Chapter 7

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

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Abstract

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

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 & 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. have studied the effect of 14 amino acids on $p70^{S6k}$ activity in intestinal epithelial cells (Nakajo *et al.*, 2005). 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). Recently, 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.

Material and Methods

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 (5000U/5000µg/ml) and 100µM non-essential amino acids. When cells were 70% confluent, the proliferation medium was replaced by a differentiation medium containing 1% horse serum. After 48h of differentiation, cells were incubated for 1h with serum-free DMEM lacking leucine and glutamine (Life Technologies). Leucine (5mM), glutamine (5mM) or both (5mM each) (Sigma) were then added to the plates.

Phosphorylation states and protein synthesis. Cells were lysed after 30min, 3h and 24h, in a buffer (pH 7.0) containing 20mM Tris, 270mM sucrose, 5mM EGTA, 1mM EDTA, 1% Triton X-100, 1mM sodium orthovanadate, 50mM sodium β-glycerophosphate, 5mM sodium pyrophosphate, 50mM sodium fluoride, 1mM 1,4-dithiothreitol and a protease inhibitor cocktail (Roche Applied Science). The supernatants (10000g, 10min) 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 500µl TRIZOL® after 1h, 3h and 24h 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 1µg/µl using RNase-free water.

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 1h 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.

Protein kinase B activity

Protein kinase B (PKB) activity was measured by the phosphorylation of a synthetic peptide after immunoprecipitation (100µg 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 50µl in the presence of 10mM MOPS (pH 7.0), 0.5mM EDTA, 10mM Mg-acetate, 0.1% β-mercaptoethanol, 0.1mM

Mg-[γ-^{32}P] ATP (specific radioactivity 1000cpm/pmol, Amersham Biosciences) and 0.25mM substrate peptide RPRAATF (Alessi *et al.*, 1996). The reaction was continued for 20min at 30°C. The supernatant was then spotted on to P81 phosphocellulose paper, followed by washes in cold 75mM phosphoric acid. ^{32}P incorporation was counted in a scintillation counter (LS 6500, Beckman) for 1min.

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 (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).

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

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

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 1µg of total RNA in a reaction volume of 20µl (4µl iScript reaction mix 5X, 1µl iScript reverse transcriptase, 1µl RNA template, 14µl RNase-free water). Primers were designed (Table 7.1) for mouse desmin, myogenin, caveolin 3 and myosin heavy chain type II (MHC II) and β-actin.

Table 7.1. *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' to 3') used for mRNA quantification by real-time RT-PCR. MHC II, myosin heavy chain type II.

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: 10min at 95°C, followed by 40 cycles of 1min at 60°C and 15s at 95°C. For each gene, real time RT-PCR was conducted in duplicate. The ΔCt values were calculated in every 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 (ΔΔCt) 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 $2^{-\Delta\Delta Ct}$.

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.

Results

Opposite effects of leucine and glutamine on the mTOR pathway

Incubation of C_2C_{12} cells for 30min in the differentiation medium supplemented with 5mM leucine increased more than 10 fold the phosphorylation state of $p70^{S6k}$ on Thr 389 ($P<0.01$, Fig. 7.1A) and almost doubled the percentage of 4E-BP1 in the phosphorylated 'gamma' form ($P<0.001$, Fig. 7.1B). This is in relation to the increase in the phosphorylation state of mTOR on both Ser 2448 ($P<0.01$, Fig. 7.1C) and Ser 2481 ($P<0.01$, Fig. 7.1D).

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. 7.1E). By contrast, 5mM glutamine decreased the phosphorylation state of $p70^{S6k}$ by 80% ($P<0.001$, Fig. 7.1A) and the percentage of 4E-BP1 in 'gamma' form by 40% ($P<0.01$, Fig. 7.1B). The inhibition induced by glutamine was also observed on mTOR but only on Ser 2448 ($P<0.05$, Fig. 7.1C) and not on Ser 2481 (Fig. 7.1D).

When leucine and glutamine were combined, the effect of leucine was predominant, overtaking the inhibition exerted by glutamine. Indeed, after the addition of 5mM leucine and 5mM glutamine, the phosphorylation state of $p70^{S6k}$ was increased more than 10 fold ($P<0.001$, Fig. 7.1A) and the percentage of the 'gamma' form of 4E-BP1 was nearly doubled ($P<0.001$, Fig. 7.1B). Similar results were obtained on mTOR phosphorylation, on both Ser 2448 ($P=0.06$, Fig. 7.1C) and Ser 2481 ($P<0.01$, Fig. 7.1D) and on S6 ($P<0.05$, Fig. 7.1E).

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. 7.1F).

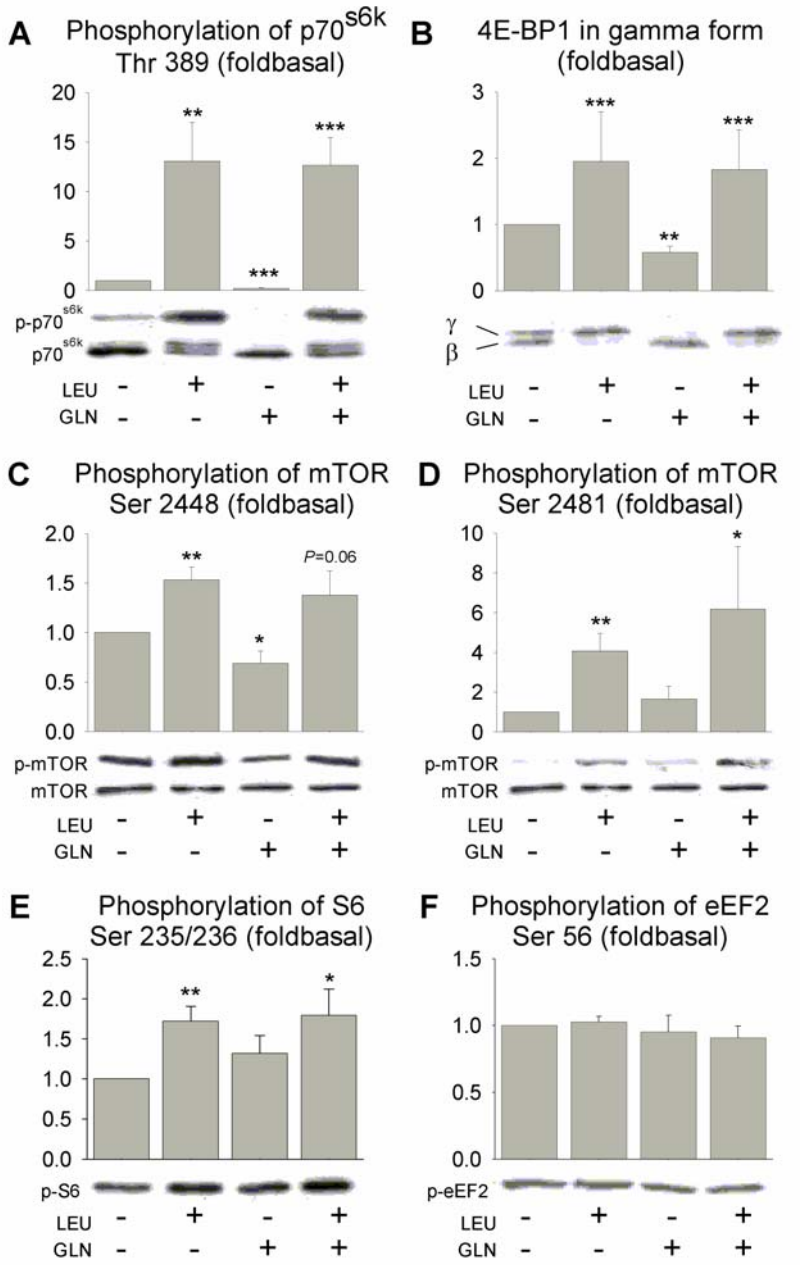

Fig. 7.1. *Effect of leucine, glutamine, alone or in combination, on the phosphorylation state of the mTOR pathway.* Phosphorylation state of $p70^{S6k}$ (A), 4E-BP1 (B), mTOR (C and D), S6 (E) and eEF2 (F) after incubation with leucine (LEU) and/or glutamine (GLN) for 30min. Results are expressed as 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.

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 15-20mM (Fig. 7.2A). Inversely, the more glutamine, the larger inhibition on $p70^{S6k}$ was observed (Fig. 7.2B).

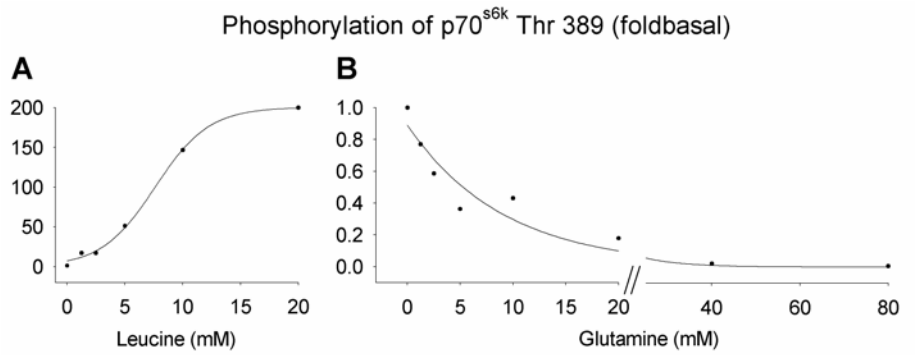

Fig. 7.2. *Dose-response curves.* Effect of increasing doses of leucine (0-20mM) and glutamine (0-80mM) on the phosphorylation state of $p70^{S6k}$ after 30min.

Leucine and glutamine, alone or in combination, did not affect the activity of PKB (Fig. 7.3A) and AMPK (Fig. 7.3B). 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 100nM rapamycin (Fig. 7.3C). In all conditions, the $p70^{S6k}$ phosphorylation was totally abolished in the presence of rapamycin, indicating that at least the action of leucine is rapamycin-dependent.

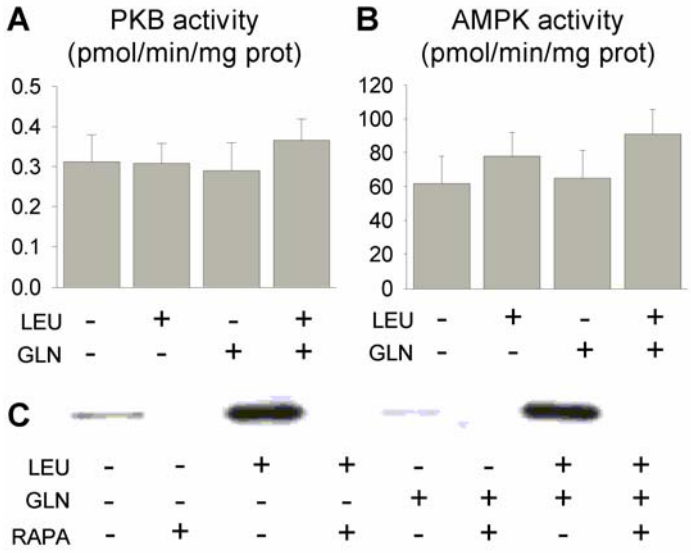

Fig. 7.3. *Effect of leucine, glutamine, alone or in combination, on proteins regulating the mTOR pathway.* PKB (A) and AMPK (B) activity. Results are expressed as the means ± SEM (n=9 for PKB; n=6 for AMPK). (C) Effect of rapamycin on the phosphorylation state of $p70^{S6k}$. Cells were preincubated with rapamycin (100nM) for 15min before being incubated for 30min as indicated. LEU, leucine; GLN, glutamine; RAPA, rapamycin.

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. 7.4A). The concentration used for each amino acid was 5mM. 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. 7.4A). 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. 7.4B) and 4E-BP1 ($P<0.05$, Fig. 7.4C).

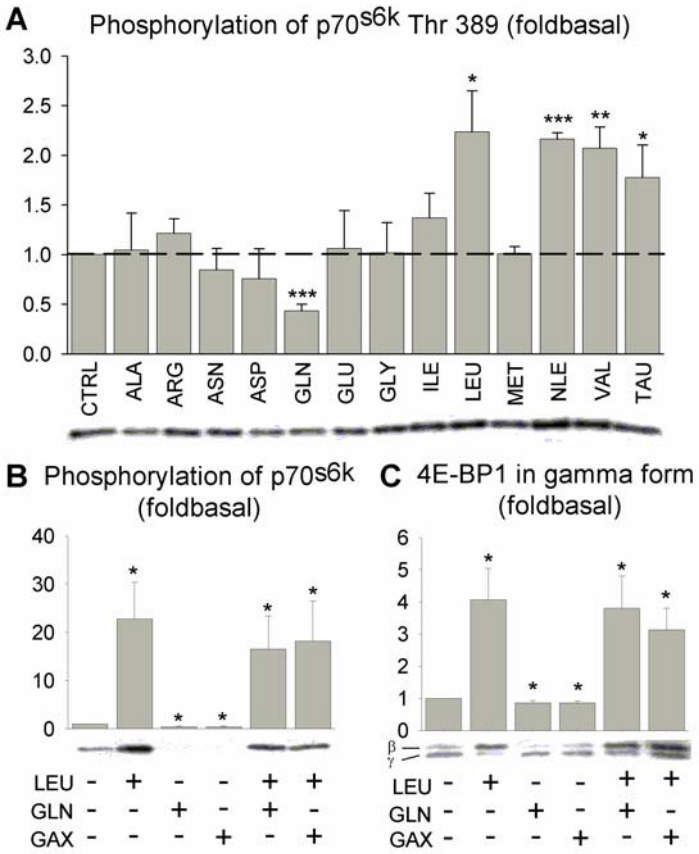

Fig. 7.4. *Specificity of glutamine.* (A) Effect of different amino acids on the phosphorylation state of $p70^{S6k}$ for 30min. CTRL, control conditions; ALA, alanine; ARG, arginine; ASN, asparagine; ASP, aspartic acid; GLN, glutamine; GLU, glutamic acid; GLY, glycine; ILE, isoleucine; LEU, leucine; MET, methionine; NLE, norleucine; VAL, valine; TAU, taurine. Results are expressed as the means ± SEM (n=3). (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 30min. Results are expressed as the means ± SEM (n=3). **P*<0.05, ***P*<0.01, ****P*<0.001 *vs.* control.

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, figure 7.5 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 30min and it returned to basal level after 3h and 24h (Fig. 7.5A). 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. 7.5C). In contrast to what happened with the $p70^{S6k}$ phosphorylation state, the incorporation of [^{35}S] labelled methionine/cysteine was decreased by 25% after 30min in cells incubated with 5mM leucine alone ($P<0.05$, Fig. 7.5A) and it was not altered by glutamine alone or by the combination of leucine and glutamine.

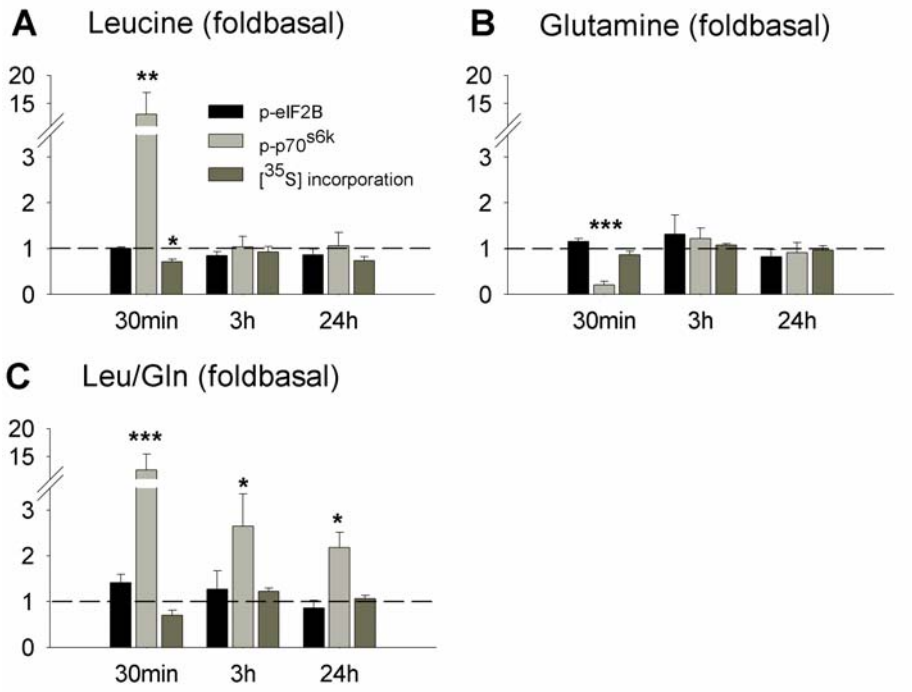

Fig. 7.5. *Effect of leucine, glutamine, alone or in combination on the incorporation of labelled [^{35}S] methionine/cysteine and the phosphorylation state of $p70^{S6k}$ and eIF2B.* The incorporation of labelled [^{35}S] methionine/cysteine (n=4) and the phosphorylation state of $p70^{S6k}$ on Thr 389 and eIF2B on Ser 535 (n=3) were quantified after 30min, 1h and 24h of incubation with 5mM leucine (A), 5mM glutamine (B) and with both 5mM leucine (Leu) and 5mM 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.

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 *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 1h by leucine (-60%) and glutamine (-40%) alone as well as by both amino acids together (-50%) (P<0.05, Table 7.2). After the same period of incubation, the combination of leucine and glutamine also repressed the expression of desmin mRNA (-30%, P<0.05). After 3h, 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 24h, 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.

Table 7.2. *Effect of leu and/or gln on mRNA levels of muscle-specific genes.*

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

Effect of 5mM leucine (LEU) and/or 5mM 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.

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. 7.6). 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 3h, the phosphorylation state of this latter returned to basal values (Fig. 7.5). 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.

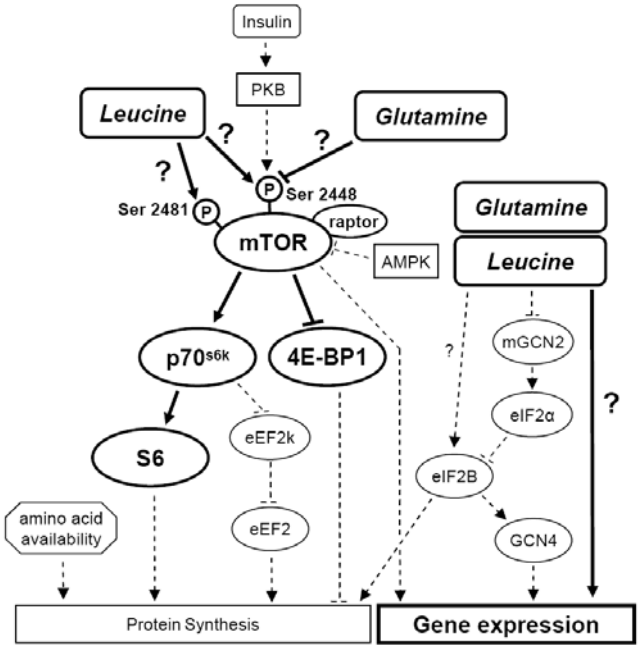

Fig. 7.6. *Model for the control of the mTOR pathway, protein synthesis and gene expression by leucine and glutamine in C_2C_{12} cells.* AMPK, AMP-activated protein kinase; eEF2k, eukaryotic elongation factor 2 kinase; mGCN2, mammalian general control non-derepressible kinase 2; eIF2α, eukaryotic initiation factor 2 alpha; 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.

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. 7.4A). 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. 7.4A). 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. 7.4B 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.

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. 7.6). 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. 7.3A 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. 7.3C). 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. 7.5). We observed a 25% decrease in labelled methionine/cysteine incorporation induced by leucine after 30min, 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. 7.1E 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. 7.5). 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 30min. 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. 7.6). 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 7.2). 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 this 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 & Barbosa-Tessmann, 2002). However, eIF2B was not affected by additional leucine and/or glutamine (Fig. 7.5), 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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Chapter 8

Discussion, perspectives and conclusion

Summary

Discussion and perspectives

An important development in research focusing on skeletal muscle protein metabolism has been the observation that nutrition affects skeletal muscle protein synthesis during the recovery period after exercise. This effect has been viewed as an additive response of physical activity and nutrition. Although correct, this view has recently been further refined. Physical activity would trigger an adaptive response to which nutrition would provide the necessary building blocks for an optimal response. Nutrition would thus be necessary to take advantage of an adaptive environment created by exercise (Miller, 2007).

Whereas the anabolic effect of exercise and nutrition is commonly accepted (Wolfe, 2000; Rennie *et al.*, 2004), the cellular mechanisms involved are insufficiently described. The purpose of the present work was thus to clarify some of them. A broad framework of pathways has been proposed to mediate the signalling induced by exercise and nutrition, amongst which the MAPK and the Akt/PKB-mTOR pathways seem to be key cascades (Widegren *et al.*, 2001; Deldicque *et al.*, 2005b; Bodine, 2006).

Exercise is known to activate the MAPK pathway leading to muscle fibre adaptations. While the activation of the Akt/PKB-mTOR pathway by exercise is controversial, its activation by nutrients is well-established. Whether the MAPK pathway is modulated by nutrients is only beginning to be tested. To investigate further the molecular mechanisms induced by nutrients and exercise, we used both C_2C_{12} myogenic cells and human skeletal muscle biopsies as experimental models.

1. Exercise

Exercise itself is known to result in a negative net protein balance. During exercise, protein synthesis is either unchanged or repressed, whereas protein breakdown is increased (Wolfe, 2000; Rennie *et al.*, 2004). Due to technical difficulties, the cellular signalling turned on during resistance exercise has not yet been studied in human skeletal muscle. However, the effect of exercise lasts on during the recovery period. During this period, contractile activity has been shown to activate multiple signal transduction pathways and to modulate transcriptional and translational processes. By taking biopsies within the 30s following the last muscle contraction, we tested if the signalling pathways involved post-exercise, i.e. the Akt/PKB and the MAPK cascades were already modulated during exercise (Fig. 8.1 and 8.2).

We found that, immediately after a high intensity resistance exercise performed in the fasted state, the Akt/PKB pathway was inhibited, whereas the p38 and the ERK1/2 MAPK pathways were greatly activated (chapter 6). These results indicate that the MAPK pathway can be triggered by contractile activity alone and it may be suggested that the Akt/PKB pathway requires nutrients to be activated.

The inhibition of Akt/PKB was unexpected as previous studies reported an increase or no modification of the phosphorylation state of Akt/PKB. However, this result fits well with the repression of protein synthesis and the activation of protein breakdown during an exercise bout in the fasted state (Wolfe, 2000). It is likely that the nutritional status of the subjects is not the origin of this drop in Akt/PKB phosphorylation. Blomstrand et al. also found that immediately after resistance exercise, the phosphorylation state of Akt/PKB was decreased to the same extend in the placebo group and in the group receiving branched chain amino acids (Blomstrand et al., 2006). In contrast, after low intensity resistance exercise, the phosphorylation of

Akt/PKB has been found to increase (Creer et al., 2005). One could postulate that high intensity (>80% 1RM) and low intensity resistance exercise induce opposite responses on Akt/PKB. The type of exercise, concentric vs eccentric, could also be an explanation for the discrepancies observed after exercise on Akt/PKB. Eccentric exercise tended to decrease Akt/PKB phosphorylation compared to concentric exercise (Eliasson et al., 2006). In our protocol, the eccentric component was very important. The intensity, the duration and the type of exercise are likely major determinants in the phosphorylation state of Akt/PKB.

AMPK has been shown to suppress protein synthesis in rat skeletal muscle through a decrease in the phosphorylation state of Akt/PKB on Ser 473, mTOR on Ser 2448, $p70^{s6k}$ on Thr 389 and 4E-BP1 on Thr 37 (Bolster et al., 2002). The activation of AMPK by resistance exercise in humans (Dreyer et al., 2006; Koopman et al., 2006) is one likely mechanism inhibiting Akt/PKB signalling immediately after exercise.

Moreover, Akt/PKB could be inhibited by skeletal muscle cell damage occurring with high-intensity exercise, coupled with degradation of cellular components and increased enzyme activity (Gissel & Clausen, 2001). The rictor (rapamycin-insensitive companion of mTOR)-mTOR complex directly phosphorylates Akt/PKB on Ser 473 in vitro and facilitates Thr 308 phosphorylation by PDK1. A reduction in rictor or mTOR expression inhibits Akt/PKB (Sarbassov et al., 2005). High-intensity exercise is associated with an increase in sarcoplasmic calcium concentration and increased phospholipase A2 activity which is known to inhibit Akt/PKB on Thr 308 (Haq et al., 2003).

Another possible explanation for the decrease in the phosphorylation state of Akt/PKB could be the drop in pH observed during high-intensity exercise. Severe ischemia inhibits insulin signalling by decreasing pH (Beauloye *et*

al., 2001). Although we did not measure pH during our exercise session, high-intensity resistance exercise is known to decrease the intracellular pH value next to 6.6-6.5 (Sahlin *et al.*, 1976; Miller *et al.*, 1988), which is the critical threshold for inhibiting PKB activity in vitro (Beauloye *et al.*, 2001). However, in the fasted state, exercise-induced changes in pH are less than in the fed state (Lunt *et al.*, 1986). Therefore, the 'pH' hypothesis is likely not involved in the decrease in the phosphorylation state of Akt/PKB.

Contractile activity is also detected at the level of the cell membrane by mechanosensors, e.g. integrins, which activate focal adhesion proteins and their downstream targets (Ingber, 2006). This could be the way by which exercise activates ERK1/2 and p38 in vivo. Indeed, in human fibroblasts, MAPK are activated by mechanical strain regulating the small G proteins rac-1 and rhoA which control focal and adhesion complexes at the membrane level (Laboureau et al., 2004).

Twenty-four hours post-exercise, two volunteers presented a large increase in the phosphorylation state of $p70^{s6k}$ on Thr 389 (chapter 6, Fig. 6.2), in opposition to the other subjects. The exercise task performed in this study comprised a significant eccentric component. It is possible that the muscles of some individuals are more sensitive to eccentric contractions and suffer greater damage and thus a greater proteolysis induced by exercise could have increased the free intracellular amino acid pool and in turn activated $p70^{s6k}$ via mTOR (Beugnet *et al.*, 2003)

The results presented in chapter 6 suggest that there is a crosstalk between p38-ERK1/2 and $p70^{s6k}$ on Thr 421/Ser 424 during exercise (Fig. 8.1). We postulated that this priming of $p70^{s6k}$ on Thr 421/Ser 424 via MAPK during exercise could be an important step in potentiating $p70^{s6k}$ on Thr 389, and thereby likely protein synthesis, with feeding during the recovery period. Indeed, when subjects were in the fasted state, $p70^{s6k}$ on Thr 389 tended to

be inhibited immediately after exercise and was not different from basal during the recovery period (chapter 6). On the other hand, when subjects received large amounts of amino acids and carbohydrates immediately after exercise, $p70^{s6k}$ on Thr 389 was highly phosphorylated 3h after exercise (chapter 3). In other words, the pre-activation of $p70^{s6k}$ on Thr 421/Ser 424 during exercise could be an important step of the adaptive environment induced by exercise.

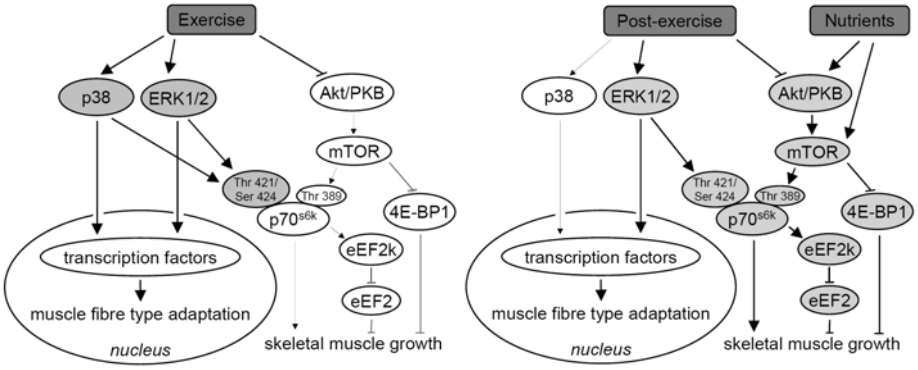

Fig. 8.1. *Hypothetical model for the activation of the p38 and ERK1/2 MAPK and the Akt/PKB pathways during (left) and after (right) exercise.* Grey, activated proteins; white, repressed or unaffected proteins.

The adaptive environment induced by exercise also occurs at the transcriptional level (Fig. 8.2). Exercise controls gene expression via activation of muscle specific transcription factors (chapter 5). This transcriptional regulation is very rapid since at the end of the exercise, i.e. after about 40min of intermittent contractile activity, a few mRNA levels were already modified. Immediately after exercise, MAFbx, MHC IIA, PGC-1α, PCNA and IL-6 mRNA were increased. Since the nuclear expression of MEF-2 was also increased at this time, it may have contributed to the regulation of these genes by exercise.

In humans, the satellite cell pool can increase as early as 4 days following a single bout of exercise. It is maintained at higher levels following several weeks of training (Crameri *et al.*, 2004; Kadi *et al.*, 2005). We found that PCNA was increased in a biphasic way, immediately post-exercise and after 3 days of recovery, indicative of a high proliferation state in muscle cells at these time points. Although we did not directly assess their activation and their proliferation state, our results suggest that a single bout of resistance exercise could already stimulate satellite cells, which does not necessarily imply that they will further differentiate into muscle cells. Indeed, activated and proliferating satellite cells may withdraw form differentiation (Kadi *et al.*, 2005). It has previously been shown that one bout of exercise was not sufficient for the satellite cell to undergo terminal differentiation (Crameri *et al.*, 2004).

PCNA mRNA and MEF-2 protein in the nucleus followed a similar expression pattern following exercise. However MEF-2 transcription factor does not seem to regulate PCNA mRNA expression (Black & Olson, 1998) and MEF-2 does not appear to be a PCNA interacting-protein (Maga & Hubscher, 2003). It is thus likely that these two factors affected different cellular processes independently from each other: MEF-2 regulating muscle-specific gene expression (Black & Olson, 1998), whereas PCNA playing a coordinating role for numerous proteins, such as polymerases and cyclins, involved in both DNA replication and repair and in cell cycle control (Maga & Hubscher, 2003).

Strength training in humans has been shown to result in MHC type IIB to type IIA transitions without affecting MHC type I percentage (Adams *et al.*, 1993). Since the changes in the amounts of the different MHC mRNA isoforms precede the corresponding changes at the protein level (Jaschinski *et al.*, 1998), our data suggest that one bout of exercise already contributes to the synthesis of MHC IIA.

Twenty-four hours post-exercise, the degeneration phase induced by exercise would be progressively replaced by a regeneration period. Indeed, at this time, mRNA for MAFbx and myostatin were both depressed (chapters 5 and 6, Fig. 8.2). Myostatin is a negative regulator of muscle mass and regulates the expression of MAFbx to modulate ubiquitin-dependent proteolysis (McFarlane *et al.*, 2006). It seems that the ubiquitin/proteasome pathway is suppressed for some time after exercise. It is likely that such a depression is associated with supporting hypertrophy induced by chronic resistance exercise.

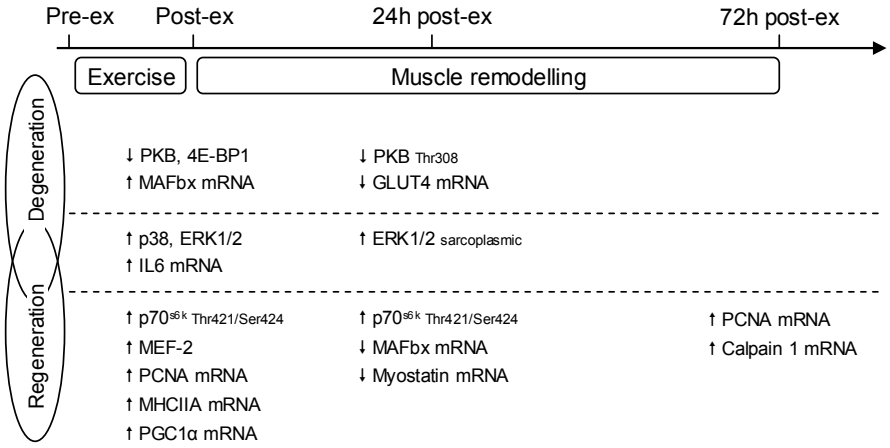

Fig. 8.2. *Summary of the effects of exercise on the MAPK and the Akt/PKB pathways and on gene expression.* Based on data from chapter 5 and chapter 6.

Muscle remodelling after resistance exercise also implicates changes in metabolic genes. GLUT-4 mRNA abundance decreased by 24h after exercise. The physiological significance of this decrease is difficult to explain, and it does not necessarily reflect a decrease in GLUT-4 protein. PGC-1α, a coactivator of PPARγ, plays an important role in the transcriptional control of mitochondrial biogenesis and in the shift in muscle fibre type from fast to slow (Lin et al., 2002). It was thus surprising to observe an increase in PGC-1α but not in MHC I mRNA although there was a trend to up-regulation in placebo conditions. PGC-1α mRNA has already

been reported to increase after resistance exercise peaking about 3h after exercise (Pilegaard *et al.*, 2003). We observed a significant increase immediately post-exercise suggesting that this coactivator takes part in the early response to exercise. This suggestion is in agreement with the observation that p38 is activated and MEF-2 is more abundant in the nucleus immediately after exercise, since the transcriptional regulation of PGC-1α partially depends on the activation of both p38 and MEF-2 (Akimoto *et al.*, 2005; Wright *et al.*, 2007).

In summary, exercise represents a serious perturbation of cell homeostasis and can, in itself, induce a favourable environment for nutrients to improve muscle anabolism during the recovery period. The adaptive environment thus provoked by exercise may partially occur via a crosstalk between the MAPK and $p70^{s6k}$ as well as the up-regulation of the expression of MEF-2 and the expression of some genes involved in muscle remodelling such as PCNA, MAFbx, MHC II, PGC-1α and IL-6.

Future research should be directed towards the description of the potential crosstalk between MAPK and $p70^{s6k}$ during exercise. Elucidating the mechanisms of this crosstalk will contribute to the understanding of the signalling induced during exercise.

2. Amino acids

The importance of nutrition during the recovery period after exercise has been highlighted in the previous section. Although, it seems obvious that nutrients, and more particularly amino acids, are required to trigger protein synthesis and to favour muscle anabolism, the mechanisms of action are not clear. The present work has gathered data from myogenic C_2C_{12} cells that help clarify the molecular mechanisms induced by amino acids, and more particularly leucine and glutamine, two key amino acids in skeletal muscle.

Amino acids have initially been viewed as the building blocks for protein synthesis but, currently, it is known that they also regulate signalling pathways controlling protein metabolism and gene expression. Amino acids are able to modulate the activity of the mTOR pathway, which regulates protein synthesis through several initiation factors (for review see Proud, 2004; Deldicque *et al.*, 2005b; Stipanuk, 2007). The mechanisms of mTOR regulation by amino acids remain unknown, although different candidates such as Rheb, TSC1/TSC2 or PP2A have been proposed (chapter 2).

We hypothesized that leucine and glutamine would activate the mTOR pathway in myogenic cells, as in hepatocytes (Krause et al., 2002), but we observed that these two amino acids exerted opposite effects (Fig. 8.3.). Leucine is already known to be a powerful activator of this pathway in L6 myogenic cells (Kimball et al., 1999; Peyrollier et al., 2000), and this was confirmed with C_2C_{12} cultures, whereas glutamine has not yet been tested in myogenic cells. Unexpectedly, glutamine inhibited the mTOR pathway whereas glutamine has been found to have some anabolic effects on protein synthesis in human skeletal muscle (MacLennan *et al.*, 1987; Rennie *et al.*, 1994). The inhibition exerted by glutamine on the mTOR pathway did not result in any reduction of protein synthesis. In the same way, the 10-fold increase in phosphorylation of $p70^{s6k}$ by leucine did not augment protein

synthesis. If anything, there is a decrease in the incorporation of labelled methionine/cysteine after 30min by leucine, which is in contradiction with the well-accepted relation between the mTOR pathway and protein synthesis. Our observation questions this relation and raises the consideration that other important pathways are involved in the control of protein synthesis in the C_2C_{12} model.

It could be argued that the presence of high concentrations of leucine and/or glutamine might have decreased transport of the labelled amino acid leading to an underestimation of protein synthesis. However, it seems unlikely that the transport of methionine (A) and cysteine (ASC) would have been affected, since their transporters are different from those of leucine (L) and glutamine (Nm). There is no reason to believe that the transport of the tracers is different from that of the tracees. Therefore, it is unlikely that high concentrations of leucine and/or glutamine would have affected the rate of methionine/cysteine incorporation into the protein pool.

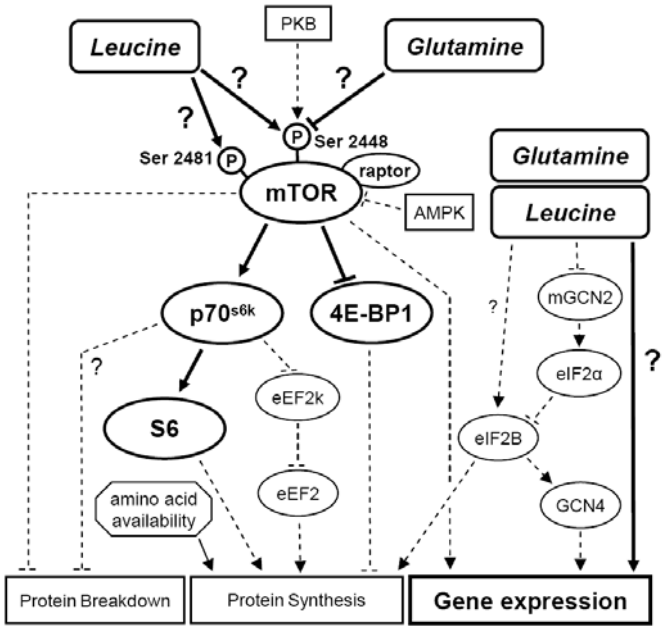

Fig. 8.3. *Model for the regulation of protein metabolism and gene expression by leucine and glutamine.* Bold lines, effect of leucine and/or glutamine. Dashed lines, no effect of leucine and/or glutamine or untested.

Since the mTOR pathway has also been implicated in the regulation of protein breakdown (Hay & Sonenberg, 2004; Deldicque *et al.*, 2005b), we tested if leucine and glutamine could alter protein catabolism. It was not affected by either amino acid (unpublished data).

It is of note that the phosphorylation state of $p70^{s6k}$ was only transiently affected by leucine or glutamine alone (30 min), whereas it remained elevated at 24h when leucine and glutamine were combined. This observation indicates that both leucine and glutamine are required to stimulate $p70^{s6k}$ at long-term. It also suggests that short-term and long-term effects are possibly mediated by different mechanisms (transporters vs receptors, see below).

The regulation of gene expression by amino acids is a recent growing area of research. Amino acid starvation leads to the up-regulation of the expression of a series of genes necessary to cope with this lack of amino acids (transporters, transaminases,…) whereas others are down-regulated (protein synthesis, turnover and folding) (Peng et al., 2002). Whether a high dose of amino acids induces the opposite response is not known. Given that amino acids have been shown to control gene expression and that the mTOR/$p70^{s6k}$ pathway is regulated by amino acids, we tested if increased leucine and glutamine concentrations could induce gene expression through the mTOR/$p70^{s6k}$ cascade. Although, the mTOR pathway is vital during myogenesis (Park et al., 2005), its involvement in the regulation of muscle-specific gene expression by amino acids has not yet been tested.

After 1h, leucine and glutamine decreased MHC II and desmin mRNA levels and a general trend towards inhibition for caveolin 3 was observed. This down-regulation in gene expression was partially reversed after 3h, with desmin and myogenin being even up-regulated. After 24h, MHC II mRNA level increased more than 6-fold with the combination of leucine and

glutamine. The short-term down-regulation is surprising since we postulated that amino acid supplementation would increase markers of myogenesis according to previous data showing the importance of amino acids in myogenic development (Erbay et al., 2003). It is of note that not all genes studied were down-regulated after 1h. Indeed, myogenin mRNA level was not modified by leucine and/or glutamine at this time. This result indicates that the regulation induced by amino acids is specific to some genes rather than a general process.

In chapter 7, we discussed the possible role of the hexosamine pathway and the role of the endoplasmic reticulum stress signalling in the control of gene expression by leucine and glutamine, but they do not seem to participate to this response. Although the mTOR pathway has been proposed as a good candidate in the regulation of gene expression by amino acids (Peng et al., 2002; Erbay et al., 2003), it is not involved in C_2C_{12} cells, since its regulation by leucine and glutamine does not fit with the observations made on gene expression. The mechanisms of regulation of muscle-specific gene expression by leucine and glutamine remain thus to be elucidated.

One major question still under debate is how amino acids induce intracellular signalling and subsequent regulation of processes like protein synthesis and breakdown and gene expression. Two theories exist and are not exclusive to each other. In the first, intracellular amino acids would induce cellular signalling after being transported inside the cells by transporters specific for different families. Several intracellular candidates, i.e. Rheb, TSC1/TSC2, PP2A, have been proposed to be regulated by amino acids and to initiate the downstream cascades leading to protein synthesis and gene expression. The second theory proposes that extracellular amino acids would be sensed by a receptor at the level of the membrane. But the existence of such a receptor for amino acids is still controversial in mammalian cells.

In yeast, the amino acid permease homologue Ssy1p is a sensor of external amino acids, which couples availability of amino acids to transcriptional events (Iraqui et al., 1999). This pathway induced by external amino acids does not interfere with the TOR pathway (Abdel-Sater et al., 2004), suggesting that two independent pathways are induced by amino acids in yeast. If the same model found in yeast might be applied or partially applied in mammalian cells, then we may not exclude the presence of a receptor linking amino acids and gene expression. The presence of such a receptor would represent a second mechanism, together with mTOR, regulated by amino acids. As observed in yeast, this mechanism could be independent of mTOR since the effects of leucine and glutamine on gene expression were not related to those on the mTOR pathway (Fig. 8.3).

Vps34, the Class III PI3K, has been proposed to be another sensor for amino acids, dependent on the mTOR pathway. In humans, Vps34 is a critical component of the nutrient sensing apparatus, together with Vps15, an associated protein kinase (Byfield *et al.*, 2005). Vps34p was first identified in yeast for its role in vesicular trafficking (Herman & Emr, 1990) and autophagy in response to nutritional deprivation (Kihara *et al.*, 2001). The same roles have been observed for mammalian Vps34 (Siddhanta *et al.,* 1998; Petiot *et al.,* 2000). In addition, human Vps34 and Vps15 kinases regulate $p70^{s6k}$ since overexpression of one of these kinases activates $p70^{s6k}$ (Byfield *et al.*, 2005). hVps34 is not part of the insulin input to $p70^{s6k}$, as it is not stimulated by insulin. However, it is inhibited by amino acid or glucose starvation, suggesting that it lies on the nutrient-regulated pathway to $p70^{s6k}$, probably upstream of mTOR. It has thus been suggested that hVps34 is a nutrient-regulated lipid kinase that integrates amino acid and glucose inputs to mTOR and $p70^{s6k}$ (Byfield *et al.*, 2005).

In summary, our results highlight the need to take with caution conclusions made on protein synthesis on basis of $p70^{s6k}$ phosphorylation. An increase in the phosphorylation on Thr 389 state of $p70^{s6k}$ does not necessarily lead to a subsequent increase in protein synthesis. Although technically much more complex to carry out, it seems that measuring protein synthesis is the only way to draw definite conclusions.

Other interesting data are those on the regulation of muscle-specific gene expression by leucine and glutamine, and thereby the regulation of myogenesis in vitro. It is possible that, in vivo, amino acids not only favour muscle anabolism but also improve muscle cell differentiation and thereby muscle remodelling during the recovery period after exercise. Repeated high-intensity exercises are known to induce a primary degeneration phase. Whether amino acid supplementation could help to limit the muscle degenerative phase and favour the subsequent regeneration period could be the purpose of future study leading to useful training and nutritional strategies for athletes involved in high-intensity exercises. For example do extra amino acids potentiate the effect of exercise on MHC and PCNA mRNA level? Amino acids would not only be seen as agents to increase muscle mass but also to improve muscle 'quality' and remodelling after repeated training sessions. The same issue about muscle remodelling applies to creatine, the mechanisms of which will be clarified in the next section.

Future research should try to determine how glutamine negatively regulates the mTOR pathway in C_2C_{12} cells at short-term, and what subsequent effects this down-regulation has on cellular functions. One piece of response could be found by comparing the metabolic fates of glutamine in the skeletal muscle and in the liver. Whereas the skeletal muscle is a net exporter of glutamine, the liver is a net consumer. Glutamine is mostly synthesised by transamination in the muscle, whereas it is principally degraded in the liver for urea synthesis and neoglucogenesis. It is possible that these different

metabolic fates partially explain the opposite effects of glutamine on the phosphorylation state of $p70^{s6k}$ in both tissues, although the mechanisms linking these events remain to be elucidated.

Future research should also be directed towards the huge increase in the phosphorylation state of $p70^{s6k}$ by leucine without affecting protein synthesis. Does leucine affect other cellular events through the mTOR pathway (or independently of mTOR), e.g. regulation of cell cycle, cell proliferation, myokine production, …?

Another interesting question to resolve is the involvement of an external amino acid sensor such as hVps34 in the signalling induced by leucine and/or glutamine in skeletal muscle cells.

3. Creatine

Creatine is used by athletes as an ergogenic aid to improve muscle recovery from high intensity and short repetitive exercises and to induce muscle hypertrophy (for review see Terjung *et al.*, 2000; Volek & Rawson, 2004; Bemben & Lamont, 2005). Since exercise and nutrition are already known to exert additive effects, we were interested in testing if creatine could further increase muscle anabolism (Fig. 8.4).

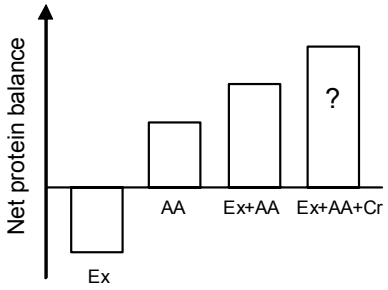

Fig. 8.4. *Net protein balance after exercise (ex), amino acids (AA) ingestion and creatine (Cr) supplementation.*

In two previous studies, the protein synthetic rate of human muscle was not modified after creatine supplementation whether at rest or post-exercise, in the fasted or in the fed state (Louis *et al.*, 2003b). Several ways of interpretating of these results are possible. First, it is possible that creatine has no anabolic effect on muscle, which would, however, not be in line with previous observations showing an increase in lean body mass (Francaux & Poortmans, 1999; Volek *et al.*, 1999). Secondly, creatine might have an effect, but beyond the time frame of the previous experiments (<3.5 h). Thirdly, the effect of creatine could be small and below the detection limits of the previously used methods. Creatine supplementation has been reported to increase muscle fibre cross-sectional area by 35% over 12 weeks of resistance training, whereas the increase was only 11% under placebo (Volek *et al.*, 1999). Therefore, even when creatine was combined with resistance exercise, it induced a change of only approximately 2% per week. The population SD of the methods used are about 14% for myofibrillar synthesis (tracer [^{13}C]leucine incorporation) and 30% for forearm protein breakdown

(a combination of tracer dilution and blood flow). Any creatine-induced effects smaller than these could not have been detected, unless much larger groups of subjects were studied. If there had been a change in synthesis or breakdown of 15%, 27 and 58 subjects would have been required, respectively, to detect them with a power of 85% and a probability of 5% as evaluated by Louis (2003b).

Therefore, in the study described in chapter 3, the hypothesis was tested that creatine could have measurable effects on several factors known to regulate anabolic processes such as IGF and two downstream effectors of the signalling induced by IGF, namely $p70^{s6k}$ and 4E-BP1. We choose to analyze the level of IGF mRNA in human since it has previously been shown to be increased by creatine in myogenic C_2C_{12} cell cultures (Louis *et al.*, 2004).

The amount of IGF-I was higher in resting muscle after creatine intake and IGF-II mRNA showed an increasing trend (chapter 3). Although the increases in IGF mRNA were only 30-40%, they may help to enhance the anabolic milieu in skeletal muscle which could explain the increase in muscle mass reported after creatine supplementation (Balsom *et al.*, 1993; Francaux & Poortmans, 1999). Exercise did not induce a further change in IGF-I and IGF-II mRNA when the subjects were supplemented with creatine. This indicates that both exercise and creatine increase the expression of IGF, but that these effects are not additive.

In the same study, we observed a massive increase in the phosphorylation state of $p70^{s6k}$ and 4E-BP1 3h post-exercise (chapter 3) which was not due to exercise alone, but probably by both acute exercise (Bolster *et al.*, 2003) and feeding (Liu *et al.*, 2001). At the same time, 5 days of creatine supplementation had no potentiating effect on the activation of $p70^{s6k}$ and 4E-BP1, confirming that creatine did not enhance protein synthesis within a short timeframe (Louis *et al.*, 2003b; Louis *et al.*, 2003c). However, 24h

post-exercise, the phosphorylation state of 4E-BP1 was found to be more elevated indicating that creatine supplementation could act to stimulate muscle growth, but not by a rapidly responding control system, as observed after exercise plus feeding, but rather by a late-response enhancement of the anabolic status of the cell involving IGFs.

The next studies thus aimed at determining the signalling induced by creatine to enhance the anabolic status of the cell. For this purpose, the myogenic C_2C_{12} cell model was used. Since creatine increased IGF mRNA both in vitro (Louis *et al.*, 2004) and in vivo (chapter 3), the implication of three major pathways known to be activated by IGF and to be involved in muscle adaptation and growth were tested: the calcineurin, the MAPK and the PI3K pathways (Fig. 8.5). A few papers have already pointed out several targets affected by creatine like GLUT-4 (Op 't Eijnde *et al.*, 2001; Ju *et al.*, 2005), MHC (Ingwall *et al.*, 1972; Willoughby & Rosene, 2001) or IGF-I (Louis *et al.*, 2004; Deldicque *et al.*, 2005a) but the present work is the first to report one whole potential signalling cascade induced by creatine. Based on the data obtained in cell cultures and in vivo, we hypothesized that IGF-I could be an important mediator in creatine signalling. To support the implication of IGF-I, picropodophyllin, an inhibitor of the receptor of IGF-I (IGF-IR), was used in C_2C_{12} cells in the presence of creatine. When having inhibited IGFI-R, creatine could still increase MHC II content, indicating that IGF-I is not the primary inducer of creatine signalling.

The importance of IGF-I in muscle cell hypertrophy has recently been reviewed by Spangenburg (2007) in the case of mechanical loading in mice. It seems that IGF-I is not the key inducer of Akt/PKB signalling after exercise since, as observed for creatine (data not shown), Akt/PKB was still activated despite an inhibition of IGF-IR. Alltogether, these results suggest that other, undetermined factors contribute to the activation of the Akt/PKB signalling.

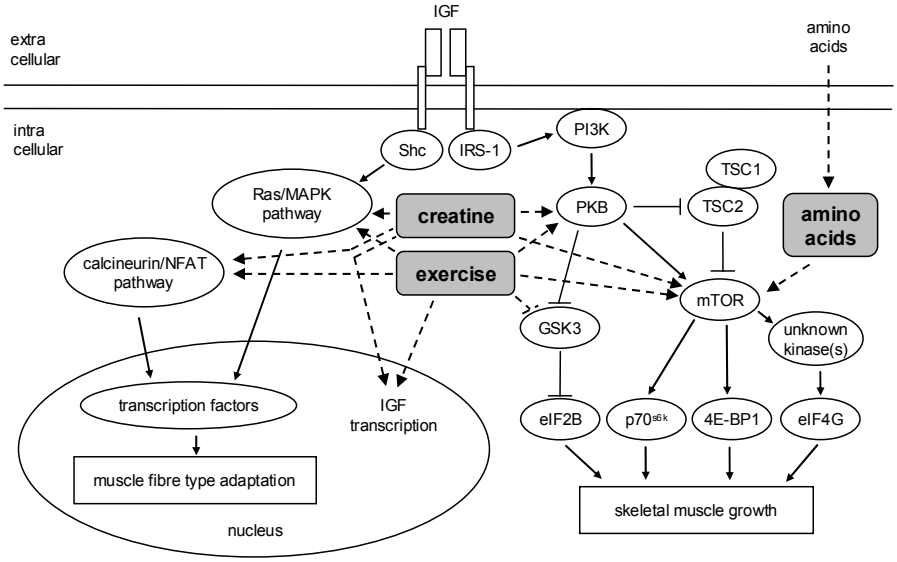

Fig. 8.5. *Summary of the potential cellular cascades affected by exercise, amino acids and creatine.* Dashed lines, potential targets for exercise, amino acids and creatine.

The major findings concerning the signalling induced by creatine have been described in chapter 4 where it was shown that creatine enhances differentiation of myogenic C_2C_{12} cells by activating both p38 and Akt/PKB pathways. After having ruled out a role of the calcineurin cascade by using its inhibitor, cyclosporin A, we proposed the following model (Fig. 8.6): creatine first activates p38, which in turn activates MEF-2, MyoD and probably other undetermined transcription factors. Through this cascade, creatine promotes the fusion of the myoblasts and the expression of muscle-specific genes (MHC II, troponin T, titin, …). At the end of the differentiation, creatine also increases the phosphorylation state of ERK1/2 which is known to promote myotube growth (Wu *et al.*, 2000). Secondarily, p38 stimulates the Akt/PKB pathway, but the signal is inhibited by AMPK at the level of mTOR, so that the higher phosphorylation state of $p70^{s6k}$ must be explained by a crosstalk with p38. The activation of $p70^{s6k}$ could serve to prime the machinery required to accelerate the translation of specific mRNA increased by creatine through the MAPK pathway.

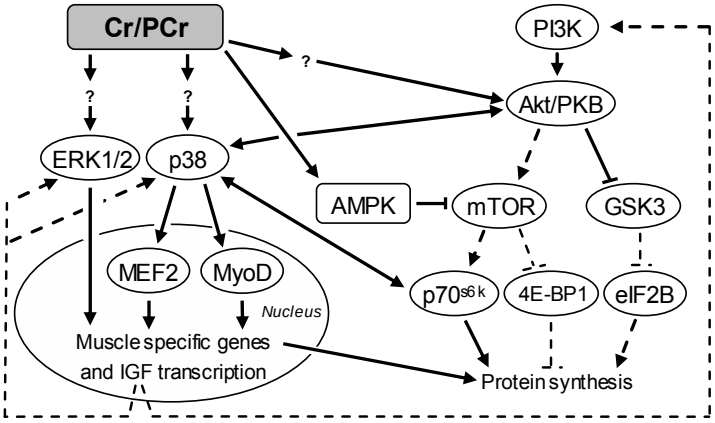

Fig. 8.6. *Model for creatine signalling.* Bold, effect of creatine; dashed, no effect of creatine or untested.

Although a major implication of IGF-I and a change of ATP level were ruled out, as well as an osmotic effect of creatine, the question remains as to how creatine activates the MAPK and the Akt/PKB pathways. Which of free creatine or phosphorylcreatine or maybe both of them is responsible for inducing this signalling?

It is of note that the signalling induced by creatine is a longer term and less acute process compared to the signalling induced by amino acids. For example, we observed that leucine increased the phosphorylation state of $p70^{s6k}$ by more than 10-fold within 30 minutes (chapter 7) whereas several days are needed to measure a 50% increase with creatine (chapter 4). Although often structurally related to them due to the three N-terminals, the mode of action of creatine is different from that induced by amino acids.

The results obtained in myogenic cell cultures could not be confirmed in human skeletal muscle (chapter 5). Five days of creatine supplementation did not affect MAPK pathway and only slightly the Akt/PKB signalling, which was not in favour of muscle mass accretion. Creatine decreased the phosphorylation state of PKB (Thr 308) at rest and of 4E-BP1 (Thr 37/46)

24h post-exercise. The decrease in the phosphorylation state of 4E-BP1 24h post-exercise in chapter 5 was surprising, since an increase in the phosphorylation state of this protein by creatine at the same time was reported in chapter 3. Since creatine supplementation protocol was identical, it is not likely to explain this discrepancy. In chapter 5, the exercise type was different, with a more pronounced eccentric phase, and the test subjects did not receive large amounts of amino acids immediately after the completion of the exercise. Nutrients administered immediately post-exercise that could still have interacted with creatine signalling 24h later.

Another explanation might be found in the antibody used to analyze the phosphorylation state of 4E-BP1. In chapter 3, the antibody recognized all phosphorylated forms of 4E-BP1 between 19 and 25kDa. The antibody used in chapter 5 only recognized the phosphorylation state on Thr 37 and Thr 46, which are the first sites to be phosphorylated followed by Thr 70 and Ser 65 (Gingras *et al.*, 2001). It is possible that creatine specifically increases the phosphorylation state of the first sites, Thr 37 and Thr 46, 24h post-exercise (chapter 5) without affecting the subsequent sites. If this is the case, the proportion of the gamma form, corresponding to the highest phosphorylation state, compared to the total amount of all phosphorylated forms would be decreased (chapter 3). However this is difficult to verify since the different phosphorylated forms of the proteins (α, β and γ) do not correspond to one specific phosphorylation site. To summarize, in humans creatine only seems to affect the Akt/PKB pathway in a minor way.

Since cellular signalling is only slightly altered by creatine, a new hypothesis was formulated: the creatine-plus-exercise effects are manifested after a 'priming' of muscle gene expression. The stimulation of transcriptional changes in muscle gene expression might occur as a result of increased availability of creatine, and associated ATP/ADP concentration or Ca^{2+} concentration changes during or after contractile activity. The result of these

transcriptional changes, in terms of protein accretion, would not be seen for days to weeks after the initial stimuli. In support of the notion for the modulation of gene expression by creatine, there has been evidence of increased MHC and IGF-I and II mRNA in human subjects (Willoughby & Rosene, 2001; Deldicque *et al.*, 2005a). We found that 5 days of creatine supplementation alone, without performing any contractile activity, increased the level of MHC I, collagen 1(α1) and GLUT-4 mRNA. When combined with an exercise bout, creatine supplementation only potentiated the effect of exercise on MHC II mRNA expression indicating that creatine and exercise have no short-term additive effects in the expression of the genes studied.

This is the first study to show an increase in collagen mRNA after creatine supplementation. Collagen is the most abundant protein of the extracellular matrix of connective tissues. It connects tissues and plays a key role in force transmission and tissue structure maintenance in tendons, ligaments, bone and muscle (Kjaer, 2004). Collagen type 1 dominates the intramuscular collagen content (Light & Champion, 1984). Increasing muscle collagen content could be one mechanism by which creatine allows improved muscle strength to be developed. Our data tend to favour the hypothesis that creatine per se induces a favourable environment for increasing lean body mass enabling a larger work load instead of this increase in lean body mass being subsequent to a greater training volume. It is possible that creatine first affects the extracellular matrix of muscle fibres, on which activated satellite cells would find a favourable structure to develop and differentiate into mature skeletal muscle fibres.

One could raise the question whether a single dose of creatine (7g) could have induced the same changes in gene expression as those observed after 5 days of supplementation. Although we did not address this issue in the present research, it is likely that a single dose of creatine does not increase

skeletal muscle content significantly. Moreover, comparatively with the effects of one exercise session, short-term creatine supplementation (5 days) only weakly modifies gene expression. We could speculate that the effects of one dose would be even smaller and probably undetectable. It seems that the most obvious effects of creatine are observed after several weeks of supplementation in combination with a resistance exercise program. A recent study reported that four to sixteen weeks of creatine supplementation potentiates the increase in satellite cell number and their activation induced by exercise training (Olsen *et al.*, 2006). Although the mechanisms of activation of the satellite cells by creatine remain unknown, it is a promising area of research for future studies. However, since it has been postulated that muscle hypertrophy may occur without activation of satellite cells (McCarthy & Esser, 2007), it will be essential to determine if the hypertrophic effect of creatine necessarily requires the recruitment of satellite cells.

Of all compounds tested in the myogenic C_2C_{12} cell culture model, creatine has revealed itself as the most potent for enhancing differentiation and favouring myotube growth. However, we found no great effect of creatine in healthy subjects, mostly because changes induced by creatine in vivo are small and thus technically difficult to detect and because creatine has a long term action. The greatest effects of creatine have been observed in patients suffering from myopathies (Louis *et al.*, 2003a), in muscle regeneration after immobilization (Hespel *et al.*, 2001) or in aging (Brose *et al.*, 2003). Creatine supplementation seems thus to have more favourable effects in ageing and in pathological muscles.

Conclusion

The present work aimed at defining the molecular mechanisms by which exercise and nutrients regulate myogenic cell differentiation and growth (Fig. 8.7). Our results underscored the importance of the MAPK and the Akt/PKB pathways in this signalling both in vitro and in vivo. We clearly showed that nutrients were necessary to fully activate these two pathways after exercise and that creatine supplementation did not further enhance the signalling induced by nutrients and exercise. However, we found that creatine was able to induce the expression of a few genes involved in muscle remodelling as soon as after five days of supplementation.

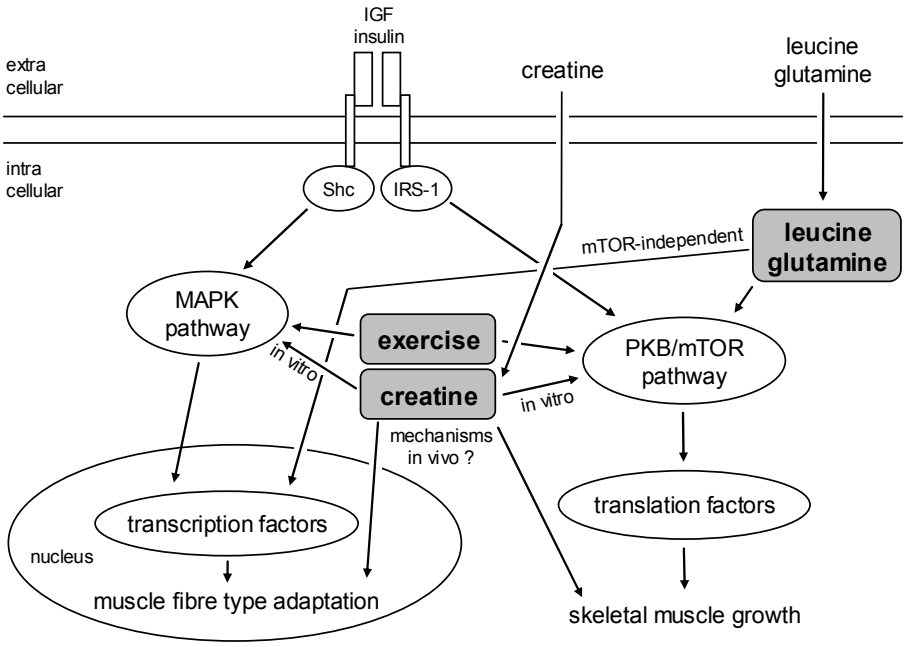

Fig. 8.7. *Summary of the signalling induced by exercise, creatine, leucine and glutamine.*

Summary

A significant advance in understanding skeletal muscle adaptation to physical training has been the observation that nutrients and exercise work in synergy to enhance muscle protein synthesis. Physical activity triggers an adaptive response to which nutrition provides the necessary building blocks for an optimal response.

The aim of the present work was to contribute to the understanding of the molecular events induced by exercise or nutrients (creatine and amino acids) to create the adaptive environment and to induce the cellular adaptation and growth, respectively. From a methodological point of view, two experimental models were used: muscle biopsies taken from the vastus lateralis of human volunteers and myotubes cultured from C_2C_{12} cells.

The transcription of a series of genes involved in muscle remodelling (MAFbx, MHCIIA, PGC-1α, PCNA and IL-6) was increased immediately after the completion of a resistance exercise session performed in the fasted state. The phosphorylation state of p38 and ERK1/2 was also increased, whereas the Akt/PKB pathway was negatively regulated. This contrasted with the high phosphorylation state observed on $p70^{s6k}$ and 4E-BP1 when subjects received a large amount of amino acids during the recovery period. Our results suggest that the MAPK pathway can be triggered by contractile activity alone, whereas the Akt/PKB pathway requires nutrients to be activated.

Certain amino acids regulate the phosphorylation state of mTOR and its downstream targets, as demonstrated by one of our *in vitro* studies. However, that modulation did not lead to a systematic modification in the rate of protein synthesis. Amino acids were also able to influence the expression of muscle-specific genes, highlighting their importance in the control of muscle protein synthesis.

Protein anabolism was largely enhanced and cell differentiation was accelerated by creatine in our *in vitro* model. We have identified the p38 and Akt/PKB pathways as mediators of these effects. Nevertheless, we were unable to confirm the existence of similar events in human skeletal muscle *in vivo*.

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