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Antagonistic effects of leucine and glutamine on the mTOR pathway in myogenic C_2C_{12} cells

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Summary. This study compared the effects of leucine and glutamine on the mTOR pathway, on protein synthesis and on muscle-specific gene expression in myogenic C_2C_{12} cells. Leucine increased the phosphorylation state of mTOR, on both Ser2448 and Ser2481, and its downstream effectors, $p70^{S6k}$, S6 and 4E-BP1. By contrast, glutamine decreased the phosphorylation state of mTOR on Ser2448, $p70^{S6k}$ and 4E-BP1, but did not modify the phosphorylation state of mTOR on Ser2481 and S6. Whilst the phosphorylation state of the mTOR pathway is usually related to protein synthesis, the incorporation of labelled methionine/cysteine was only transiently modified by leucine and was unaltered by glutamine. However, these two amino acids affected the mRNA levels of desmin, myogenin and myosin heavy chain in a time-dependant manner. In conclusion, leucine and glutamine have opposite effects on the mTOR pathway. Moreover, they induce modification of muscle-specific gene expression, unrelated to their effects on the mTOR/$p70^{S6k}$ pathway.

Keywords: Amino acids – $p70^{S6k}$ – 4E-BP1 – protein synthesis – gene expression

1. Introduction

Amino acids are the building blocks for protein synthesis which they are also able to regulate by modulating signalling pathways. In many cell types, amino acids increase the phosphorylation state of p70 ribosomal S6 kinase ($p70^{S6k}$) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), two proteins downstream of the mammalian target of rapamycin (mTOR) and playing a key role in the control of protein synthesis initiation (for review see Proud, 2002; Jefferson and Kimball, 2003; Deldicque et al., 2005). The way by which amino acids stimulate $p70^{S6k}$ and 4E-BP1 is not fully understood but clearly involves the mTOR pathway, which senses nutrients and regulates transcription, translation and protein degradation (Rohde et al., 2001).

Leucine, a branched chain amino acid, seems to be a particularly effective anabolic agent. It is known to activate $p70^{S6k}$ in hepatocytes (Krause et al., 2002), pancreatic beta cells (Xu et al., 1998) and adipocytes (Fox et al., 1998). Leucine also increases the phosphorylation state of $p70^{S6k}$ in proliferating L6 (Kimball et al., 1999) and C_2C_{12} myogenic cells (Du et al., 2007). Recently, Nakajo et al. (2005) have studied the effect of 14 amino acids on $p70^{S6k}$ activity in intestinal epithelial cells. In this cell type, both leucine and arginine activated $p70^{S6k}$ whereas glutamine did not modify its activity. By contrast, glutamine reversed the activation of $p70^{S6k}$ induced by leucine and arginine when it was combined with one of these two amino acids. Unlike in hepatocytes (Krause et al., 2002), in which glutamine is able to activate $p70^{S6k}$ alone or in combination with leucine, glutamine negatively regulated the mTOR pathway in intestinal epithelial cells (Nakajo et al., 2005).

In addition to controlling protein translation and more particularly $p70^{S6k}$ and 4E-BP1, evidence that amino acids may also induce or repress gene transcription is growing. Amino acid deprivation upregulates genes involved in nutrient catabolism and energy production and downregulates genes participating in lipid and nucleotide synthesis and in protein synthesis, turnover and folding (Peng et al., 2002). In myogenic cells, amino acid sufficiency is required to activate IGF-II transcription via the mTOR pathway leading to enhanced differentiation (Erbay et al., 2003). Recent-

ly, the mTOR pathway has been found to be critical in the cardiac transcriptional response to glutamine. The addition of glutamine to cultured rat cardiomyocytes increased abundance of the mRNAs encoding contractile proteins and metabolic enzymes through the protein kinase A and mTOR cascades (Xia et al., 2003).

Since the effect of amino acids on the phosphorylation state of $p70^{S6k}$ has been reported in several cell types, but only a few papers present data supporting a stimulation of protein synthesis (Kimball et al., 1999; Mordier et al., 2000; Du et al., 2007), the purpose of the present work was to analyze the effect of leucine and glutamine on several proteins constituting or regulating the mTOR signalling pathway and their ability, separately or in combination, to modify the protein synthesis rate in differentiating myogenic C_2C_{12} cells. Since labelled methionine/cysteine incorporation was only affected to a minor extent by leucine, the second purpose of this work was to test if the changes observed in $p70^{S6k}$ phosphorylation could alter gene expression.

2. Materials and methods

2.1 Cell culture

C_2C_{12} murine skeletal muscle myoblasts (ATCC) were seeded in Petri culture dishes. They were grown in DMEM (Dulbecco's modified Eagle's medium, Life Technologies) supplemented with 10% fetal bovine serum, penicillin/streptomycin (5000 U/5000 µg/ml) and 100 µM non-essential amino acids. When cells were 70% confluent, the proliferation medium was replaced by a differentiation medium containing 1% horse serum. After 48 h of differentiation, cells were incubated for 1 h with serum-free DMEM lacking leucine and glutamine (Life Technologies). Leucine (5 mM), glutamine (5 mM) or both (5 mM each) (Sigma) were then added to the plates.

Phosphorylation states and protein synthesis. Cells were lysed after 30 min, 3 h and 24 h, in a buffer (pH 7.0) containing 20 mM Tris, 270 mM sucrose, 5 mM EGTA, 1mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM 1,4-dithiothreitol and a protease inhibitor cocktail (Roche Applied Science). The supernatants (10,000 g, 10 min) were stored at −80 °C and protein concentration was determined using the DC protein assay kit (Bio-Rad Laboratories) with BSA as standard.

mRNA levels. Cells were homogenized in 500 µl TRIZOL® after 1, 3 and 24 h of incubation with leucine and/or glutamine. Total RNA was extracted according to the instructions provided by the manufacturer (Invitrogen). RNA was quantified by spectrophotometry (260 nm) and its concentration adjusted to 1 µg/ µl using RNase-free water.

2.2 SDS/PAGE and immunoblotting

Proteins were separated by SDS/PAGE and transferred to a PVDF (Polyvinylidene Difluoride) membrane for immunoblot analysis except for 4E-BP1, which was transferred to a nitrocellulose membrane. Membranes were then incubated in a 5% Blotto solution. The following phospho-specific antibodies were added and incubated overnight at 4 °C in Blotto or in TBST (Tris-buffered saline with Tween-20) containing 1% BSA: mTOR (Ser 2448, Ser 2481; Cell Signalling), $p70^{S6k}$ (Thr 389; Santa Cruz), 4E-BP1 (Calbiochem), S6 (Ser235/236; Cell Signalling), eEF2 (Thr 56, provided by V. Stroobant from the Ludwig Institute, Brussels, Belgium) and eIF2B (Ser 535, Biosource) (Some abbreviations are defined in the legends to Figs. 4 and 6). Membranes were then incubated for 1 h at room temperature in secondary antibody conjugated to horseradish peroxidase (1:10000) in a 5% Blotto solution. Chemiluminescent detection was carried out using an ECL Western blotting kit (Amersham Biosciences). Then, the membranes were stripped and re-probed with a total antibody (total mTOR, Cell Signalling; total $p70^{S6k}$, Santa Cruz) to verify the relative amount of the analyzed proteins through the experiment. The films were scanned on an ImageScanner using the Labscan software (Amersham Biosciences) and quantified with the Image Master 1D Image Analysis Software (Amersham Biosciences). The results represent the phosphorylated form of the protein.

2.3 Protein kinase B activity

Protein kinase B (PKB) activity was measured by the phosphorylation of a synthetic peptide after immunoprecipitation (100 µg of protein extract) with a total PKB antibody recognizing the PH-domain (Upstate Cell Signalling) as previously described (Bertrand et al., 1999). To summarize, the assay was performed in a final volume of 50 µl in the presence of 10mM MOPS (pH 7.0), 0.5 mM EDTA, 10 mM Mg-acetate, 0.1% β-mercaptoethanol, 0.1 mM Mg-[γ-^{32}P] ATP (specific radioactivity 1000 cpm/pmol, Amersham Biosciences) and 0.25 mM substrate peptide RPRAATF (Alessi et al., 1996). The reaction was continued for 20 min at 30 °C. The supernatant was then spotted on to P81 phosphocellulose paper, followed by washes in cold 75 mM phosphoric acid. ^{32}P incorporation was counted in a scintillation counter (LS 6500, Beckman) for 1 min.

2.4 Adenosine monophosphate-activated protein kinase activity and acetyl-CoA carboxylase phosphorylation

Total adenosine monophosphate-activated protein kinase (AMPK) activity was measured according to the method described by Marsin et al. (2000). Acetyl-CoA carboxylase (ACC) phosphorylation, which is the best known substrate of AMPK, was assessed by immunoblotting to confirm the results on the AMPK activity (anti-phospho specific Ser 79 ACC was a generous gift from D. G. Hardie, Dundee, Scotland).

2.5 Incorporation of labelled [^{35}S] methionine/cysteine

Cells were grown in serum-free DMEM for 1 h before the experiment. Cells were then preincubated with 1 mM unlabelled methionine/cysteine and with either 5 mM leucine or 5 mM glutamine, alone or in combination for 15 min. After the preincubation period, 20 µl of a protein labelling mix (EasyTag™ Express Protein Labelling Mix, [^{35}S] methionine/cysteine mixture, NEN Life Science Products) were added to each plate. Cells were lysed after 30 min, 3 h and 24 h, centrifuged and proteins were precipitated with 10% trichloroacetic acid. The precipitated proteins were dissolved in 0.1 N NaOH and precipitated again. The final pellet was resuspended in 800 µl of formic acid. 5 ml of scintillating liquid (Ultima-gold, Perkin Elmer) was then added and ^{35}S incorporation was counted in a scintillation counter (LS 6500, Beckman) for 1 min.

2.6 Real time RT-PCR analyses

Reverse transcription (RT) was performed using the iScript synthesis kit (Bio-Rad) on a MyIQ thermal cycler (Bio-Rad) with 1 µg of total RNA in a reaction volume of 20 µl (4 µl iScript reaction mix 5×, 1 µl iScript reverse transcriptase, 1 µl RNA template, 14 µl RNase-free water). Primers were designed (Table 1) for mouse desmin, myogenin, caveolin 3 and myosin heavy chain type II (MHC II) and β-actin. Quantitative real-time polymerase chain reaction (PCR) was performed with the Sybr Green® PCR Core kit or master mix (Eurogentec) on a MyIQ thermal cycler (Bio-Rad) using the following cycle conditions: 10 min at 95 °C, followed by 40 cycles of 1 min at 60 °C and 15 sec at 95 °C. For each gene, real time RT-PCR was conducted in duplicate. The ΔCt values were calculated in every

Table 1. Primer sequences

	Forward	Reverse
Desmin	CAGGACCTGCTCAATGTGAA	GCTGGTTTCTCGGAAGTTGA
Myogenin	GTGCCCAGTGAATGCAACTC	ACGATGGACGTAAGGGAGTG
Caveolin 3	GCTCGGATCATCAAGGACAT	ACCTTCCATACACCGTCGAA
MHC II	GAACCCTCCCAAGTACGACA	GCATAACGCTCTTTGAGGTTG
β-actin	TCCTGAGCGCAAGTACTCTGT	CTGATCCACATCTGCTGGAAG

Sequences of primers (5′–3′) used for mRNA quantification by real-time RT-PCR. MHC II, myosin heavy chain type II

sample for each gene of interest as follows: $Ct_{\text{gene of interest}} - Ct_{\text{reporter gene}}$, with β-actin as the reporter gene. Calculation of relative changes in the expression level of one specific gene ($\Delta\Delta Ct$) was performed by subtraction of ΔCt from the control conditions to the corresponding ΔCt from the leucine and/or glutamine conditions. The mRNA results are presented as $2^{-\Delta\Delta Ct}$.

2.7 Statistical analysis

The effect of leucine and glutamine was tested by unpaired Student's *t*-test. The significance threshold was set to $P < 0.05$. The results are presented as the means ± SEM.

3. Results

3.1 Opposite effects of leucine and glutamine on the mTOR pathway

Incubation of C_2C_{12} cells for 30 min in the differentiation medium supplemented with 5 mM leucine increased more than 10 fold the phosphorylation state of $p70^{S6k}$ on Thr 389 ($P < 0.01$, Fig. 1A) and almost doubled the percentage of 4E-BP1 in the phosphorylat-

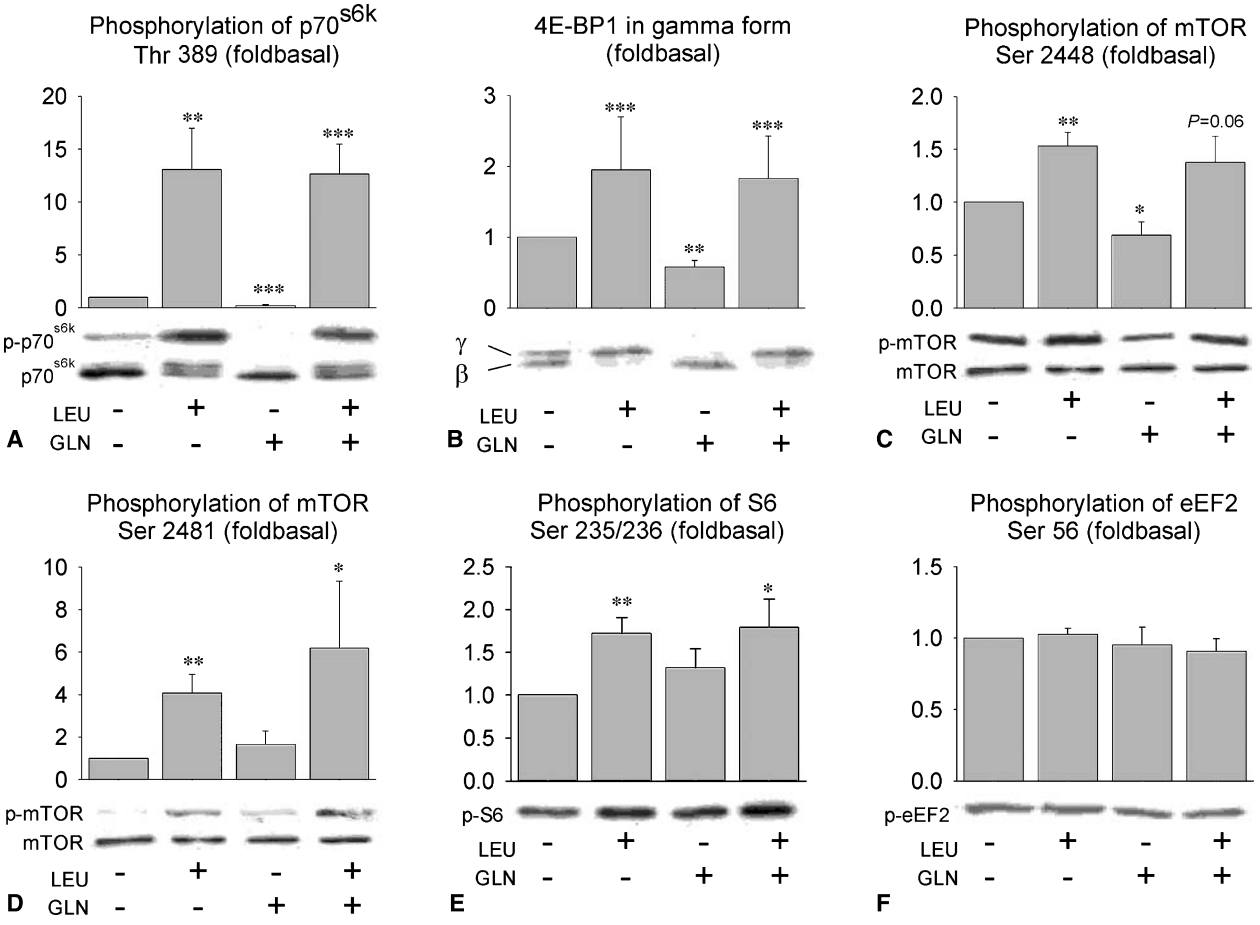


Fig. 1. Effect of leucine, glutamine, alone or in combination, on the phosphorylation state of the mTOR pathway. Phosphorylation state of $p70^{S6k}$ (**A**), 4E-BP1 (**B**), mTOR (**C** and **D**), S6 (**E**) and eEF2 (**F**) after incubation with leucine (*LEU*) and/or glutamine (*GLN*) for 30 min. Results are expressed as the means ± SEM ($n = 8$ for $p70^{S6k}$ and 4E-BP1; $n = 5$ for mTOR, S6 and eEF2). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control

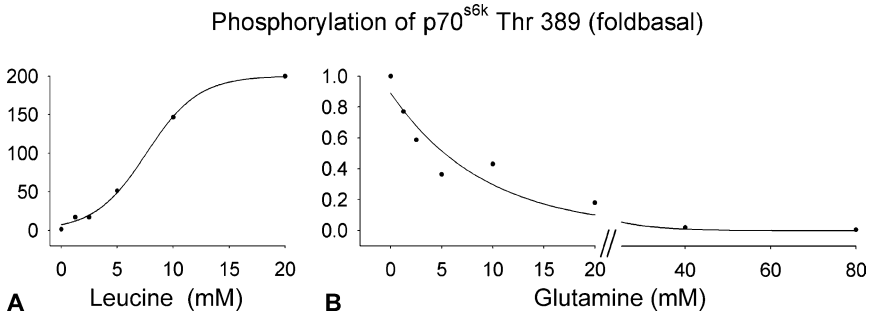


Fig. 2. Dose-response curves. Effect of increasing doses of leucine (**A**, 0–20 mM) and glutamine (**B**, 0–80 mM) on the phosphorylation state of $p70^{S6k}$ after 30 min

ed 'gamma' form ($P<0.001$, Fig. 1B). This is in relation to the increase in the phosphorylation state of mTOR on both Ser 2448 ($P<0.01$, Fig. 1C) and Ser 2481 ($P<0.01$, Fig. 1D). The leucine-induced $p70^{S6k}$ activation resulted to the expected increase in phosphorylation state of S6 on Ser 235/236 (+70%, $P<0.01$, Fig. 1E). By contrast, 5 mM glutamine decreased the phosphorylation state of $p70^{S6k}$ by 80% ($P<0.001$, Fig. 1A) and the percentage of 4E-BP1 in 'gamma' form by 40% ($P<0.01$, Fig. 1B). The inhibition induced by glutamine was also observed on mTOR but only on Ser 2448 ($P<0.05$, Fig. 1C) and not on Ser 2481 (Fig. 1D). The effects of leucine and glutamine on the phosphorylation state of $p70^{S6k}$ were dose-dependent. The more leucine was added to the medium, the more $p70^{S6k}$ was phosphorylated reaching a maximum at about 15–20 mM (Fig. 2A). Inversely, the more glutamine, the larger inhibition on $p70^{S6k}$ was observed (Fig. 2B).

When leucine and glutamine were combined, the effect of leucine was predominant, overtaking the inhibition exerted by glutamine. Indeed, after the addition of 5 mM leucine and 5 mM glutamine, the phosphorylation state of $p70^{S6k}$ was increased more than 10 fold ($P<0.001$, Fig. 1A) and the percentage of the 'gamma' form of 4E-BP1 was nearly doubled ($P<0.001$, Fig. 1B). Similar results were obtained on mTOR phosphorylation, on both Ser 2448 ($P=0.06$, Fig. 1C) and Ser 2481 ($P<0.01$, Fig. 1D) and on S6 ($P<0.05$, Fig. 1E).

In contrast with the mTOR-mediated pathway, the phosphorylation state of eEF2 on Thr 56 was unaffected by either amino acid or the combination of both (Fig. 1F).

3.2 Effect of leucine and glutamine on proteins regulating the mTOR pathway

Leucine and glutamine, alone or in combination, did not affect the activity of PKB (Fig. 3A) and AMPK (Fig. 3B).

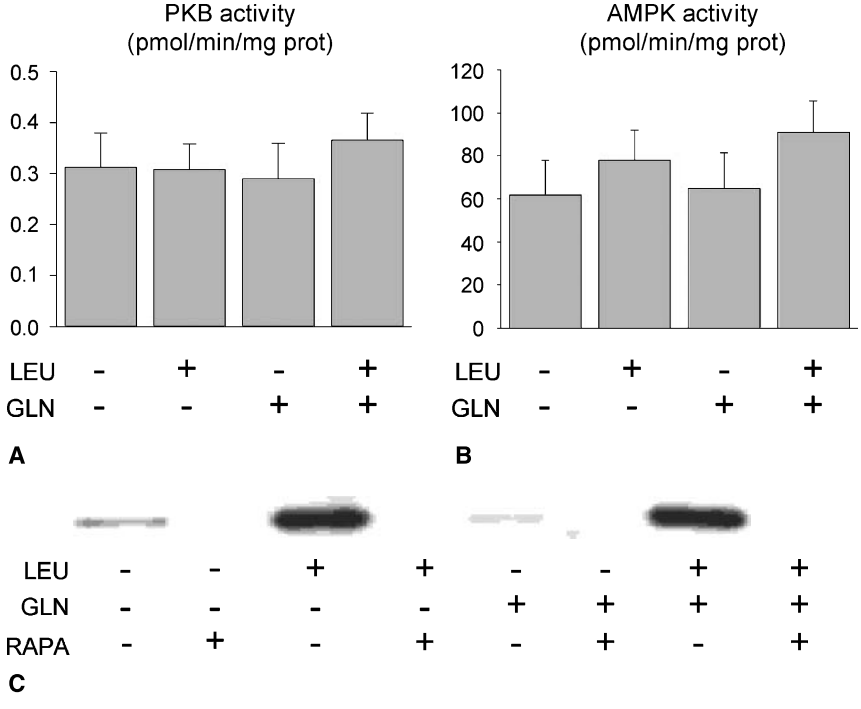
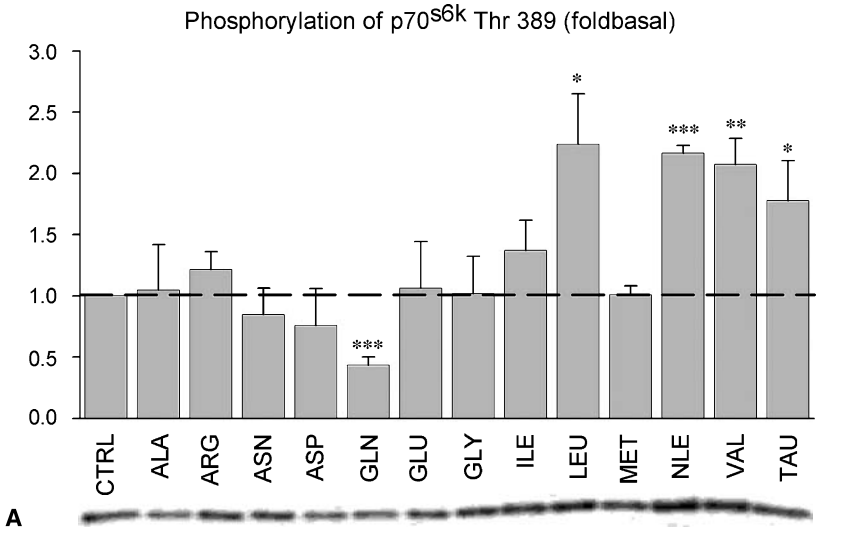


Fig. 3. Effect of leucine, glutamine, alone or in combination, on proteins regulating the mTOR pathway. PKB (**A**) and AMPK (**B**) activity. Results are expressed as the means ± SEM ($n=9$ for PKB; $n=6$ for AMPK). (**C**) Effect of rapamycin on the phosphorylation state of $p70^{S6k}$. Cells were preincubated with rapamycin (100 nM) for 15 min before being incubated for 30 min as indicated. *LEU* leucine; *GLN* glutamine; *RAPA* rapamycin

The absence of modification of AMPK activity was confirmed by an unchanged ACC phosphorylation state (Ser 79) (data not shown). To evaluate the implication of the rapamycin-sensitive part of the mTOR pathway in the response to leucine and glutamine, C_2C_{12} cells were incubated with 100 nM rapamycin (Fig. 3C). In all conditions,



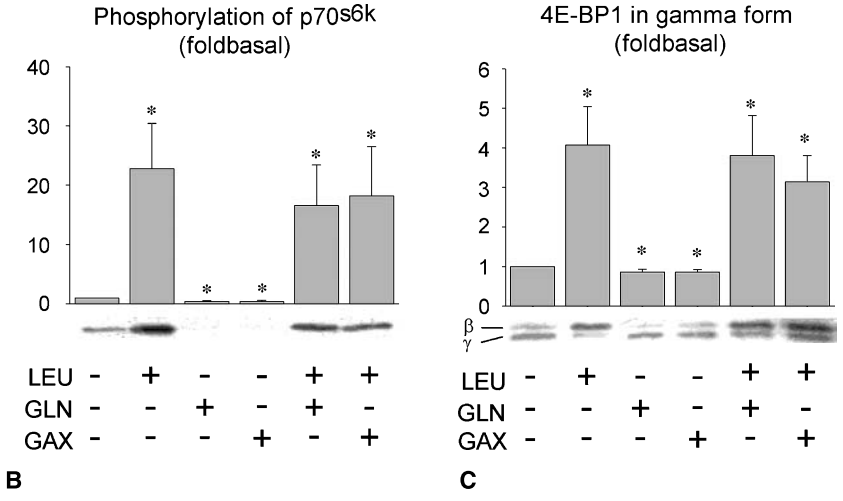


Fig. 4. Specificity of glutamine. (**A**) Effect of different amino acids on the phosphorylation state of $p70^{S6k}$ for 30 min. *CTRL* control conditions; *ALA* alanine; *ARG* arginine; *ASN* asparagine; *ASP* aspartic acid; *GLN* glutamine; *GLU* glutamic acid; *GLY* glycine; *ILE* isoleucine; *LEU* leucine; *MET* methionine; *NLE* norleucine; *VAL* valine; *TAU* taurine. Results are expressed as the means ± SEM ($n = 3$) and relative to the control conditions represented by the dashed line. (**B**, **C**) Effect of leucine (*LEU*), glutamine (*GLN*), GlutaMAX™ (*GAX*), alone or in different combinations, on the phosphorylation state of $p70^{S6k}$ on Thr 389 (**B**) and on the percentage of 4E-BP1 in gamma form (**C**) after an incubation period of 30 min. Results are expressed as the means ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control

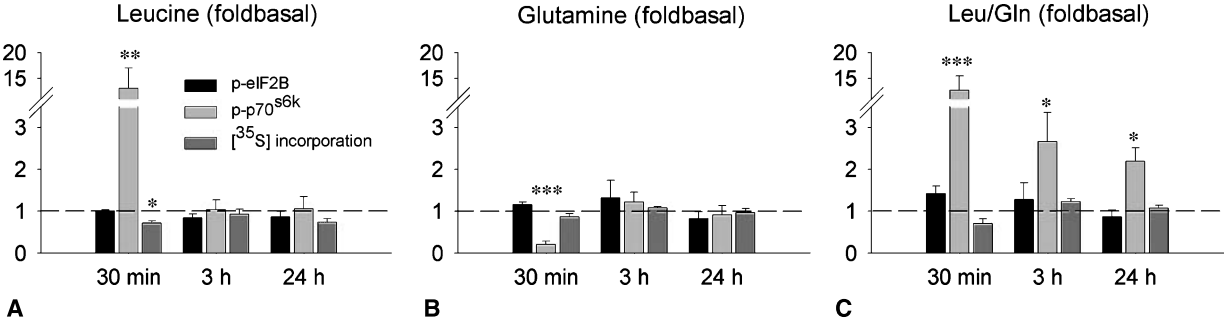


Fig. 5. Effect of leucine, glutamine, alone or in combination on the incorporation of labelled [^{35}S] methionine/cysteine and the phosphorylation state of $p70^{S6k}$ and eIF2B. The incorporation of labelled [^{35}S] methionine/cysteine ($n = 4$) and the phosphorylation state of $p70^{S6k}$ on Thr 389 and eIF2B on Ser 535 ($n = 3$) were quantified after 30 min, 1 h and 24 h of incubation with 5 mM leucine (**A**), 5 mM glutamine (**B**) and with both 5 mM leucine (Leu) and 5 mM glutamine (Gln) (**C**). Results are expressed as the means ± SEM and relative to the control conditions represented by the dashed line. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control same time

the $p70^{S6k}$ phosphorylation was totally abolished in the presence of rapamycin, indicating that at least the action of leucine is rapamycin-dependent.

3.3 Effect of other amino acids

C_2C_{12} cells were also incubated with alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, isoleucine, methionine, norleucine, valine and taurine in order to test if other amino acids could also modify the phosphorylation state of $p70^{S6k}$ (Fig. 4A). The concentration used for each amino acid was 5 mM. None of them decreased the $p70^{S6k}$ phosphorylation state, whereas valine ($P<0.01$), taurine ($P<0.05$) and norleucine ($P<0.001$), but not isoleucine, were able to increase it (Fig. 4A). In the same way, the effect of GlutaMAX™ (Life Technologies, Merelbeke, Belgium), a dipeptide composed of glutamine and alanine which reduces glutamine degradation into glutamic acid and ammonia, was studied. Like glutamine, GlutaMAX™ strongly decreased the phosphorylation state of $p70^{S6k}$ ($P<0.05$, Fig. 4B) and 4E-BP1 ($P<0.05$, Fig. 4C).

3.4 Effect of leucine and glutamine on [^{35}S] labelled methionine/cysteine incorporation

Whereas the phosphorylation state of $p70^{S6k}$ is often used as a marker of protein synthesis, Fig. 5 clearly shows that the incorporation of [^{35}S] labelled methionine/cysteine did not follow the phosphorylation state of $p70^{S6k}$ in the present study. The phosphorylation state of $p70^{S6k}$ was increased by leucine alone (12 fold, $P<0.01$) and was decreased by glutamine alone (−80%, $P<0.001$) after an incubation period of 30 min and it returned to basal level after 3 and 24 h (Fig. 5A). In the presence of both leucine and glutamine, $p70^{S6k}$ was more phosphorylated than in control conditions at each time point studied ($P<0.05$, Fig. 5C). In contrast to what happened with the $p70^{S6k}$ phosphorylation state, the incorporation of [^{35}S] labelled methionine/cysteine was decreased by 25% after 30 min in cells incubated with 5 mM leucine alone ($P<0.05$, Fig. 5A) and it was not altered by glutamine alone or by the combination of leucine and glutamine.

3.5 Effect of leucine and glutamine on muscle-specific mRNA levels

Since the mTOR pathway has also been shown to be involved in myogenesis (Park et al., 2005) and in the regulation of gene transcription by amino acid (Erbay

Table 2. Effect of leucine and/or glutamine on the mRNA level of muscle-specific genes

	1 h				3 h				24 h			
	CTRL	LEU	GLN	L/G	CTRL	LEU	GLN	L/G	CTRL	LEU	GLN	L/G
Desmin	1.0 ± 0.06	0.9 ± 0.12	0.8 ± 0.07	0.7 ± 0.06*	1.0 ± 0.06	1.3 ± 0.03*	1.3 ± 0.08*	1.3 ± 0.14	1.0 ± 0.21	1.1 ± 0.19	1.2 ± 0.41	1.5 ± 0.39
MHC II	1.0 ± 0.12	0.4 ± 0.02*	0.6 ± 0.05*	0.5 ± 0.03*	1.0 ± 0.12	0.7 ± 0.13	0.9 ± 0.16	0.7 ± 0.07	1.0 ± 0.24	1.1 ± 0.17	1.4 ± 0.38	3.7 ± 1.10*
Myogenin	1.0 ± 0.12	1.1 ± 0.19	1.0 ± 0.18	0.9 ± 0.06	1.0 ± 0.13	1.5 ± 0.03*	1.1 ± 0.07	1.3 ± 0.15	1.0 ± 0.19	1.3 ± 0.21	1.0 ± 0.34	0.8 ± 0.21
Caveolin3	1.0 ± 0.25	0.7 ± 0.22	0.6 ± 0.09	0.5 ± 0.04	1.0 ± 0.21	1.0 ± 0.24	1.0 ± 0.08	1.4 ± 0.08	1.0 ± 0.20	1.0 ± 0.19	1.1 ± 0.26	1.0 ± 0.27

Effect of 5 mM leucine (*LEU*) and/or 5 mM glutamine (*GLN*) on the mRNA for desmin, myosin heavy chain type II (*MHC II*), myogenin and caveolin 3. Results are expressed as the means ± SEM ($n=3$) and relative to the control conditions. * $P<0.05$ vs. control same time

et al., 2003), we analyzed the effect of leucine and glutamine on the expression of several muscle-specific genes. The mRNA level for MHC II was decreased after 1 h by leucine (−60%) and glutamine (−40%) alone as well as by both amino acids together (−50%) ($P<0.05$, Table 2). After the same period of incubation, the combination of leucine and glutamine also repressed the expression of desmin mRNA (−30%, $P<0.05$). After 3 h, the level of desmin mRNA was increased by leucine and glutamine alone (+30%, $P<0.05$) and the level of myogenin mRNA by leucine (+50%, $P<0.05$). After 24 h, the mRNA level for MHC II was increased by 3.7 fold when leucine and glutamine were combined ($P<0.05$). Caveolin 3 mRNA was not altered by leucine and/or glutamine.

4. Discussion

In this study, it is reported that leucine and glutamine have opposite effects on the mTOR pathway in myogenic C_2C_{12} cells (Fig. 6). Like in other cell types (Fox et al., 1998; Xu et al., 1998; Kimball et al., 1999; Krause et al., 2002), leucine increased the phosphorylation state of $p70^{S6k}$ and 4E-BP1 in our C_2C_{12} cells. However, leucine only transiently affected $p70^{S6k}$ since, after 3 h, the phosphorylation state of the latter returned to basal values (Fig. 5). More strikingly, glutamine exerted an opposite transient effect, reducing the phosphorylation state of $p70^{S6k}$ and 4E-BP1, whereas this amino acid is known to be a major activator of $p70^{S6k}$ in hepatocytes (Krause et al., 2002). Therefore, the action of glutamine on the phosphorylation state of $p70^{S6k}$ seems to be specific to the cell type studied, glutamine being able to exert opposite effects in two different cell types. Moreover, none of the other amino acids tested decreased the phosphorylation state of $p70^{S6k}$, suggesting that glutamine has a specific action in C_2C_{12} cells (Fig. 4A). The dephosphorylation induced by glutamine was probably not caused by its transformation into glutamate in the medium. Even if glutamate could have been transported into muscle cells (Rennie et al., 1996), it did not alter the phosphorylation state of $p70^{S6k}$ (Fig. 4A). Moreover, GlutaMAX^{TM}, which is a stabilized form of glutamine with the structure of a dipeptide L-alanyl-L-glutamine, mimicked the effect of glutamine by reducing the phosphorylation of $p70^{S6k}$ and 4E-BP1 (Fig. 4B and C). These results strengthen the hypothesis that glutamine itself rather than the products of its degradation, e.g. glutamic acid and ammonia, is responsible for the inhibition observed on the mTOR-mediated pathway.

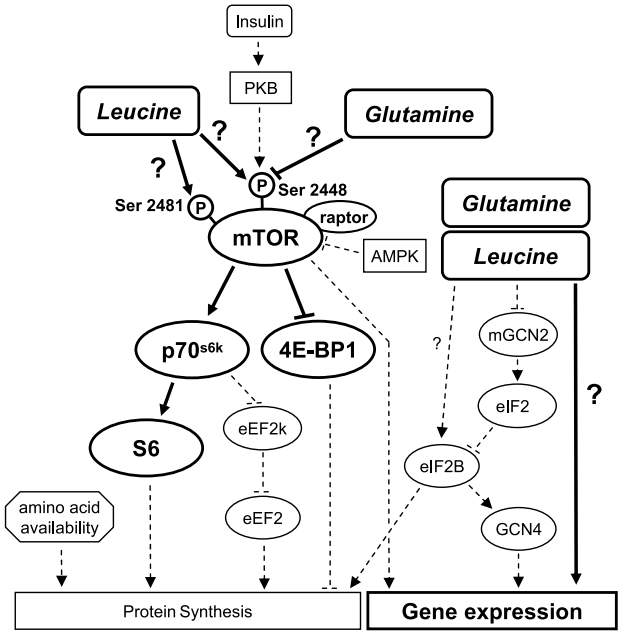


Fig. 6. Model for the control of the mTOR pathway, protein synthesis and gene expression by leucine and glutamine in C_2C_{12} cells. *PKB* protein kinase B; *mTOR* mammalian target of rapamycin; *raptor* regulatory associated protein to mTOR; *AMPK* AMP-activated protein kinase; *$p70^{S6k}$* p70 ribosomal S6 kinase; *4E-BP1* eukaryotic initiation factor 4E-binding protein 1; *eEF2k* eukaryotic elongation factor 2 kinase; *eEF2* eukaryotic elongation factor 2; *S6* ribosomal protein S6; *mGCN2* mammalian general control non-derepressible kinase 2; *eIF2*α eukaryotic initiation factor 2 alpha; *eIF2*β eukaryotic initiation factor 2B; *GCN4* general control non-derepressible kinase 4. *Bold lines* effect of leucine and/or glutamine; *dashed lines* no effect of leucine and/or glutamine or untested

Following these unexpected data, we tried to understand further how leucine and glutamine exerted their respective effect on mTOR and the physiological role of this leucine- and glutamine-induced signalling. Several proteins have been shown to regulate mTOR; PKB, AMPK and raptor being of the highest importance (Fig. 6). PKB is known to activate whereas AMPK is known to inhibit (Kimura et al., 2003) the mTOR pathway, respectively. However, neither leucine nor glutamine was able to modify the activity of these kinases, demonstrating that the signalling of both amino acids is independent of PKB and AMPK (Fig. 3A and B). Leucine signalling through mTOR is rapamycin-dependent as demonstrated by the total inhibition of the action of this amino acid by the addition of rapamycin (Fig. 3C). The binding of rapamycin to its intracellular receptor, FK506-binding protein, allows it to interact specifically with mTOR and to prevent the binding of raptor to mTOR to form the mTORC1 complex (Kim et al., 2002). Raptor is known to mediate the activation of $p70^{S6k}$ by nutrients and the increase in cell size. Our data confirm that raptor plays a

key role in the action of leucine on the mTOR pathway in C_2C_{12} cells.

As $p70^{S6k}$ is generally believed to be a key regulator of protein synthesis initiation, the present study investigated whether the activation induced by leucine and the inhibition exerted by glutamine on the phosphorylation state of $p70^{S6k}$ had any effect on the incorporation of labelled methionine/cysteine. Unexpectedly, the incorporation of labelled methionine/cysteine was only transiently affected by leucine and was not modified by glutamine (Fig. 5). We observed a 25% decrease in labelled methionine/cysteine incorporation induced by leucine after 30 min, which is contrary to its effect on the mTOR/$p70^{S6k}$ pathway. Our results on the phosphorylation of the ribosomal S6 subunit and eEF2 might partially explain those on labelled methionine/cysteine incorporation. Although both lying downstream of $p70^{S6k}$ in the regulation of protein synthesis, S6 was only slightly affected and eEF2 not modified at all by leucine and glutamine (Fig. 1E and F). Since eIF2B has also been proposed as a key regulator of protein synthesis (Kimball et al., 1998), its phosphorylation state after leucine and glutamine incubation was analyzed (Fig. 5). But, in contrast to $p70^{S6k}$, leucine and glutamine did not affect its phosphorylation state, ruling out its involvement in the decrease in labelled methionine/cysteine incorporation induced by leucine after 30 min. Taken together, our data indicate that a change in the phosphorylation state of $p70^{S6k}$ on Thr 389 does not necessarily lead to a subsequent modification of the phosphorylation of its downstream targets and to a change in amino acid incorporation. These data are noteworthy since previous experiments showing modifications in protein metabolism were based on amino acid restriction (Mordier et al., 2000) or were carried out during the proliferation phase and high protein turnover (Kimball et al., 1999; Du et al., 2007). By contrast, the present experiments were carried out during the differentiation phase characterized by a lower protein translation rate.

Since amino acid incorporation was only affected to a minor extent by leucine, the study examined whether the changes in $p70^{S6k}$ phosphorylation state could alter gene expression (Fig. 6). Moreover, amino acids have recently been shown to control IGF-II transcription and myogenesis through mTOR/$p70^{S6k}$ pathway (Erbay et al., 2003). To characterize better the possible involvement of amino acids in the regulation of gene expression and myogenesis in C_2C_{12} cells, we analyzed the mRNA level of muscle-specific genes after incubation with leucine and/or glutamine (Table 2). Although leucine and/or glutamine modified the mRNA levels of desmin, myogenin and MHC II, the changes did not follow the phosphorylation state of the mTOR/$p70^{S6k}$ pathway, contrary to our hypothesis, suggesting that the latter is probably not involved in the control of the transcription of these genes. Other pathways have been proposed to mediate the effect of amino acids on gene expression. Although they were not tested in this study, it seems likely that they do not contribute to the present results. The hexosamine signalling pathway, through glucosamine-6-phosphate and subsequent O-linked glycosylation of various proteins, seems to play an important role in regulating gene expression (Marshall, 2006). Since glutamine is used as an amino donor to convert fructose-6-phosphate to glucosamine-6-phosphate (Marshall, 2006), it plays an important role in the regulation of gene expression induced by the hexosamine signalling pathway (Brasse-Lagnel et al., 2003). However, this pathway essentially regulates the expression of genes encoding proteins that are involved in controlling the insulin-stimulated glucose transport system and triglyceride synthesis (Marshall, 2006). Amino acid deprivation has also been shown to cause ER (endoplasmic reticulum) stress and to induce gene expression via phosphorylation of eIF2α and eIF2B (Abcouwer et al., 2002; Kilberg and Barbosa-Tessmann, 2002). However, eIF2B was not affected by additional leucine and/or glutamine (Fig. 5), ruling out a possible implication of ER stress in the regulation of gene expression in the present study.

In conclusion, leucine activates the mTOR pathway in myogenic C_2C_{12} cells. By contrast to leucine, glutamine inhibits this pathway by dephosphorylating the Ser 2448 of mTOR and decreasing the phosphorylation state of $p70^{S6k}$ and 4E-BP1. Whilst the phosphorylation state of $p70^{S6k}$ is usually related to the rate of protein synthesis, the incorporation of labelled methionine/cysteine remained unaffected by glutamine and was only transiently decreased by leucine. In line with the recent conception of gene expression regulation by amino acids, leucine and glutamine affected the mRNA levels of desmin, myogenin and MHC II in a time-dependent manner but unrelated to their effects on the mTOR/$p70^{S6k}$ pathway in C_2C_{12} cells.

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