These findings support the proposition of a direct role of L-glutamine concentration on control of cellular protein content. This effect of L-glutamine availability on protein homeostasis occurs concomitantly with its essential role as a precursor for nucleotide and nucleic acids synthesis in skeletal muscles.

In summary, reduced intracellular glutamine levels decreased the cellular protein content significantly, increased protein degradation, and decreased the phosphorylated Akt content in the presence of insulin. As compared to the reference condition (2 mM), an increase in L-glutamine concentration to 8 or 16 mM was not able to further enhance the cell protein content but elevated the contents of amino acids derived from glutamine metabolism. Intracellular L-glutamine levels per se play a significant role in the control of protein content in skeletal muscle myotubes (Fig. 7).

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Disclosure Statement

The authors have no conflicts of interest to declare.

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